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COMPARISON OF DIAGNOSTIC TOOLS AND MOLECULAR BASED TECHNIQUES FOR THE RAPID IDENTIFICATION OF *ESCHERICHIA COLI* AND COLIFORMS IN CONTAMINATED RIVER WATER

by

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DECLARATION

I, Thando Ndlovu, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date** **05/03/2013**

ABSTRACT

Water is an important daily requirement and in a clean, pure form, it promotes health and well-being. In addition to South Africa being one of the driest countries in the world, water availability is also being compromised by massive pollution of remaining water sources. The Berg- and Plankenburg Rivers are two of the surface water sources in the Western Cape, South Africa, which are highly polluted by sewage, industrial and agricultural run-off. The current investigation was aimed at comparing diagnostic tools, which are employed by municipalities and food industries, and molecular based techniques to routinely monitor water for indicator organisms in time- and cost-effective manner. These rivers were sampled twice a month (July 2010 to January 2011) at the sites closest to the informal settlements of Kayamandi in Stellenbosch (Plankenburg River) and Mbekweni in Paarl (Berg River).

The contamination levels of the two river systems were evaluated by the enumeration of *Escherichia coli* and coliforms using the Colilert 18® system, Membrane Filtration (MF) and Multiple Tube Fermentation (MTF) techniques. The highest faecal coliform count of 9.2×10^6 microorganisms/100 ml was obtained in weeks 21 and 28 from the Plankenburg River system by the MTF technique, while the lowest count of 1.1×10^3 microorganisms/100 ml was obtained in week one for both river systems by the MTF technique. The highest *E. coli* count of 1.7×10^6 microorganisms/100 ml was obtained from the Berg River system (week 9) using the MTF technique, while the lowest count of 3.6×10^2 microorganisms/100 ml was obtained by the MF technique from the Plankenburg River system. The coliform and *E. coli* counts obtained by the enumeration techniques thus significantly ($p > 0.05$) exceeded the guidelines of 2000 microorganisms/100 ml stipulated by the Department of Water Affairs and Forestry (DWAF, 1996) for water used in recreational purposes.

Overall the results obtained in this study showed that the water in the Berg- and Plankenburg River systems is highly polluted, especially where these water sources are used for irrigational and recreational purposes. For the coliform and *E. coli* counts obtained using the three enumeration techniques, it was noted that the MTF technique was more sensitive and obtained higher counts for most of the sampling weeks. However, the media (Membrane lactose glucuronide agar) used in the MF technique also effectively recovered environmentally stressed microbial cells and it was also better for the routine selection and growth of coliforms and *E. coli*. While *E. coli* and total coliforms were detected utilising the Colilert 18® system, accurate enumeration values for these two indicator groups was not obtained for the entire sampling period for both river systems. It has previously been shown that dilutions (up to 10^{-3}) of highly polluted waters increase the accuracy of the Colilert 18® system to enumerate colifoms and *E. coli* in marine waters. As the results obtained utilising

the Colilert 18® system were also not comparable to the MF and MTF techniques it is recommended that highly polluted water samples be diluted to increase the accuracy of this system as a routine enumeration technique.

Water samples were directly inoculated onto MacConkey, Vile Red Bile (VRB) agar and the Chromocult Coliform agar (CCA) and single colonies were inoculated onto nutrient agar. Chromocult coliform agar proved to be more sensitive than MacConkey and VRB agar for the culturing of *E. coli* and coliforms. Preliminary identification of these colonies was done using the RapID ONE and API 20 E systems. The most isolated *Enterobacteriaceae* species by both systems, included *Klebsiella pneumoniae, Klebsiella oxytoca*, *Escherichia coli* and *Enterobacter cloacae* in both river systems. The API 20 E system was more sensitive in the preliminary identification of the various isolates, as greater species diversity was obtained in comparison to the RapID ONE system.

The Polymerase Chain Reaction (PCR) was firstly optimised using positive *Enterobacteriaceae* species. The optimised method was then applied to the analysis of river water samples, which were centrifuged to harvest the bacterial cells, with DNA extracted using the boiling method. The extracted DNA was amplified using conventional PCR with the aid of species specific primers. The *Enterobacteriaceae* species that were detected throughout the study period in both river systems include *Serratia marcescens*, *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus cereus*. Conventional PCR was the most reliable and sensitive technique to detect *Enterobacteriaceae* to species level in a short period of time when compared to RapID ONE and the API 20 E systems. Multiplex PCR was optimised using the positive pathogenic *E. coli* strains namely, Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAEC). It was then employed in river water sample analysis and enabled the detection of EAEC, EHEC, and EIEC strains in Berg River system, with only the EAEC detected in the Plankenburg River system. Real-time PCR was used to optimise the multiplex PCR in the amplification of *E. coli* strains and successfully reduced the time to obtain final results when using control organisms. Real-time PCR was found to be more sensitive and time-effective in the identification of *E. coli* strains, and also more pronounced DNA bands were observed in real-time PCR products compared to conventional-multiplex PCR amplicons.

To sustain the services provided by the Berg- and Plankenburg Rivers in the Western Cape (South Africa), these water sources should frequently be monitored, results assessed and reported according to the practices acknowledged by responsible bodies. It is therefore recommended that the enumeration techniques be used in conjunction with the very sensitive PCR technique for the accurate detection of coliforms and *E. coli* in river water samples.

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DEDICATION

This Thesis is dedicated to my family

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GLOSSARY

Abbreviation	Definition
AAF	Aggregative Adherence Fimbriae
ANOVA	Analysis of Variance
APHA	American Public Health Association
BEACH	Beaches Environment Assessment and Coastal Health
BGB	Brilliant Green Bile Broth
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
CCA	Chromocult Coliform Agar
CFU	Colony Forming Unit
DAEC	Diffuse Adhering *Escherichia coli*
DST	Defined Substrate Technology
DWAF	Department of Water Affairs and Forestry
EAEC	Enteroaggregative *Escherichia coli*
EAF	Enteropathogenic *Escherichia coli* Adherence Factor
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
elt	Heat labile enterotoxin
EPA	Environmental Protection Agency
EPEC	Enteropathogenic *Escherichia coli*
ERIC	Electronic RapID Compedium
est	Heat stable enterotoxin
ETEC	Enterotoxigenic *Escherichia coli*
HIV	Human Immunodeficiency Virus
HUS	Haemolytic Uremic Syndrome
Ipa	Invasion plasmid antigens
LTB	Lauryl Tryptose Broth
MF	Membrane Filtration
MLGA	Membrane Lactose Glucuronide Agar
MPN	Most Probable Number
MTF	Multiple Tube Fermentation
MUG	4-methylumbelliferyl-β-D-glucuronide
ONPG	o-nitrophenyl-β-D-glucuronide
PCR	Polymerase Chain Reaction

SABS	South African Bureau of Standards
STEC	Shiga toxin-producing *Escherichia coli*
stx	Shiga-like toxins
TAE	Tris-acetate-ethylenediamine tetraacetic acid
UNICEF	United Children's Fund
USEPA	United States Environmental Protection Agency
USA	United States of America
WHO	World Health Organisation

1. LITERATURE REVIEW

1.1 Introduction

Water comprises a major part of the earth's hydrosphere and is central to all life. Access to clean and safe water is one of the most important basic human rights and the habitation of a region is largely dependent on the availability of fresh water (Muller and Kfir, 2003). Economic growth and development is also influenced by the availability of water used for irrigation, domestic purposes, hydropower and fishing (Hammer and Mackichan, 1981).

South Africa is considered one of the driest countries in the world, with an average rainfall of 495 mm per annum compared to the global average of 860 mm (Burgess and Neils, 2008). Experts warn that the management of water sources in South Africa is in serious crisis as surface water sources (dams, rivers and streams) are becoming more stressed due to a higher water consumption (Ashton, 2010). In addition, the destruction of sensitive catchments, massive pollution, transfer capacity, failure to retain water distribution infrastructure and to invest in more bulk storage (catchment dams), all contribute to the water crisis in the country. An increase in economic activity and the general standard of living also leads to a significant increase in the consumption and utilisation of water (Oelofse and Strydom, 2010).

Urbanisation, industrialisation and an increase in population, then contributes to the pollution of water sources when effluents are discharged into rivers leading to the deterioration of water quality. The Department of Water Affairs and Forestry's (DWAF) [currently known as Department of Water and Environmental Affairs (DWEA)] National Water Resource Strategy Report (2004) states that, agricultural irrigation consumes more than 60% of South Africa's total water usage. Approximately 15% of the available water is also used in energy generation, mining and manufacturing (Burgess and Neils, 2008), while domestic supply and sanitation comprises the remaining 25%. According to Peter Ashton [Senior Water Researcher at the Council for Scientific and Industrial Research (CSIR)], demand already exceeds supply in 80% of South Africa's catchments (Burgess and Neils, 2008). As the constructions of dams are indefinite, alternative water sources should thus be considered in order to alleviate the water crisis in South Africa.

1.2 Water Sources

The two main water sources are surface water (rivers, dams and lakes) and groundwater (boreholes and springs). Groundwater can be defined as water found between alluvial material (clay, gravel, sand or silt), cracks and crevices underground, below the water table

(Hammer and Mackichan, 1981). About 90-95% of the rural and suburban population in the United States use groundwater as their source of drinking water (Prescott *et al*., 2005), while in South Africa approximately 15% of water consumption is from a groundwater source, with the majority of water originating from surface water sources (DWAF, 2002). Many rural populations in developing countries, including South Africa, use groundwater without any treatment due to the lack of infrastructure. A low percentage of groundwater sources is thus also utilised in South Africa, due to high levels of nitrates, calcium, magnesium, phosphates and fluorides, which renders the water unfit for human consumption (Kongolo, 2011).

1.2.1 Surface Water

Water found above the ground in dams, lakes, rivers, streams and the ocean is referred to as surface water. South Africa relies on these dams, rivers and streams as the most important sources of water for domestic, industrial, agricultural and recreational purposes, in the thermo-electric power industry to cool electricity-generated equipment as well as to naturally purify wastewater effluents (Hammer and Mackichan, 1981). In South Africa, rivers flow into dams, which then serve as catchment basins to store water. These dams usually have a total capacity of approximately 32 400 million m^3 which constitutes 70% of the total mean annual run off (Oelofse and Strydom, 2010). The total available yield is approximately 13 227 million m^3 per annum, while 12 871 million m^3 per annum was the total amount of water that was required for use in various sectors by the end of year 2000 (DWAF, 2004a).

The quality of surface water resources should be capable of supporting aquatic life, be aesthetically pleasing and should be suitable for recreational purposes such as swimming, water skiing and boating. Approximately 51% of surface water (dams and rivers) in South Africa is also used in irrigational farming (Oosthuizen, 2002). Drinking water supplies are largely secured by implementing multiple barriers from the catchment dam to the consumer to ensure that contamination is prevented or reduced to levels that are not harmful to humans (DWAF, 2005). However the quality of water sources are being threatened by contamination from industries and waste from municipality sewage treatment plants as well as the excessive use of pesticides and fertilisers in the agricultural sector (State of the Rivers Report: Berg River, 2004). Most of the urban rivers in the Cape Town area are also used as conduits for discharging treated effluents into the ocean (DWAF, 2004b). A study carried out, on the widespread invasion on surface water resources in South Africa and Lesotho by alien plants (trees and woody shrubs), showed that these alien plants are also depleting the available water, as they were found to be consuming an estimated 3 300 m^3 of water per year (Le Maitre *et al.,* 2000). In addition, water sources are

disrupted by natural disasters such as floods, catchment chemical spills and bacteriological contamination, which leads to significant health risks to consumers (DWAF, 2005).

1.2.1.1 Berg River

The Berg River is found in the northern region of Cape Town in the province of the Western Cape, South Africa. It is approximately 300 km long and rises from the Groot Drakenstein Mountains near the town of Franschhoek. It flows northwards past Paarl, Wellington, Hermon and Gouda, drains a catchment area of about 9 000 km^2 and enters the Atlantic ocean on the West Coast at Velddrif. During the winter rainy season (from June to August) the level of the water can reach 2.49 metres, which is dominated by run off (Department of Water Affairs, 2010). Approximately 50% of the catchment area is cultivated agricultural land consisting mainly of vineyards, citrus orchards and wheat fields (De Villiers, 2007). The upper catchment area of the Berg River supplies most of the Boland region and the surrounding suburbs with fresh water for various uses. In addition, it provides water for irrigational purposes along the middle and lower reaches of the river (De Villiers, 2007). A combination of dry spells, increases in population and a fast growing local economy has put severe pressure on water resources within this river system.

The Berg River system serves as an accumulation point for improperly treated wastewater from the agricultural and pig farming areas, industries, informal settlements and suburbs that border the river system (Winter and Mgese, 2011). In addition, surface run off, (especially during the rainy season) from the informal settlements bordering the river, carrying all the solid waste and grey water also leads to a negative impact on the chemical and microbial quality of water in the Berg River. A study conducted by Paulse *et al*. (2007) investigated the microbial contamination at sampling sites along the Berg River system near an informal settlement, industrial sites and close to the agricultural areas. The results for faecal coliforms and *Escherichia coli* exceeded the guidelines as stipulated by the South African Bureau of Standards (SABS, 1984) and DWAF (1996) respectively for water used for recreational, domestic and irrigation purposes (Paulse *et al.,* 2007; Ackerman, 2010).

The quality of water in the Berg River is also of great concern to farmers, fresh produce retailers and consumers when it is used for irrigation without any prior treatment (Ackerman, 2010). Use of contaminated water for irrigation may lead to the carryover of pathogenic microorganisms from water onto fresh produce such as vegetables, which are consumed raw (Ackerman, 2010). However the Berg River dam, in the Groot Drakenstein Mountains near Franschhoek, provides some relief for Cape Town's water supply problems, and also improves the quality of water in the lower reaches of this river system.

1.2.1.2 Plankenburg River

The Plankenburg River is approximately 10 km long and originates from the mountainous area in the Boland region of South Africa. It meanders past Stellenbosch and flows through the densely populated informal settlement of Kayamandi. It later joins the Eerste River, which is used by many farmers for irrigation without prior water treatment (Thom, 2002). The Plankenburg River is surrounded by clothing, winery, and dairy product factories and mechanical and spray painting workshops, which discharge their wastewater into the river system, making the water unsafe for general use (Barnes, 2003). In addition, storm drainage pipes are used to drain the waste effluents from the informal settlements directly into the river system. Farmers along the Plankenburg River also make use of fertilisers, which are washed away during the rainy season and deposited into the river system.

Water quality testing has been conducted at selected sites along the Plankenburg River for parameters such as microbial counts and metal concentrations, which were found to be higher than the recommended guidelines stipulated by the DWEA and SABS (Barnes, 2003; Jackson *et al.,* 2009; Paulse *et al.,* 2009; Ackerman, 2010). High concentrations of metals in water, which may have leached from plumbing fittings, healthcare products and galvanised structures used by informal settlement dwellers, also have a negative impact on aquatic organisms and human life.

1.3 Surface Water Quality Guidelines

Water quality standards are established to sustain public health, as well as to protect aquatic organisms such as fish, shellfish and wildlife. The analysis of water quality promotes the identification of hazards and assessment of risks in water sources (DWAF, 2005; USEPA, 2005). The United States Environmental Protection Agency (USEPA) stipulates guidelines for surface water quality to protect aquatic life and human health (USEPA, 2005). The South African Bureau of Standards (SABS, 1984) and the Department of Water and Forestry (DWAF, 1996) are recognised for establishing and monitoring the quality of water sources. These bodies ensure that water used for domestic, industrial, agricultural and recreational purposes comply with fixed rules and regulations. Limits are thus stipulated on the level of pollutants in lakes, rivers and marine waters, which are mostly used for recreational purposes such as swimming, fishing and also to protect aquatic life forms.

According to DWAF (1998) surface water sources serve domestic, recreational and agricultural uses and contribute to the economic growth of the country and the continent as a whole. Water quality guidelines for surface water sources thus ultimately aim to protect the environment and consumers by promoting the quality of products and services. **Table 1.1**

summarises all the indicators used in the quality control of the waters utilised for recreational, domestic and agricultural purposes as stipulated by SABS (1984) and DWAF (1996).

Table 1.1 Summary of Water Quality Guidelines for Microbiological Indicators (SABS, 1984; DWAF, 1996)

Microorganism	SABS (CFU/100 ml)	DWAF (CFU/100 ml)
Total coliforms	0 (for domestic purposes)	~ 5 (for domestic purposes)
Faecal coliforms	0 (for domestic purposes)	~ 2000 (for recreational purposes) ~10000 (for irrigational uses) ~ 0 (for domestic purposes)
Enterococci	Not available	~ 30 (for full contact recreational purposes) ~ 230 (for intermediate contact recreational purposes)
Escherichia coli	~ 2000 (for recreational purposes)	~ 130 (for recreational purposes) ~ 1 (for irrigational use)

Impoundments such as dams, rivers, streams or boreholes, are also primarily used for domestic, irrigation and recreational purposes. Based on the DWAF guidelines (1996), domestic water refers to water used for drinking, bathing, personal hygiene, and other general household uses. The guidelines also stipulate that when a surface water source is used for domestic purposes, total coliform counts should not exceed five total coliforms/100 ml and no faecal coliforms should be present in the water source (DWAF, 1996a). In addition, according to the SABS (1984) and DWAF (1996b) guidelines, water used for recreational purposes should not exceed *E. coli* counts of 2000 microorganisms/100 ml and 130 microorganisms/100 ml, respectively, as illustrated in **Table 1.1**, as values greater than these limits may lead to an increase in waterborne infections. Recreational waters are contaminated when a combination of evidential factors such as sewage disposal and agricultural run off are recognised or present. Recreational water environments are also largely faecally contaminated by sewage and river discharges (WHO, 2003). Groundwater, farm dams, large reservoirs, rivers, industrial and municipal effluents are the major sources of irrigational water in South Africa. The agricultural sector has to cope with low amounts of water available for irrigation as well as the deteriorating water quality of surface water sources. In addition, human and animal microbial pathogens could be transferred to crops and vegetables through irrigation with contaminated water (Lotter, 2010). **Table 1.1** also lists the acceptable Colony Forming Units (CFU) for faecal coliforms and *E. coli* cells per 100 ml in water used for irrigational purposes (DWAF, 1996c).

1.4 Pollution of Water Sources

According to the National Water Act of the Republic of South Africa (1998), water pollution is the alteration (direct or indirect) of the chemical, microbiological or physical properties of a

water source making it unfit for any beneficial use. It may be expected to be potentially harmful to the health, safety or welfare of human beings or to any aquatic or non-aquatic organisms as well as to resource quality and property (National Water Act, 1998).

Water pollution ranges from partially dissolved or suspended solids in water, to persistent pollutants such as chemical compounds, heavy metals and pesticides. According to the Department of Water affairs and Forestry (2004) for the Thukela Water Project Report, national water sources have been threatened by drought spells, as well as point and non-point source pollution (DWAF, 2004). Water pollution is the result of mainly natural and anthropogenic pollution, both of which have a negative impact on the health of people relying on the water sources (Kong *et al.,* 2009). Anthropogenic pollution is generally caused by the development of agriculture, industrialisation, increases in population and urbanisation. It is classified into two different types, namely point source and non-point source or diffuse pollution. **Figure 1.1** illustrates the possible point and non-point sources of pollution for both surface and groundwater sources.

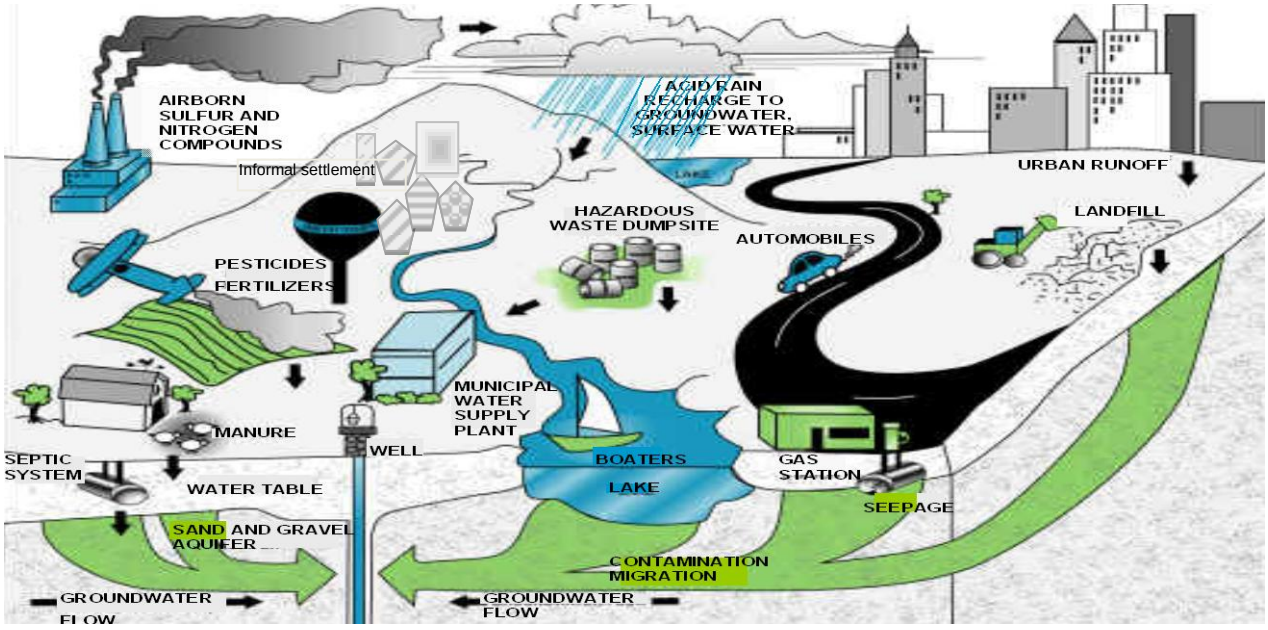

Figure 1.1 Illustration of non-point source and point source pollution of groundwater and surface waters (Adapted from The Groundwater Foundation, 2001)

The rapid development of informal urban settlements along rivers and streams poses serious challenges to municipalities and service providers. In many instances they are unable to meet the demand for sewage and waste removal, which results in untreated effluents being disposed into the river systems. In addition, leaching of metals and other contaminants, present in sewage wastes and household products from the informal settlements, suburbs, hazardous waste dumpsites and stationery effluents in treatment plants, into the river also contributes to the contamination of both surface and groundwater

sources. The degradation of water quality in surface water resources in South Africa impairs the integrity of aquatic life, and also adds to the economic burden as additional costs are incurred for the treatment of water for human consumption and for industrial and irrigational applications (Oelofse and Strydom, 2010).

1.4.1 Point Source Pollution

Point source pollution results when pollutants originate from specific locations, which are identifiable. The major sources of point source pollutants are industries, sewage treatment plants, and active abandoned underground mines. This pollution occurs mostly in urban areas, as illustrated in **Figure 1.2**, as it is fairly easy to identify and can be monitored and regulated.

Figure 1.2 Solid waste (left) and polluted drainage channels (right) within informal settlements

Most streams and rivers located adjacent to urban and agricultural areas are used as final dumping sites for inadequately treated municipal wastes and other materials (Prescott *et al.,* 1993). Malfunctioning municipal sewage treatment plants are also the main source of pollution of rivers in developing countries, where up to 98% of human waste flows into rivers and streams untreated (Hill, 2004). Wastewater is discharged from industries after process production, and may contain harmful chemicals such as acids, alkalis, oils, poisons, salts and in some cases pathogenic bacteria. The main sources of industrial pollutants are organic wastes from breweries, abattoirs and sugar refineries. Other sources of pollution such as nitrogen and phosphorus may seep into the river from domestic wastewater. Run off from agricultural fields may also contribute to the eutrophication of surface waters (Gumbo *et al*., 2003). Mines around South Africa have been associated with contamination of rivers, as acid mine drainage contains high concentrations of metals, sulphides and salts. The Loskop dam and the Olifants River catchment in particular, have been severely polluted

by the drainage from the Highveld Coalfield mine in Mpumalanga (South Africa) (Manders *et al.,* 2009).

Environmental experts propose that governmental water testing is sub-standard as most of the rivers are said to contain bacteria at levels above the standards stipulated by the SABS (1984) and DWAF (1996), placing millions of people at risk. Rivers with the highest levels of disease-causing organisms in South Africa are the Olifants River (Mpumalanga), Vaal River (Gauteng) and Umngeni River (KwaZulu-Natal). Other river systems badly affected include the Magalies River, the Jukskei River, Plankenburg River in the Cape and various lagoons along KwaZulu-Natal's coast (Transvaal Agricultural Union of South Africa, 2009).

According to the World Health Organisation (WHO) (1995), at least three million children from impoverished households die annually from drinking water contaminated with untreated or partially treated sewage waste containing infectious bacteria, viruses and parasites (Hill, 2004). Research has also shown that high population densities in informal settlements and urbanisation, create challenges in controlling the quality of the river water (Gemmell and Schmidt, 2010). **Figure 1.3** illustrates how inadequate sanitation and wastewater removal facilities in the informal settlements, which are situated on the riverbanks, contribute to the deterioration of the water quality in streams and rivers around South Africa. Faecal matter may also enter the river system from the pit latrines, which seeps below the ground surface into streams (Barnes, 2003).

Figure 1.3 Water pollution in the Plankenburg River (Stellenbosch)

1.4.2 Non-Point Source Pollution

Non-point source pollution originates from many diverse sources and sectors and is mostly dominated by surface run off. Non-point source pollution also results from land run off,

drainage, seepage, groundwater flow, precipitation and in South Africa it is largely caused by rainfall and the associated run offs mainly in urban and agricultural areas (Quibell, 2000; DWAF 2004a; Heath *et al.,* 2009) (**Table 1.2**).

Table 1.2. Non-point sources of pollution (Hill, 2004)

Sources	Pollutants
Agriculture (growing crops)	Contaminants include soil, fertilisers, pesticides
Agriculture (Animal operations)	Animal wastes with pathogens, nutrients, BOD, suspended solids
Timber cutting operations	Soil, BOD, nutrients
Mining operations	Acid (Sometimes severe), soil, metals
Construction sites	Soil, oil/grease, heavy metals, debris
Cities/suburbs with sealed surfaces	Oil, grease, metals, sand, animal wastes and debris, most of the water striking on surfaces (paved or roofed) runs off into storm drains

Water used for washing cars, watering crops and lawns and also rain water, transfer an array of contaminants including oil and sand from roadways, pesticides and herbicides from farmland as well as nutrients and toxic materials from residential areas. This run off carries oil, grease, dirt, animal waste, microorganisms and chemical pollutants, which eventually reaches the river system directly or through storm drain collection systems. **Figure 1.4** illustrates how stormwater drains situated close to informal settlements (Paarl) flow directly into the Berg River system (Western Cape).

Urban areas are the most prevailing non-point sources of pollution, which include run off from streets, and parking lots, roofs and construction sites, while rural non-point sources include agriculture, logging and mining sites (Hill, 2004). In addition, faecal run off from informal settlements and surrounding suburbs have also decreased the quality of water in river systems, which could jeopardise exports in the agricultural sector (Barnes, 2003). Large quantities of acids found in contaminated surface waters, flow from gold and coal mines, while agricultural pesticides, fertilisers and herbicides may wash into stagnant waters, streams and rivers. Coal and gold mines are said to be highest contributors to non-point source pollution due to the magnitude at which they are currently mined. The mining industry, industrial and power generating sectors thus contributes to significant non-point pollution of both surface and groundwater sources in South Africa (Heath *et al.,* 2009).

Figure 1.4 Storm drainage flow from Mbekweni informal settlement (Paarl, South Africa) into the Berg River

1.5 Microbial Pollution

Increased levels of microbial pollution are experienced during rainy seasons as large numbers of microorganisms are washed from soil, faecal wastes (from humans and animals) and decaying vegetable matter into the river systems. Waste material should thus be disposed of in a safe and effective manner in order to not contaminate water sources as it may contain high loads of microorganisms, which are pathogenic to humans (Muller and Kfir, 2003; Barnes *et al.,* 2004; Ijabadeniyi, 2010).

In urban areas the overloaded municipal sewage systems and informal settlements are the major sources of deteriorating microbiological water quality. Outbreaks in industrialised countries have highlighted human vulnerability to pathogens found in drinking water supplies (Payne, 2007), while in developing countries it is estimated that approximately 90% of health issues in children are related to water (Cheesbrough, 2006) and therefore effective water monitoring is essential in order to prevent waterborne diseases. Methods that are used by water utilities to detect and identify indicator organisms from drinking water supplies should thus be reliable and should provide a high degree of sensitivity and specificity (Payne, 2007).

In many cases the extent of pollution causes increases in the number of faecal indicator organisms to levels exceeding limits for water intended for domestic, recreational and irrigational use (**Table 1.1**) (DWAF, 2005). Microbial pollution of rivers also mostly affects the rural communities, as they extract water directly from rivers for domestic purposes without prior treatment. Faecal contamination from humans, livestock and wild animals are therefore of great concern as they contain various pathogens. *Escherichia coli* found in surface waters, originates mainly from municipal wastewater discharges, septic leachate,

agricultural or storm water run off, wildlife populations, or non-point sources of human and animal waste (An *et al.*, 2002; Barnes, 2003, Gemmell and Schmidt, 2010). Pathogens associated with faecal pollution cause human disease and economic losses in industries that utilise water directly from surface water sources.

1.5.1 Waterborne Pathogens

Enteric pathogens such as bacteria, parasites and viruses are the world's leading "silent" killers and are often anthropogenic in origin. Pathogenic microorganisms are transmitted into the water bodies in run off from storm water, improper operating septic systems or livestock operations. This problem is exacerbated by the malfunctioning of sewage works and the lack of adequate and accurate water quality testing techniques (Leclerc *et al.,* 2002; Ashton, 2010). Water then acts as an inert carrier of the pathogenic microorganisms, such as protozoa, helminths, viruses and bacteria, and humans can become infected with diseases such as diarrhoea, skin irritations, typhoid and respiratory disorders, from microbially contaminated water sources (Krantz and Kifferstein, 2003; Kong *et al.,* 2009).

Pathogenic microorganisms associated with water include *Salmonella*, *Shigella*, *E. coli*, *Klebsiella aerogenes*, *Pseudomonas fluorescens,* amongst others, which are known to cause gastroenteritis, diarrhoea and bacillary dysentery (Obasohan *et al*., 2010). *Campylobacter* species are transmitted through consumption of contaminated food and water, contact with infected animals, or anal-oral sexual activity (Muller and Kfir, 2003), while *Vibrio cholerae,* the Gram-negative causative agent of cholera, is passed through contaminated water and food (Prescott *et al.,* 2005). *Salmonella* is a food- and waterborne pathogen, which can lead to death in extreme infections (Center for Disease Control and Prevention, 2010). Protozoan cysts and viruses persist for a long time in the environment and tend to be more resistant to water treatment (Prescott *et al.,* 1996). Enteric viruses infect the gastrointestinal tract of humans and other warm blooded animals, which can enter surface water systems through the discharge of sewage contaminated water leading to the potential spread of water-related diseases (Bosch, 1998). Adenoviruses, enteroviruses, hepatitis A and retroviruses are the enteric viruses of concern in water. They cause gastroenteritis and certain strains infect the upper respiratory tract. Infected larvae of *Schistosoma* cause Schistosomiasis, by the infective cercaria, which burrows into the human skin maturing into *Schistosomula* and migrating to specific internal parts of the body, which may include the spleen, lungs, intestines, portal venous system, the bladder, etc. (WHO, 2011). An estimated 207 million people are infected with schistosoma, of which 85% are found on the African continent. *Schistosoma haematobium* and *Schistosoma mansoni* are the two main species of medical importance found in Africa (WHO, 2011).

1.5.1.1 Health Effects

Water serves as an inert carrier of pathogenic microorganisms, which presents potential human health risks. Individuals exposed to disease-causing viruses, bacteria, protozoa and helminths found in drinking and recreational water may develop waterborne disease or infection. The most common illnesses include gastro-intestinal upset, which can occur for short (72 hours) or prolonged time periods depending on the age and health of the infected individuals. Infants, the elderly and the immunocompromised are the most commonly affected and the condition may be severe, chronic or even fatal (Muller and Kfir, 2003). Bacteria (*Shigella*, *Campylobacter, Vibrio,* etc*.)*, viruses (*Norovirus*, *Hepatitis* A virus, etc.) and protozoa (*Giardia*, *Cryptosporidium,* etc*.*) are responsible for severe diarrhoea-related illnesses and some pathogens may affect the lungs, liver, central nervous system, skin or eyes. *Naegleria fowleri* is a protozoa of worldwide concern, causing amoebic meningoencephalitis, which is an infection of the central nervous system mostly affecting children and young adults (Prescott *et al.,* 2005).

Millions of people die annually in developing countries from drinking water contaminated with infectious microorganisms or parasites. Children under the age of five and elderly people are the most commonly infected and in certain cases they may die from diarrhoea-related illnesses. In South Africa, diarrhoea is responsible for 43 000 deaths per year of which approximately 20% is infants (one to five years) (Mackintosh and Colvin, 2003), while it also claims the lives of more than 500 000 infants in India per annum (Hill, 2004). The earthquake which occurred in Haiti in January 2010, disrupted available drinking water sources as potable water was severely contaminated with sewage, which led to a cholera (caused by *Vibrio cholerae*) outbreak. More than 170 000 people across all provinces of Haiti were diagnosed with cholera while more than 3 600 lives were lost by the end of 2010 (Dowell *et al.,* 2011). In 2007, Somalia experienced a major cholera outbreak with approximately 67 000 recorded cases. In 2011 these communities were still facing diarrhoeal outbreaks especially in the displaced populations living in informal settlements in and around Mogadishu, the capital city, where there is unsafe domestic water supplies and inadequate sanitation facilities (UNICEF, 2011). A major cholera epidemic also occurred in Zimbabwe from mid 2008 to mid 2009, with approximately 100 000 cholera cases and 4 000 deaths recorded, mainly due to insufficient clean water supplies and poor sanitary facilities (Reyburn *et al.,* 2011).

There is thus a need for the implementation of a national surveillance system to efficiently monitor waterborne outbreaks in South Africa and other developing countries, as diarrhoea in infants and young children still contributes to the highest number of mortality and

morbidity rates in South Africa, and very little recent data can be used to establish the impact on the whole population group (Sooka *et al.,* 2004).

1.5.2 Indicator Organisms

Indicator organisms are microorganisms that are found in large numbers in faecal matter and their presence in a water source usually indicates faecal contamination, which implies that the water is not safe to drink prior to disinfection. These harmless organisms of the faecal flora are continually excreted and persist longer in contaminated water (Okpokwasili and Akujobi, 1996). The presence of indicator microorganisms (which may not necessarily be pathogenic) indicates that other harmful microorganisms (pathogens), which may be more difficult to detect, might be present. A microorganism should thus meet specific criteria as outlined in **Table 1.3** in order to be regarded as an indicator organism (Prescott *et al.,* 2005; Myers *et al.,* 2007).

Table 1.3. Properties of an ideal indicator organism (Prescott *et al.*, 2005; Myers *et al*., 2007)

- It must be present in faecal material of humans or animals in large numbers.
- Should be suitable for the analysis as an indicator organism in water obtained from various water sources: ground, rivers and streams, ocean and sea, estuaries, tap and wastewater.
- Be present along with the pathogen of concern and not present in clean or treated uncontaminated water.
- It must be used as a surrogate for a variety of possible pathogens: Should coexist with pathogens.
- Must behave similar to the pathogen of concern when exposed to certain chemical or physical treatment process.
- Should be detected by simple and cost-effective techniques.
- It must have a high indicator pathogen of concern ratio in contaminated water.

An ongoing debate continues regarding which organisms should be used as general indicators of contamination, as currently it is suggested that no strong connection exists between indicator organisms and the pathogenic microbes they are supposed to indicate (Meay *et al*., 2004). Coliform bacteria are the dominant indicator organisms used for fresh water (Buckalew *et al.*, 2006) and currently total coliforms, faecal coliforms, *E. coli* and *Enterococci* are bacterial indicators used in water quality and health risk assessment. *Escherichia coli* and faecal coliforms are used as indicator organisms because they can be detected and enumerated by simple and more cost-effective techniques used to detect the pathogens themselves [American Public Health Association (APHA), 1998; Scott *et al*., 2002]. Enterococci is widely used to detect faecal contamination in brackish and marine waters but not in salt water, as they tend to die at a slower rate than faecal coliforms in salt water (Prescott *et al.,* 2005). The presence of an indicator organism thereby leads to further extensive water testing for the presence of possible pathogens.

1.5.2.1 Coliform Bacteria

Coliforms represent a large group of bacteria that belong to the *Enterobacteriaceae* family and have been defined as aerobic or facultatively anaerobic, Gram-negative, non spore-forming rods. They convert carbohydrates into lactic acid within 48 hours at 35-37ºC with gas production by the Multiple Tube Fermentation (MTF) technique. With advances in bacterial analyses, the presence of β-galactosidase enzyme in the members of the *Enterobacteriaceae* family is said to be specific for the coliform group (APHA, 1992). Some of the *Enterobacteriaceae* family members, who share similar coliform characteristics and are largely found in faecal matter, include the genera *Shigella, Salmonella* and *Serratia* amongst others. Coliforms come from two distinct sources namely the gastrointestinal tract of humans and other warm blooded animals (faecal origin) and from within the natural environment (non-faecal origin) (Prescott *et al.,* 2005).

General coliform testing was prescribed for determining the sanitary integrity of potable water in distribution systems, even though some coliforms have been found to be widespread in nature and do not originate from the intestinal tract of warm blooded animals (Craun, 1986; Kong *et al.,* 2009). Extensive research suggests that the presence of coliforms in drinking water may also be associated with pathogenic microorganisms but not metal contaminants (APHA, 1992; Low, 2002; Barnes *et al*., 2004; Myers *et al.,* 2007; Kong *et a*l., 2009). The coliform group of bacteria has been used as the bacterial pollution indicators in various sources, however coliforms can originate from various environments. Total coliforms proliferate in the soil, water or vegetation at low levels and are considered relatively harmless. Typical coliform genera include *Escherichia*, *Klebsiella, Enterobacter, Citrobacter, Serratia,* amongst others (Prescott *et al.,* 2005). The total coliform group is composed of approximately 60% to 90% faecal coliforms, while faecal coliforms are approximately 90% *Escherichia* species (APHA, 1992). Faecal coliforms are a subgroup of the total coliform group, which can be differentiated by their ability to grow at an elevated temperature of 44.5ºC. The thermotolerant characteristic of faecal coliforms, which is found in the gastrointestinal tract of warm blooded animals, distinguishes them from other coliforms (Craun, 1986; Myers *et al.,* 2007). Faecal coliforms constitute approximately 10% of microorganisms found in the intestinal tract of warm blooded animals including humans, and is used as an indicator organism for drinking, recreational and irrigational water (Prescott *et al.,* 1993). The human gastrointestinal tract harbours many microorganisms, which include *E. coli, P. aeruginosa, P. fluorescens, Klebsiella pneumoniae, Enterococcus faecalis, Shigella sonnei, Vibrio cholerae* and *Salmonella typhimurium.* These organisms can then be used as indicators for the faecal contamination of water or food products. Various research groups developed conventional PCR methods, which could be used to

identify each group of bacteria from various samples by the use of species specific primers targeting specific genes found in *K. pneumoniae*, *S. marcescens*, *S. sonnei*, *S. typhimurium*, *Micrococcus luteus*, *P. aeruginosa*, *P. fluorescens*, *E. coli* and *Bacillus cereus* (Liu *et al.,* 2008*;* Zhu *et al.,* 2008*;* Hsu *et al.,* 2007*;* Yeh *et al.,* 2002*;* Mukamolova *et al.,* 2002*;* Spilker *et al.,* 2004*;* Scarpellini *et al.,* 2004*;* Kong *et al.,* 1999*;* Manzano *et al.,* 2003).

1.5.2.1.1 *Escherichia coli*

Escherichia coli is a motile, non-spore forming, thermotolerant coliform, which utilises tryptophan to produce indole at a temperature of 44.5±0.5ºC within 24 hours. It is methyl-red positive, unable to produce acetyl-methyl carbinol and cannot utilise citrate (Blount *et al.,* 2008). It is the most abundant coliform in the intestinal flora of humans and other warm blooded animals and its presence in water is strongly associated with faecal contamination.

Escherichia coli is regarded as a more specific indicator than faecal coliforms, since the more general test methods for faecal coliforms also detect thermotolerant bacteria from the environment. The United States Environmental Protection Agency (USEPA) proposed that *E. coli* indicates faecal contamination better than faecal coliforms for the purpose of evaluating ambient fresh water quality (USEPA, 1986; An *et al.*, 2002). However, due to the low numbers and low infectious doses of protozoans and viral pathogens found in water, *E. coli* may not be an appropriate indicator for these pathogens. It should also be noted that the absence of *E. coli* in water sources does not necessarily indicate the absence of disease-causing organisms and for many of these pathogens, no suitable microbiological indicators are currently known.

Although most strains of the *E. coli* group are non-pathogenic members of the normal intestinal flora, some can cause various health-related illnesses such as urinary tract infections, diarrhoea, respiratory illnesses and pneumonia, by several mechanisms. Lateral gene transfer from pathogenic microorganisms to *E. coli* can also lead to the emergence of new pathogenic strains of *E. coli* (Sooka *et al.,* 2004). Pathogenic strains of *E. coli* can cause different types of diarrhoeal diseases due to the presence of specific genes associated with pathogenicity, colonisation factors and other virulence factors that are generally absent in non-pathogenic strains of *E. coli.* Depending on the virulence factors concerned, pathogenic strains of *E. coli* have been classified into Enteroaggregative *Escherichia coli* (EAEC), Enterohaermorrhagic *Escherichia coli* (EHEC), Enteroinvasive *Escherichia coli* (EIEC), Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC) and the recently proposed group Diffuse adhering *Escherichia coli* (DAEC), which has not

been significantly established (Hunter, 2003; Sooka *et al.,* 2004; Prescott *et al.,* 2005; O'Sullivan *et al.,* 2007).

1.5.2.1.1.1 Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* is regarded as a major cause of *E. coli*-mediated diarrhoea in humans worldwide. It is usually related to two major clinical syndromes namely, weaning diarrhoea among impoverished children in developing countries, and traveller's diarrhoea, which is common among people of all ages visiting parts of the world with inadequate sanitation infrastructure. Upon the ingestion of food or water containing ETEC, symptoms such as cramps, vomiting, and profuse diarrhoea are experienced within 10 to 72 hours and can persist for up to five days. This *E. coli* strain has important implications in the agricultural industry where it is a major pathogen of animals such as cattle and weaning piglets (Daniels, 2006). Enterotoxigenic *E. coli* strains associated with humans induce mild or severe watery diarrhoea without pus, mucus or blood (Kothary and Babu, 2001) by producing a heat labile enterotoxin (LT1), which resembles the cholera-toxin in structure and heat stable enterotoxins (ST1a and/or ST1b). Contaminated seafood and salads are the most common vehicles of infection in outbreaks of ETEC infections. Enterotoxins produced by ETEC stimulate the secretion of chlorides and inhibition of sodium absorption thereby leading to watery diarrhoea (O'Sullivan *et al.,* 2007). In a study conducted by Geyer *et al. (*1993) at Ga-Rankuwa hospital (Gauteng province, South Africa) it was shown that 38.5% of paediatric patients diagnosed with gastroenteritis had ETEC, which was mostly due to the consumption of contaminated water and food.

1.5.2.1.1.2 Enteropathogenic *Escherichia coli*

Enteropathogenic strains of *E. coli* adhere to the brush borders of the intestinal epithelial cells, thereby causing effacing lesions. The EPEC induce profuse watery, sometimes bloody diarrhoea with mucus, fever and dehydration. Bloody diarrhoea is associated with the attachment of the EPEC strain to the epithelial cells of the intestines leading to its physical alteration by the formation of attaching and effacing lesions, which interfere with the host cell signal transduction (O'Sullivan *et al.,* 2007). Virulence genes are located on the EPEC adherence factor (EAF) and enables the localised adherence of bacteria to intestinal walls with no toxins produced (Todar, 2008). The destruction of the epithelial cells leads to subsequent diarrhoea and it is more common in children residing in developing countries (Prescott *et al.,* 2005). Food and water contaminated with EPEC are known sources of infection, with raw beef and chicken being the most implicated food groups in outbreaks of EPEC. A study conducted in Shongwe hospital (Mpumalanga province, South Africa),

between February 1985 and January 1986 on paediatric patients with diarrhoea, revealed that 27.6% of cases were caused by the EPEC strain (Weggerhof, 1987).

1.5.2.1.1.3 Enteroinvasive *Escherichia coli*

The EIEC strains cause a syndrome that is similar to shigellosis with high fever and profuse diarrhoea (Todar, 2008). It causes severe diarrhoea by penetrating and multiplying within the intestinal epithelial cells producing an enterotoxin or cytotoxin that destroys the enterocytes of the colonic mucosa (Prescott *et al*., 2005). The EIEC have the ability to introduce themselves into epithelial cells, disseminating from cell to cell by the use of genes clustered on a 220 kb invasion plasmid, which is also present in *Shigella* species. The expression of a variety of plasmid-encoded proteins is mandatory for complete virulence of EIEC and these invasion plasmid antigens (Ipa) are encoded in the Ipa operon (O'Sullivan *et al.,* 2007). Isolates are non-motile, lactose-negative and lysine decarboxylase-negative. Enteroinvasive *E. coli* has also been implicated in food-borne outbreaks as the causative agent of diarrhoea amongst travellers. Previous studies conducted by Taylor *et al*. (1986) and Kalnauwakul *et al.* (2007) in Thailand showed that *E. coli* was the enteropathogenic bacteria isolated most often from stool samples of diarrhoeal patients, with EIEC constituting 5% and 0.2% of the infections, respectively.

1.5.2.1.1.4 Enteroaggregative *Escherichia coli*

Enteroaggregative *E. coli* strains have been recognised as agents of diarrhoea in industrialised and developing countries. Many of the EAEC strains attach to Hep-2 cells in an aggregative adherence pattern, which lead to cytotoxic effects on the intestinal mucosa. They are associated with watery diarrhoea in young children residing in developing countries (Prescott *et al*., 2005). The EAEC is differentiated from other strains of *E. coli* by the presence of Aggregative Adherence Fimbriae (AAF) encoded for by *aggR* genes, which may cause acute or chronic diarrhoea in children. The EAEC produces an enteroaggregative heat-stable toxin, which is similar to the heat-stable enterotoxin produced by ETEC. It is encoded on a plasmid by *astA* genes and this toxin is thought to be responsible for the symptoms of infection (O'Sullivan *et al.,* 2007).

Wanke *et al.* (1998) revealed that EAEC was more frequently found in HIV-positive patients' stool with diarrhoea and they appeared to be symptomatic when the HIV disease was more advanced. It has also been identified as a major cause of traveller's diarrhoea in Mexico, Jamaica and India. A study conducted in these three regions revealed that EAEC was the second most common pathogen isolated from patients with diarrhoea (Adachi *et al.,* 2001).

1.5.2.1.1.5 Enterohaemorrhagic *Escherichia coli*

Enterohaemorrhagic *E. coli* is a worldwide cause of many infections in animals and humans. In certain animals, no symptoms are present, while in humans infections may range from mild diarrhoea to haemorrhagic colitis, haemolytic uremic syndrome (HUS) or death. The EHEC is also known as Shiga toxin-producing *E. coli* (STEC), Verotoxin- or Verocytotoxin-producing *E. coli* (VTEC). It is differentiated from other *E. coli* strains by its ability to produce a verocytotoxin/shiga toxin and also possesses a 60-megadalton (MDa) EHEC plasmid, which enables it to form attaching and effacing lesions on epithelial cells. A wide range of warm blooded animals harbour EHEC, but cattle are found to be the main reservoir. Enterohaemorrhagic *E. coli* produces one or more of the shiga-like toxins (*stx* I and *stx* II), which cause diseases in humans by inhibition of protein synthesis of host cells, leading to cell death. Some strains also form attaching and effacing lesions, which are characterised by the destruction of brush border microvilli and the attachment of the bacteria to the enterocyte membrane forming a cup-like pedestal (O'Sullivan *et al.,* 2007). The occurrence of potential EHEC in processed and unprocessed water from surface water sources in most towns of India has been found to be a significant health concern, as they depend on this water for domestic and drinking purposes. Recently, the insufficient treatment of surface waters utilised for drinking purposes, malfunctioning of municipal sewage systems and the inefficient water dispersion pipelines, have led to contamination of potable water by faecal bacteria in India (Ram *et al.*, 2008).

The EHEC infection occurs mainly via the faecal-oral route and the O157:H7 is the most dominant strain, which is associated with eruption and sporadic disease cases by producing a variety of clinical symptoms including diarrhoea, haemorrhagic colitis and HUS. Waterborne transmission of EHEC mainly occurs through swimming in contaminated water, direct contact with faecal matter in recreational waters and also drinking contaminated water. *Escherichia coli* O157:H7 is a major cause of mortality and morbidity among children in developing countries such as South Africa (WHO, 1997). The World Health Organisation Consultations and Workshops (1997) identified three cases of O157:H7 in South Africa since 1988. In the agricultural sector of South Africa, there were also ten isolates of EHEC, which were obtained from pigs with haemorrhagic colitis (WHO, 1997). An outbreak of *E. coli* O157:H7 also occurred in a water supply system in Canada (Walkerton's small farming community) resulting in seven deaths with 2300 illnesses (Hrudey *et al.,* 2003). The Northern parts of Germany also experienced a large HUS and bloody diarrhoea outbreak, which was caused by a rare O104:H4 shiga-producing *E. coli* strain, leading to 39 deaths and about 3222 recorded cases by mid-June 2011, which spread across Europe (Frank *et al.,* 2011). The O104:H4 strain was detected on bean sprouts and it was also

found to be affecting mostly women, unlike the other pathogenic bacteria, which affects children, the elderly and immunocompromised (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011).

1.5.2.2 Enterococcus

Enterococcus species are a group of bacteria found in the intestinal tract of warm blooded animals. In a study conducted by the United States Environmental Protection Agency (USEPA) (1986), the presence of enterococci in both marine and freshwater samples had a very high correlation with the presence of human pathogens. The USEPA therefore recommended enterococci as the best indicator of health risk for marine recreation waters. Enterococci have been found to be directly correlated to swimmer-associated gastroenteritis, and this has led to the concept of using it as an alternative to *E. coli* for freshwater monitoring. As a result of the Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000, the USEPA mandated that state governments and authorised bodies adopt enterococci bacteria, a subgroup of the faecal streptococci group, as an indicator of human health risk in coastal recreational waters. Saltwater enterococci standards are based on criteria citing epidemiological studies, which concluded that enterococci counts of 35 Colony-Forming Units (CFU)/100 ml (mean of at least five samples over a 30-day period) and 104 CFU/100 ml (single sample maximum) result in a risk in which 19 of 1 000 people would develop gastrointestinal illness. Since the publication of the study, enterococci bacteria have been used in the USA as an indicator for human health risk (Buda *et al.*, 2003). The *Enterococcus faecalis* account for the majority of human enterococcal infections. Liu *et al.* (2005) developed a PCR method, which could be used to distinguish *E. faecalis* from other bacteria by the use of species specific primers targeting the putative transcriptional regulator gene found in *E. faecalis*.

1.6 Overview of Techniques Employed in Water Analysis

Numerous methodologies are currently available to monitor the quality of raw and treated water sources and the efficiency of treatment and disinfection processes. The three methods predominantly used for the routine detection and enumeration of microorganisms in the water industry, include the Multiple Tube Fermentation (MTF) and Membrane Filtration (MF) techniques as well as the Colilert 18® system (Prescott *et al.,* 2005). The MTF is commonly used to determine the Most Probable Number (MPN) of gas-producing bacteria in food products, soil and water (Food and Drug Administration, 2001). Goetz and Tsuneishi then developed the Membrane Filtration (MF) technique in 1951 and used it to detect indicator

organisms in water. Differential media used in the MF technique depends on the indicator organism being detected and varies from M-Endo medium to Membrane Lactose Glucuronide Agar (MLGA), which is being used by various municipalities such as the Paarl Municipality in the Western Cape (South Africa). This technique was found to be more accurate, as well as time-, labour- and cost-effective for the routine testing of drinking water (Buckalew *et al*., 2006), and was consequently used as an alternative to the MTF technique. The Colilert 18® system has been found to be cost-effective to perform routine analysis, with the final results being obtained after 18 hours of incubation without any requirement for a confirmation step (Niemela *et al.,* 2003). Current techniques used to identify indicator organisms in water and food are however, time consuming, labour intensive, and are often not sensitive enough to obtain accurate results. Assessment methods for the detection and enumeration of indicator organisms such as faecal bacteria and *E. coli* in water, food, wastewater effluents and soils have thus evolved from the Multiple Tube Fermentation technique (MTF) to the Polymerase Chain Reaction (PCR) (Buckalew *et al.*, 2006).

Different media composed of essential nutrients promote the growth of specific organisms. These types of media also contain reagents that inhibit the growth of non-target organisms. In addition, the media may contain indicators which allow the target organism to be easily identified, often by a colour change in the medium. For instance, MacConkey (with crystal violet) (Biolab Diagnostics, SA) allows for the growth of Gram-negative organisms, Chromocult Coliform Agar (CCA) (Merck, Germany) allows for coliform and *E. coli* growth, while Violet Red Bile (VRB) agar (Biolab Diagnostics, SA) also allows for the growth of Gram-negative microorganisms. However, there are only a few selective media available for the cultivation of specific microorganisms, and limitations encountered include length of incubation, interference of competitive organisms non-specific to the coliform group and a poor level of detection of sluggish or strained coliforms.

The PCR technique, which was developed between 1983 and 1985 to synthesise large quantities of Deoxyribonucleic Acid (DNA) without cloning (Mullis, 1990; Prescott *et al.,* 2005), is an extremely sensitive technique as it can detect one Colony Forming Unit (CFU) of indicator organism per 100 ml of water. The PCR technique thus amplifies minute genetic material samples within a few hours, allowing for the rapid and reliable detection of genetic markers in each microorganism present in the sample (Mullis, 1990; Valasek and Repa, 2005). Conventional PCR involves the replication of single specific genetic material (Toze, 1999), while multiplex PCR enables the detection of multiple genes from the same or different organisms, by the use of multiple primer sets in a single reaction tube to produce amplicons of various sizes in samples of different origins (Tantawiwat *et al.,* 2005). Real-time PCR measures and distinguishes between specific

nucleic acid sequences from a variety of samples or within a sample, while also enabling the quantification of microbial loads (Valasek and Repa, 2005).

1.6.1 Enumeration Techniques

1.6.1.1 Multiple Tube Fermentation Technique

The MTF [also known as Most Probable Number (MPN)] has been used for over 80 years to determine the most probable number of gas-producing microorganisms that utilise lactose in liquid media in food products, soil and water (Food and Drug Administration, 2001; Rompre *et al.,* 2002). Each group of microorganisms requires a different type of medium; for example, when enumerating coliforms, lauryl tryptose broth (Merck, Darmstadt, Germany) is used as a preliminary presumptive test (Prescott *et al.,* 2005). In the second (confirmation) part, Brilliant Green Bile (BGB) broth (Merck, Germany) is used to confirm faecal coliforms with tryptone water used (Biolab diagnostics (Pty) Ltd, South Africa) for *E. coli*. In a study conducted on the Plankenburg-, Berg- and Diep Rivers (Western Cape, South Africa) (Barnes, 2003; Paulse *et al.,* 2007), the MTF was used to detect and enumerate faecal coliforms and *E. coli* in river water samples. The results obtained in these studies significantly exceeded ($p \leq 0.05$) the maximum level of microbial numbers as stipulated by DWAF (1996) and the SABS (1984). The MTF was also compared to the Membrane Filtration (MF) and the Defined Substrate Technology (DST) media in the identification of waterborne coliform bacteria, *E. coli* and Enterococci from a variety of water sources in Southern Sweden (Eckner, 1998). Results showed that the Colilert method was more sensitive in the detection of *E. coli* in drinking water. However, the MTF was best in enumerating cells in highly turbid water samples (Eckner, 1998).

1.6.1.2 Membrane Filtration Technique

Goetz and Tsuneishi projected and developed the Membrane Filtration technique (MF) in 1951 and applied it to water testing (Buckalew *et al*., 2006). It is mainly used for routine testing of natural and drinking water and also wastewater from various industries (APHA, 1998). It is widely used on waters that have low levels of non-coliform microorganisms, heavy metals and turbidity (Prescott *et al.,* 2005). The MTF and MF techniques are ideal reference techniques used for water testing by municipalities and food industries to assess the effectiveness of treatment technologies in eliminating microorganisms in products intended for human consumption. Membrane filtration is considered better than the MTF when a resuscitation medium is used prior to filtering the sample and when culturing on selective medium (Prescott *et al.,* 2005).

However, the set back with this technique is the amount of time taken to obtain the final results, which requires at least 48 hours to complete the confirmation of typical colonies (Yanez *et al.,* 2006). The commonly used media in the MF technique are m-Endo and m-FC media in the US and Canada, respectively (APHA, 1998) and Tergitol-TTC medium in Europe. Most of the media used in MF technique lack specificity and coliform confirmation is required, which further delays the results by two to three days. In addition the presence of antagonistic heterotrophic bacteria has shown to reduce the recovery of coliform bacteria (Hill, 2004).

Turco and Nienow (2006) used the Membrane Filtration method to identify *E. coli* and *Enterococcus* species from river water. The modified m-TEC agar used contained sodium lauryl sulphate and sodium desoxycholate, which in turn inhibits the growth of Gram-positive bacteria. *Escherichia coli* and *Enterococcus* colonies were transferred onto a nutrient agar slant respectively and into a tube of tryptose soy broth for verification. A colony was verified as *E. coli* if the bacterium was cytochrome Oxidase-negative, produced gas in *Escherichia coli* broth (EC broth), did not utilise citrate and was indole positive (Turco and Nienow, 2006). These enzymes were selected as indicators as conventional coliform monitoring is based on the detection of β-galactosidase and because the gene encoding the β-glucuronidase enzyme (uidA) was found to be specific for *E. coli* identification. In a study conducted by Maheux *et al.* (2008) the four commercial β-galactosidase and β-glucuronidase-based test media [Colilert, Readycult, Chromocult and [4-Methylumbelliferyl-β- D-galactopyranoside (MUGal) and Indoxyl-β-D-glucuronide (IBGD) (MI agar)] were compared using a collection of bacteria representing strains of different geographical origins and serotypes obtained in both faecal and environmental settings. The results showed that all the media were highly specific in the identification of environmental total coliforms (Maheux *et al.,* 2008). Fricker *et al.* (2008) used MLGA to detect coliforms and *E. coli* in natural waters and compared it with the Colilert 18® system, Chromocult agar, MI agar and coliscan medium. The medium failed to detect 15.6% of cultures that were positive for β-D-glucuronidase activity when compared to the other media. It showed a poor sensitivity in the detection of β-D-glucuronidase activity in *E. coli* isolated from contaminated water because of its pH sensitivity and acid produced from fermentation by *E. coli* from the lactose present in MLGA (it is found to have a higher source of fermentable lactose when compared to other media, resulting in significant amounts of acids during fermentation thereby reducing the pH). Membrane lactose glucuronide agar contains lauryl sulphate (which inhibits Gram-positive organisms), 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (BCIG) (which is cleaved by enzyme glucuronidase found in most of *E. coli* to give a distinctive green colour) and lactose and

phenol red (which is utilised by all coliforms releasing acid and yields yellow coloured colonies).

1.6.1.3 Colilert 18® System

The Colilert 18® system (IDEXX Laboratories, USA) is an example of defined substrate technology medium that utilises two substrates namely, 4-methylumbelliferyl-β-D-glucuronide (MUG), which screens for β-D-glucuronide from *E. coli* and o-nitrophenyl-β-D-galactopyranoside (ONPG), which screens for β-D-galactosidase from coliforms and *E. coli*. The Colilert 18® system has been certified by the United States Environment Protection Agency (USEPA) as a viable method for the microbial assessment of surface waters (USEPA, 2003). Coliforms and *E. coli* have the enzyme β-D-galactosidase, which enables these microorganisms to degrade ONPG yielding o-nitrophenol which appears yellow on the media. *Escherichia coli* has an additional enzyme β-D-glucuronide, which degrades MUG resulting in the fluorescent product 4-methylumbelliferone, thereby making it easier for the determination of coliforms and *E. coli* from the same source simultaneously. Eckner (1998) concluded that it was easier to use the Colilert system, as it saves time by eliminating the confirmation step.

Edberg *et al.* (1990) used the Colilert system in the analysis of water from natural water resources and found it to be as sensitive as the MTF method. In this study the efficiency of the Colilert system was compared to standard methods such as the MTF for the detection and enumeration of total coliforms and *E. coli* from surface water (Edberg *et al.*, 1990). The Colilert system was found to be similarly sensitive to MTF testing by chi-square, regression, t-test, and the fraction analyses. It was then concluded that the Colilert system could be used for the enumeration of source water samples as the benefits previously described for the distribution of water testing sensitivity included specificity, time- and cost-efficiency. The technique can also be used by persons with minimal training and will still provide reliable results (Kampfer *et al*., 2008).

The Colilert 18® system detects coliforms, which originate from faeces and the environment in general. It provides rapid (18 hours) results, and has been reported to detect coliforms and *E. coli* in potable water samples with a higher degree of accuracy (Pisciotta *et al*., 2002; Kampfer *et al*., 2008). The Colilert 18® system gives higher total coliform counts than the traditional methods (Kampfer *et al*., 2008), for *E. coli* and total coliforms in fresh water as many Gram-negative and Gram-positive bacteria, besides *E. coli,* produce β-galactosidase. It can be employed for waters with high turbidity and allows greater resuscitation of coliform microorganisms.

1.6.2 Culturing Techniques

1.6.2.1 MaConkey Agar

MacConkey agar was the first solid differential and selective media formulated for the isolation and differentiation of bacteria belonging to the family *Enterobacteriaceae* from faeces, urine, foodstuffs and water (Allen, 2010). MacConkey agar contains crystal violet and bile salts, which inhibit the growth of Gram-positive and fastidious Gram-negative bacteria, which include *Neisseria* and *Pasteurella*. In addition, it has neutral red and lactose, which enables the differentiation of lactose fermenters within the Gram-negative bacteria group (Allen, 2010). In a study conducted by Finney *et al*. (2003), chromocult coliform agar (CCA) was compared with MacConkey agar for the isolation and enumeration of *Enterobacteriaceae* from human faecal samples. The results showed that the CCA medium was as effective as MacConkey and it had an advantage that it had the ability to differentiate *E. coli* from other coliforms.

1.6.2.2 Violet Red Bile Agar

The presence of crystal violet and bile salts in Violet Red Bile agar enables the inhibition of Gram-positive bacterial flora. Fermentation of lactose in VRB results in the acidification of the medium which is indicated by the colour change and precipitation of bile acids around colonies. Mahale *et al.* (2008) used VRB agar in the enumeration of coliforms from street vended fruit juices in India. The effectiveness in the recovery of stressed cells of *Enterobacter sakazakii* in infant milk was also tested for and results obtained showed that those bacterial cells could not be resuscitated (Al-Holy *et al.,* 2008).

1.6.2.3 Chromogenic Media

Chromogenic media are defined as microbiological growth media that contain enzyme substrates associated with a chromogen, which yields a clear distinguishable colour, thereby also allowing for clear differentiation and identification. There are two major groups of chromogenic media and these include chromocult (i.e. agar) and flourocult (i.e. broth) media (Yanez *et al*., 2006). In chromocult coliform agar, red and salmon colonies are counted as coliforms while the blue colonies represent *E. coli*. In spite of its advantages, the main hindrance of chromogenic media is the cost for routine analyses where a large number of samples are analysed (Yanez *et al*., 2006). Methods based on the enzymatic properties of coliforms have been developed and these include Colilert (IDEXX laboratories, Westbrooks, ME, USA), Readycult coliforms 100 (Merck KGaA, Darmstadt, Germany), Chromocult

Coliform Agar Enhanced Selectivity (Merck, Germany) and MI agar [a combination of 4-methylumbelliferyl-D-galactopyranoside (MUGal-flourogen) and Indoxyl-D-glucuronide (IBDG-chromogen)] agar (Becton, Dickson and Company, USA).

1.6.2.3.1 Chromocult Coliform Agar

Chromocult Coliform Agar is one of the chromogenic media commonly used for the determination of total coliforms and *E. coli* in potable water and food. Rapid growth of colonies and the recovery of injured coliforms are authenticated by the interaction of selected peptones, pyruvate, orbital and phosphate buffer. The growth of Gram-positive as well as non-target Gram-negative bacteria is largely inhibited by the addition of Tergitol 7, which does not interfere with the growth of the coliform bacteria. The substrate X-glucuronide is also used for the detection of β-D-glucuronide, which is typical of *E. coli*. *Escherichia coli* cleaves salmon-GAL and X-glucuronide and positive colonies assume a dark-blue to violet colour. These are well differentiated from the rest of the coliforms, which display a salmon to red colour (Finney *et al*., 2003). Byamukana *et al*. (2000) used chromocult coliform agar to quantify *E. coli* in contaminated stream water. This media proved to be efficient and feasible for the determination of faecal pollutants within 24 hours after sampling.

1.6.3 Identification Techniques

1.6.3.1 API 20 E System

The API 20 E system is a standardised method using miniaturised biochemical tests for the identification of *Enterobacteriaceae* species. It has been widely used for the identification of pathogens isolated from agricultural products and water samples (Sundram *et al.,* 2000; Sabae and Rabeh, 2007). Sabae and Rabeh (2007) used the API 20 E system to identify microorganisms of the family *Enterobacteriaceae* along the Nile River at the Damietta branch in Egypt to evaluate the microbial water quality. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the most prevalent organisms constituting 16%, 14% and 12%, respectively of all the bacteria isolated and identified using the API 20 E system (Sabae and Rabeh, 2007). All the microorganisms were grown on nutrient agar and incubated at 37°C for 18-24 hours before being inoculated onto the API 20 E test strips.

1.6.3.2 RapID™ ONE System

The RapID™ ONE system is a qualitative method which uses both conventional and chromogenic substrates for the identification of *Enterobacteriaceae*, other Oxidase-negative

and Gram-negative rods. The system is based on the ability of microorganisms to degrade specific substrates detected by various indicator systems. According to Kitch *et al.* (1994) the RapID™ ONE system can be used to identify Oxidase- and Gram-negative microorganisms belonging to the family *Enterobacteriaceae* to the species level without additional tests. Valenzuela *et al.* (2009) used the RapID™ ONE and RapID NF systems for the biochemical identification of enteric bacteria in the assessment of the microbiological contamination of groundwater in the rural area of Chile. The rapid systems enabled the identification of 123 strains that were isolated from groundwater sources, and results showed that there were abundant *Enterobacteriaceae* species present during the rainy season (Valenzuela *et al.,* 2009).

1.6.3.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular based technique which copies and amplifies DNA from various sample sources with the use of DNA polymerases (Powledge, 2004). It was developed by Kary Mullis in the 1980s, and it enables the production of a large number of copies of a specific nucleic acid segment. This technique is highly sensitive which makes it suitable for the identification of a range of microorganisms from a single water sample. In addition PCR can specifically detect a desired microorganism within mixed populations in water sources without culturing (Mullis, 1990; Prescott *et al.,* 2005). Molecular based techniques have also been utilised for testing of different water sources by targeting the 16S rRNA region in coliforms (Prescott *et al.,* 2005; Khan *et al.,* 2007). Thus the PCR technique serves as a substitute for detecting microbial indicators in water and food samples based on the isolation of DNA and RNA from target microorganisms (Tantawiwat *et al*., 2005). There are a variety of PCRs which include conventional PCR, multiplex PCR and real-time PCR which can also be used for the identification of *Enterobacteriaceae* species from environmental samples.

Various PCR methods have been previously developed to detect and quantify *E. coli* from environmental samples (Ibekwe *et al.,* 2004; Heijnen and Medema, 2006; Kong *et al.,* 2009). Ibekwe *et al*. (2004) used real-time PCR for the identification and quantification of *E. coli* O157:H7 from soil and leaf surfaces of irrigated plants and they further suggested that it can be a useful technique to quantify O157:H7 in water used for irrigation. Heijnen and Medema (2006) also used real-time PCR in quantifying the O157:H7 strain in wastewater and the results proved that real-time PCR was more sensitive than culture based methods. Kong *et al*. (1999) developed a multiplex PCR and successfully used it to detect *E. coli* and three pathogenic strains (ETEC, EHEC and EPEC) from sea water samples.

1.6.3.3.1 Conventional Polymerase Chain Reaction

Conventional PCR is a technique that allows for the quick replication of a single specific gene by enabling the rapid and accurate detection of genetic markers e.g. a specific microbial pathogen in a single reaction tube (Toze, 1999). The PCR method does not distinguish between a viable and non-viable organism, which implies that it is sensitive and will detect all organisms present in a sample of water. Conventional PCR was used to detect heat-stable and heat-labile enterotoxin genes found on the Enterotoxigenic *E. coli* (ETEC) strain isolated from domesticated animals (Ahmadi *et al.,* 2009). Results indicated that 36% of the *E. coli* possessed heat-labile genes, while no heat-stable genes were detected. Paulse *et al*. (2009) used universal primers in the conventional PCR to detect and identify bacterial pollutants from the Plankenburg- and Berg- Rivers in the Western Cape, South Africa. The results showed that the primers used amplified the 16S rRNA regions of Gram-negative and Gram-positive bacterial strains found in the contaminated river water.

1.6.3.3.2 Multiplex Polymerase Chain Reaction

Multiplex PCR involves the targeting of multiple genes from the same or different organisms by the use of multiple primer sets in a single reaction tube to produce amplicons of different sizes (Burgart *et al.,* 1992; Edwards and Gibbs, 1994). It has been widely used for pathogen identification from different sources such as clinical, water (including other environmental samples) and food samples (Edwards and Gibbs, 1994; Toma *et al.,* 2003; Tantawiwat *et al.* 2005, Watterworth *et al.,* 2005; Madoroba *et al.,* 2009). Tantawiwat *et al.* (2005) developed a multiplex PCR, which enabled the detection of total coliform bacteria, *E. coli* and *Clostridium perfringens* in potable water. Electrophoresis enabled the detection of 876 bp DNA band representing the *lacZ* gene found in all coliform bacteria, a DNA band of 280 bp and 876 bp for the *uidA* and *lacZ* genes, respectively, of all strains of *E. coli*. The addition of a pre-enrichment step in test samples has been found to be effective, sensitive and rapid for the simultaneous identification of microorganisms in potable water (Tantawiwat *et al.*, 2005). Toma *et al. (*2003) developed a multiplex PCR to identify the five human diarrheoagenic *E. coli* strains and it was found to be successful when DNA samples from human faeces was used. Lorusso *et al.* (2011) developed a multiplex to detect Verocytotoxin-producing *E. coli* in raw ground beef and milk, by detection of the *wzx, stx* I and *stx* II genes. The technique was applied to samples inoculated with the *E. coli* O26 strain, which is positive for the named genes, and also to un-inoculated samples where it was shown to be highly sensitive. The authors concluded that the developed multiplex could

be further applied to clinical and environmental samples for the detection of the same *E. coli* strain, and the major pollutants were successfully identified.

1.6.3.3.3 Real-time Polymerase Chain Reaction

Real-time PCR is a reliable technique for the identification and measurement of amplicons generated during each cycle of the PCR process, which directly corresponds to the starting concentration of the template (Gizinger, 2003; Valasek and Repa, 2005). The main aim of real-time PCR is to measure and distinguish between specific nucleic acid sequences from a variety of samples or within a sample, enabling quantification of microbial loads. It uses intercalating dyes (Sybr green is the widely used dye and the Evagreen dye which is a newly emerging for use in real-time PCR) which binds to the minor groove of double stranded DNA (dsDNA), with the Sybr green absorbing light at 480 nm and emitting light at 520 nm. The fluorescent signal of an intercalating dye increases with the increasing amount of dsDNA, therefore any non-specific amplification of DNA in the reaction mixture will be measured. Real-time PCR distinguishes the amount of DNA before, during and after the reaction by measuring the product formation which correlates with the amount of starting nucleic acid template (Valasek and Repa, 2005). The specificity of dyes can be checked by analysing the dissociation curve to determine the melting curve point which is unique for each gene. If one peak is present it suggests that the amplification was specific to target DNA (Valasek and Repa, 2005).

Gene expression is quantified using the amplification graph curves generated by specific software present in real-time cyclers to help determine the time at which fluorescence reaches a threshold level. It is also called quantitative PCR because of its sensitivity in the detection and quantification of gene expression levels in samples with low nucleic acid concentrations (Pfaffl, 2004). Ibekwe *et al*. (2004) used real-time PCR to identify and quantify *E. coli* O157:H7 from the phyllosphere. It was concluded that it was more sensitive than MUG-based defined substrate tests, which are sensitive enough to detect about 10-fold, while real-time PCR can detect as little as 2-fold change. Apart from providing fast, precise and accurate results, real-time PCR is designed to accumulate information as the reaction is progressing, which is more accurate for nucleic acid quantification and there is no need for laborious post PCR methods.

Absolute and relative quantifications are the two methods used in real-time PCR (Yuan *et al.,* 2006). Absolute quantification involves the use of a positive control of known quantity which is diluted to generate a standard curve and test samples are then compared to this curve. Relative quantification involves the measurement of the steady-state change levels in a gene under investigation in relation to a known control gene. The quantity of

internal standard will be unknown and housekeeping genes must be used so that the quantity of the relative gene is easier to determine. This technique is mostly used on physiological and pathological studies (Yuan *et al.,* 2006).

Absolute quantification accuracy is dependent on the quantity of the known positive control, but inhibitors which may be in the test sample cannot be compensated or detected by the external standard. It is used mostly if the concentration of target nucleic acid is directly proportional to the number of bacteria in a sample. It is currently used in many fields within molecular diagnostics, clinical research, cell culture systems, genotyping knockout, it also facilitates epidemiological studies, can detect microbial load and disease progression and the efficacy of antiviral therapies (Nadkarni *et al.,* 2002; Martinon *et al.,* 2011). Reischl *et al. (*2004) developed a real-time PCR for the identification of the heat labile enterotoxin and the heat stable enterotoxin from ETEC of human origin using a Roche light cycler. They used gene specific primers and hybridisation probes during the reaction and they found the assays to be 100% sensitive and specific for both genes. The hybridisation probe enabled the melt curve analysis to be performed after amplification. The quality of the template used in real-time PCR is the most important determinants of its reliability and reproducibility. Inhibitory components, which are used for nucleic acid extraction or which are simultaneously purified along with the nucleic acids from the samples, decrease the efficiency of real-time PCR (Gizinger, 2003; Guescini *et al.,* 2008).

1.7 OBJECTIVES OF THE RESEARCH

Diagnostic tools applied by municipalities are time- and cost-effective in the identification of *E. coli* and coliform bacteria in river water samples, and can accurately enumerate coliform and *E. coli* cells. An alternative to this hypothesis states that molecular based techniques are faster in the identification of *E. coli* and coliforms from contaminated river water. Accurate identification and enumeration techniques that are time- and cost-effective and are more species sensitive thus need to be implemented for the routine monitoring of water samples in the river systems.

The main aim of the study was to compare the diagnostic tools and molecular based techniques for the rapid identification of *E. coli* and coliforms in contaminated water in the Berg- and Plankenburg Rivers. This aim was achieved by:

a) The collection of five litres of water samples twice a month (for seven months) in the Berg- and Plankenburg Rivers at points closest to the informal settlements of Kayamandi (Plankenburg River) and Mbekweni (Berg River) (July 2010 to January 2011);

b) The detection and enumeration of coliforms and *E. coli* using the Multiple Tube Fermentation technique, Colilert 18® system and the Membrane Filtration techniques. Results obtained for the three techniques were compared based on the number of microorganisms detected and accuracy. In addition the cost and time taken to perform each technique was compared;

c) The culturing of water samples on Chromocult Coliform agar, MacConkey and Violet Red Bile agar and isolating single colonies for preliminary identification using the API 20 E and RapID ONE identification systems;

d) The isolation of DNA directly from river water by firstly concentrating microbial cells and then using the boiling method to extract the nucleic acids;

e) The optimisation and application of conventional PCR in the identification of *E. coli*, *K. pneumoniae*, *E. faecalis*, *S. marcescens*, *S. sonnei*, *S. typhimurium*, *M. luteus*, *B. cereus*, *P. aeruginosa*, *P. flourescens* and *V. cholerae* directly from river water using species specific primers targeting conserved gene regions within these microorganisms;

f) The optimisation and application of multiplex PCR in the identification of EHEC, EPEC, ETEC, EIEC and EAEC from river water, using primers targeting conserved genes in each strain;

g) The optimisation and application of the real-time PCR to identify EHEC, EPEC, ETEC, EIEC and EAEC strains of *E. coli* from river water samples, and ultimately compare the expense, sensitivity and time taken to obtain the final results between multiplex and real-time PCR.

2. MATERIALS AND METHODS

2.1 Sample Collection

2.1.1 Sampling Sites

Sampling was conducted along the Berg- and Plankenburg River systems in the Western Cape, South Africa from July 2010 to January 2011 (samples were collected every two weeks). Based on previous research (Paulse *et al.,* 2007; Paulse *et al.,* 2009) one sampling site was identified along the Plankenburg River (Stellenbosch) location [Site A1 –Informal Settlement of Kayamandi (**Figure 2.1**)] and another sampling site was identified along the Berg River (Paarl) [Site A2 –Plot 8000 at Mbekweni Informal Settlement (**Figure 2.2**)]. These sites were selected as significantly high total cell counts ($p < 0.05$) were obtained throughout the sampling period in the studies conducted by Paulse *et al.* (2007; 2009).

2.1.2 Collection of Water Samples

A five litre water sample was collected by immersing a sterile Nalgene bottle into the river at the point closest to the informal settlements of Kayamandi (Plankenburg River) and Mbekweni (Berg River), respectively, and was transported to the laboratory on ice to maintain a low temperature. The temperature and pH of the river water at the sampling locations were measured using an YSI 100 portable pH/temperature meter (YSI Environmental Inc). The water samples collected from Site A1 (Kayamandi) and Site A2 (Mbekweni), throughout the study period, were analysed using the enumeration techniques (**Section 2.2**), culturing techniques (**Section 2.3**) and identification techniques (**Section 2.5**) outlined below.

2.2 Enumeration Techniques

Diagnostic tools such as the multiple tube fermentation (MTF), membrane filtration (MF) and Colilert 18® system are classical reference methods used by municipalities and food industries to assess the effectiveness of various treatment technologies in eliminating microorganisms in human consumed products (Prescott *et al.,* 2005). These diagnostic tools were used for the detection and enumeration of microorganisms from the Berg- and Plankenburg Rivers as outlined below.

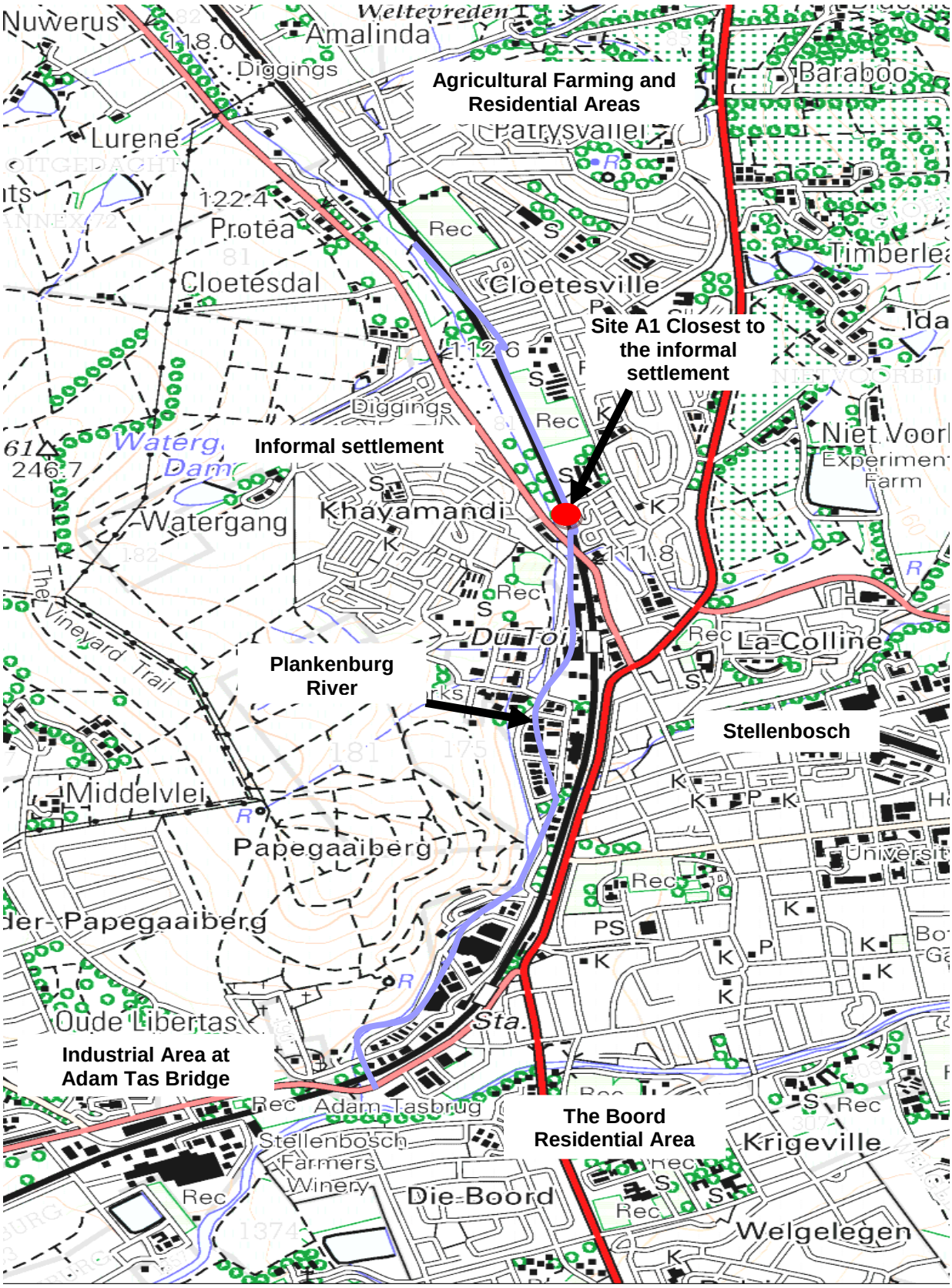

Figure 2.1 Map of the Plankenburg River indicating the sampling point Site A1 - closest point to the informal settlement of Kayamandi (Paulse *et al.*, 2007)

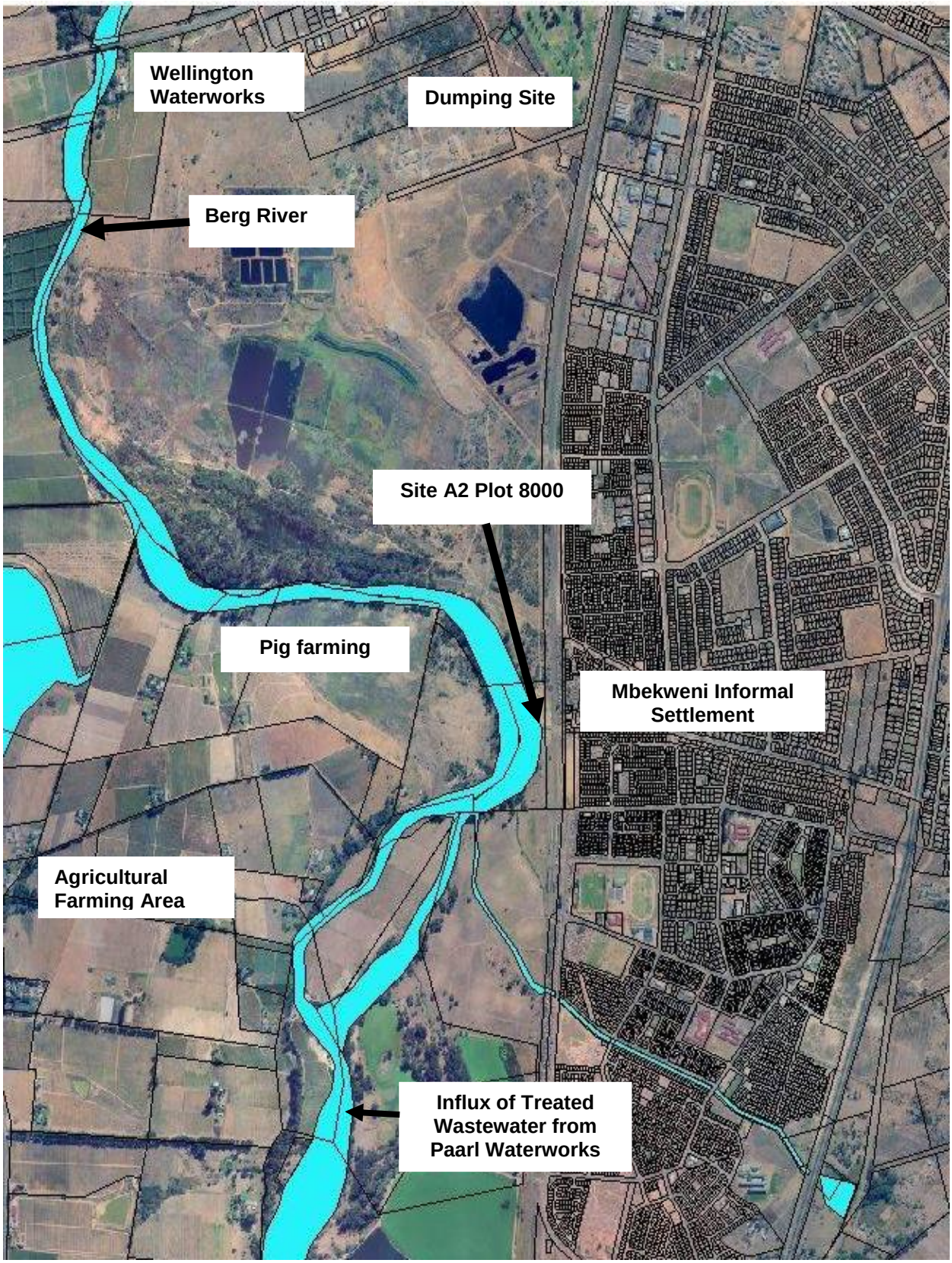

Figure 2.2 Map of the Berg River indicating the sampling point Site A2 (plot 8000) - closest to the informal settlement of Mbekweni (Paulse *et al.*, 2009)

2.2.1 Multiple Tube Fermentation Technique

All the media employed in the multiple tube fermentation technique [Lauryl tryptose broth (LTB) (Biolab Diagnostics, SA), brilliant green bile (BGB) broth (Merck, Germany), tryptone water (Biolab Diagnostics, SA)] were prepared according to manufacturer's instructions. The experimental procedure was performed as previously modified by Barnes (2003) and employed by Paulse *et al.* (2007), by completing a 10^{-1} to 10^{-5} serial dilution of the water samples before inoculation into LTB tubes containing durham tubes (durham tubes enable the visualisation of possible gas production). One millilitre of each diluted and undiluted water sample were inoculated into 5 single strength LTB tubes, while the double strength LTB tubes were inoculated with 5 ml of undiluted samples. The LTB tubes were incubated at 37ºC for 48 hours. All tubes positive for gas-formation, indicative of the presence of gas-producing microorganisms, were regarded as positive presumptive tests and were read off the De Mans tables (American Public Health Association, 1992, 1995). One millilitre of the positive LTB tubes were re-inoculated into BGB broth and tryptone water tubes, respectively, and incubated in a 44.5ºC waterbath for 24 hours. Gas-production in BGB tubes (indicating faecal coliforms) was compared to the growth in the tryptone water tubes (indicating possible presence of *E. coli*). The addition of Ehrlich's reagent into the tryptone water tubes confirmed the presence of *E. coli* when a colour change occurred from clear to pink or red. The number of positive tubes for each dilution was recorded and read off the De Mans tables to obtain the most probable numbers.

2.2.2 Membrane Filtration Technique

The Membrane Filtration technique experimental procedure was performed as stipulated by the Paarl Municipality facility where membrane lactose glucuronide agar (MLGA) (Oxoid Limited, UK) was used as the culturing medium. For the first six sampling dates, water samples were diluted in a sterile 100 ml bottle by adding 50 ml of sterile water to 50 ml sample. This however resulted in the overgrowth on the membrane filter and water samples were then diluted 100 times before filtering for the last seven sampling dates. Subsequently, the diluted sample was filtered through a 47 mm diameter, 0.45 µm nominal pore sized nitro-cellulose based membrane filter (Separations Scientific (Pty) Ltd, SA). The filter was aseptically transferred onto 65 mm petri dishes containing MLGA, incubated at 30ºC for 3 hours and thereafter incubated at 37ºC overnight (18-21 hours). The total number of colonies typical for coliforms and *E. coli* were counted and recorded as per 100 ml after multiplying by the dilution factor.

2.2.3 Colilert 18® System

The Colilert 18® system procedure was performed according to manufacturer's instructions and as utilised by the Paarl Municipality Laboratory. During the first six sampling dates, 100 ml of sample was analysed (Paarl Municipality tested the river water without diluting water sample) and a Colilert-18® snap pack media was added and dissolved by shaking. Due to the consistency in the coliforms and *E. coli* counts obtained for the first six sampling dates, dilutions were subsequently performed for each sample collected in the last seven sampling dates, i.e. a 10X (10 ml water sample and 90 ml sterile distilled water) and 100 times (1 ml water sample and 99 ml sterile distilled water). The contents were then dispensed into a sterile Quanti-tray™ 2000 (IDEXX Laboratories, USA), which was heat-sealed before incubating the trays (upward direction) in a 37°C incubator for 18 hours. After incubation the number of yellow wells visible in normal light (Coliforms) and the number of yellow wells that fluoresce under UV (365 nm) light (*E. coli*) were counted. These counts were then read from the appropriate MPN table and recorded as the number of microorganisms per 100 ml.

2.3 Culturing Techniques

Three selective media were used for culturing *Enterobacteriaceae* species from the contaminated river water. MacConkey (Biolab Diagnostics, SA), Violet Red Bile (VRB) agar (Biolab Diagnostics, SA) and Chromocult Coliform Agar (CCA) (Merck, Germany) were used to culture microorganisms directly from water samples and the subsequent growth of coliforms and *E. coli* was observed.

2.3.1 Culturing of Water Samples

Serial dilutions (10^{-1} and 10^{-2}) were performed in duplicate on the water samples collected. The dilutions were then spread plated directly onto MacConkey, CCA and VRB plates, which were incubated at different temperatures (CCA and MacConkey plates were incubated at 37ºC for 18-24 hours, while VRB plates were incubated at 30ºC for 24 hours) as prescribed by the manufacturer's instructions. After the incubation period, all plates were observed for growth and 1 to 5 colonies exhibiting typical *Enterobacteriaceae* characteristics were restreaked onto nutrient agar (NA) and incubated for 18-20 hours. The Oxidase test was performed on these colonies prior to being identified using the RapID ONE and API 20 E identification systems. The RapID ONE system identifies Oxidase negative species only while the API 20 E system is utilised for Oxidase negative and Oxidase positive organisms.

2.4. Statistical Analysis

Repeated measures for analysis of variance (ANOVA) (RMA) was performed on all data obtained from the enumeration techniques as outlined in Dunn and Clark (1987), using Statistica™. In each RMA, the residuals were analysed to determine whether they were normally distributed. In all hypothesis tests, a significant level of 5% was used as standards.

2.5 Identification Techniques

2.5.1 Identification of *Enterobacteriaceae* Species Using the API 20 E System

Microorganisms identified using the API 20 E system were obtained from the selective media (CCA, MacConkey and VRB agar), which were used for culturing isolates from water samples. Single isolated colonies which resembled the *Enterobacteriaceae* strains were then restreaked onto NA plates. The API 20 E test strip was then prepared according to manufacturer's instructions (BioMérieux, South Africa) by adding 5 ml of distilled water into the combed wells of the tray. A single isolated colony was suspended in 5 ml of sterile saline solution and emulsified to obtain a homogenous bacterial suspension, thereafter it was added to each tube of the strip while avoiding the formation of bubbles. The test strip was then incubated at 37ºC for 18-24 hours. After incubation the strip was read by referring to the manufacturer's instruction table for colour changes, the results were recorded and the organisms were identified using the Apiweb™ identification software (BioMérieux, South Africa).

2.5.2 Identification of *Enterobacteriaceae* Species Using the RapID™ ONE System

The RapID™ ONE experimental procedure was performed according to manufacturer's instructions (Remel Inc, USA). Positive *Enterobacteriaceae* colonies isolated from selective media (CCA, MacConkey and VRB agar), which were cultured from the river water samples, were utilised as test organisms. Single isolated colonies were then grown on NA for 18-24 hours prior to identification using the RapID™ ONE system. Colonies from the NA plates were suspended in RapID Inoculation Fluid (2 ml) and compared to the number 2 McFarland standard for turbidity. The mixture was then inoculated into the panels of the RapID™ ONE system and incubated at 37ºC for 4 hours. After incubation, 2 drops of RapID™ ONE reagent were added to cavities 15 to 17 and 2 drops of spot indole were added to cavity 18 on the strip (allowed for colour development for not more than 2 minutes). Colour changes were read and recorded in an appropriate box of the report form. The online

Electronic RapID Compendium (ERIC) program was used to identify the specific organisms isolated.

2.5.3 Polymerase Chain Reaction

2.5.3.1 Genomic DNA Extraction from Control Microorganisms

The reference strains used in a conventional singleplex Polymerase Chain Reaction (PCR) are listed in **Table 2.1**. These strains were obtained from the laboratories of the Departments of Biotechnology and Biomedical Sciences at the Cape Peninsula University of Technology. The Laboratory cultures routinely used (without ATCC numbers) were first tested and confirmed positive using biochemical tests and PCR.

Table 2.1 List of *Enterobacteriaceae* species used as reference strains in the conventional PCR procedure

Organism	Strain number	Source
Bacillus cereus	ATCC 13061	Biotechnology
Enterococcus faecalis	ATCC 29212	Biotechnology
Escherichia coli	ATCC 25922	Biotechnology
Klebsiella pneumoniae	ATCC 10031	Biomedical Sciences
Micrococcus luteus	ATCC 4696	Biotechnology
Pseudomonas aeruginosa	ATCC 27853	Biotechnology
Pseudomonas fluorescens	Lab strain	Biotechnology
Salmonella typhimurium	Lab strain	Biomedical Sciences
Serratia marcescens	ATCC 14756	Biomedical Sciences
Shigella sonnei	Lab strain	Biotechnology
Vibrio cholerae	Lab strain	Biomedical Sciences

The extraction and purification of DNA from the reference strains was performed using the boiling method adapted from Watterworth *et al.* (2005) and the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). For the boiling method, pure cultures of the microorganisms were grown on NA at 37°C for 18-24 hours, and a single colony was inoculated onto Luria Bertani (LB) broth and incubated under the same conditions. One millilitre of broth aliquot was centrifuged at 14 000 rpm for 10 minutes, the supernatant was discarded and the pellet was resuspended in 100 µl of sterile double distilled water and boiled in a 95°C water bath for 15 minutes. The suspension was then cooled on ice for 10 minutes, and centrifuged at 14 000 rpm for 5 minutes with the supernatant (DNA is contained in the supernatant) transferred into a sterile 1.5 ml eppendorf tube. Deoxyribonucleic acid was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). One times concentration Tris-acetate-ethylenediamine

tetraacetic acid (TAE) electrophoresis buffer was used and the gel was run for 1 hour at 90 volts. Lambda DNA/PstI ladder was used in DNA analysis on agarose gels. Deoxyribonucleic acid extracted using the boiling method was also quantified using the Qubit™ fluorometer according to the manufacturer's instructions. Deoxyribonucleic acid samples were stored at -20°C until they were used for PCR analysis. All the reagents used for DNA analysis and PCR products were prepared as shown in **Appendices A and B**. The DNA extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) was done according to the instructions of the manufacturer. Deoxyribonucleic acid was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide. One times concentration TAE electrophoresis buffer was used and the gel was run for 1 hour at 90 volts. Lambda DNA/PstI ladder was used in DNA analysis on agarose gels. Deoxyribonucleic acid extracted using the High Pure Template Preparation Kit was also quantified using the Qubit™ fluorometer according to the manufacturer's instructions. Deoxyribonucleic acid samples were stored at -20°C until they were used for PCR analysis.

2.5.3.2 Genomic DNA Extraction from Water Samples

Microbial cells were harvested from river water samples (**Section 2.1.1**) by centrifuging 500 ml of river water at 7 000 rpm for 20 minutes. The pellet was then incubated in 2 ml of LB broth for 6 hours at 37°C (Kong *et al*., 1999). Extraction of DNA was performed using the boiling method adapted from Watterworth *et al*. (2005) as described in **Section 2.5.3.1**. Deoxyribonucleic acid was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). One times concentration TAE electrophoresis buffer was used and the agarose gel was run for 1 hour at 90 volts. The 1 kb Generuler^{TM} (Fermentas) was used in DNA analysis on agarose gels. Deoxyribonucleic acid samples were stored at -20°C until they were used for PCR analysis.

2.5.3.3 Optimisation of Conventional Polymerase Chain Reaction

Table 2.2 lists the species specific primer sequences and the primers that were used by various authors to identify different areas of the *Enterobacteriaceae* species utilised in the singleplex PCRs. After extraction of DNA (boiling method and the High Pure PCR Template Preparation Kit) from control *Enterobacteriaceae* microorganisms (**Section 2.5.3.1**), reaction mixtures and cycling conditions for the respective microorganisms were completed from the protocol used by previous authors (**Table 2.2**). **Table 2.3** shows how the concentrations and volumes of PCR reagents were adjusted and optimised and the cycling programs were altered for each member of the *Enterobacteriaceae* family. **Table 2.3.1** provides an overview

of what the final reaction volumes (in a final volume of 50 µl) were for each reference strain, while **Table 2.3.2** provides the final cycling conditions for each of the reference strains.

Table 2.2 Primer sequences and predicted size of Polymerase Chain Reaction amplicons

Bacteria	Primer –Sequence(5'-3')	Target area	Product size (bp)	Reference
Bacillus cereus	BCFW1- GTTTCTGGTGGTTTACATGG BCrevnew- TTTTGAGCGATTTAAATGC	*gyrB* gene	374	Manzano *et al.,* 2003
Enterococcus faecalis	Ef0027F- GCCACTATTTCTCGGACAGC Ef0027R- GTCGTCCCTTTGGCAAATAA	Putative transcriptional regulator gene	518	Liu *et al*., 2005
Escherichia coli	PhoF-GTGACAAAAGCCCGGACACCATAAATGCCT PhoR-TACACTGTCATTACGTTGCGGATTTGGCGT	*PhoA* gene	903	Kong *et al.,* 1999
Klebsiella pneumoniae	Pf- ATTTGAAGAGGTTGCAAACGAT Pr1- TTCACTCTGAAGTTTTCTTGTGTTC	Internal transcribed spacer region	130	Liu *et al.,* 2008
Micrococcus luteus 1	27F- AGAGTTTGATCMTGGCTCAG 27R- TACGGYTACCTTGTTACGACTT	16S rRNA region	1600	Current paper
Micrococcus luteus	MLF- GGTCGAACGCGACTCAGGTC MLR- TTCATGTCCCAGGTGCCGTT	*Rpf* gene	610	Mukamolova *et al.,* 2002
Pseudomonas aeruginosa	PA-SS-F- GGGGGATCTTCGGACCTCA PA-SS-R- TCCTTAGAGTGCCCACCCG	16S rDNA region	956	Spilker *et al*., 2004
Pseudomonas fluorescens	SPSEfluF- TGCATTCAAAACTGACTG SPSER- AATCACACCGTGGTAACCG	16S rRNA region	850	Scarpellini *et al.,* 2004
Salmonella typhimurium	fimY1- GAGTTACTGAACCAACAGCT fimY2- GCCGGTAAACTACACGATGA	*fimY* gene	526	Yeh *et al*., 2002
Serratia marcescens lux gene	FluxS1- GCTGGAACACCTGTTCGC RluxS2- ATGTAGAAACCGGTGCGG	*luxS* gene	102	Zhu *et al.,* 2008
Serratia marcescens pfs gene	Fpfs1- CCGGCATCGGCAAAGTCT Rpfs2 ATCTGGCCCGGCTCGTAGCC	*Pfs* gene	193	Zhu *et al.,* 2008
Shigella sonnei	IS1SS- ATGCCGGGCAACTGCA IS1SR3- CTGCGTATATCGCTTG	Insertion sequence 1 region	369	Hsu *et al.,* 2007
Vibrio cholerae	ompWF- CACCAAGAAGGTGACTTTATTGTG ompWR- GAACTTATAACCACCCGCG	*OmpW* gene	588	*Nandi et al.,* 2000

The PCR volumes and concentrations applied to the river water samples were that of the optimised conventional PCR as listed in **Table 2.3.1**, while the cycling conditions were performed as shown in **Table 2.3.2**. *Klebsiella pneumoniae*, *Serratia marcescens (pfs* gene*) and Micrococcus luteus* 1 primer sets could not be used in water samples as no positive results after using the Basic Local Alignment Search Tool (BLAST) were obtained. Polymerase chain reaction amplicons were confirmed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml). One times concentration TAE electrophoresis buffer was used and the gel was run for 1 hour at 90 volts. Lambda DNA/PstI or Generuler 1 kb DNA ladders were used in analysis of PCR products.

Table 2.3 Optimisation of the conventional PCR for *Enterobacteriaceae* species

Organism	PCR optimisation	Cycling program
B. cereus	Primer concentration was increased from 0.2 µM to 0.5 µM while the concentration of $MgCl_2$ reduced from 3.5 mM to 1.5 mM.	The cycling program was used as previously described by Manzano *et al.* (2003).
E. faecalis	Total reaction volume was increased from 25 µl to 50 µl, the concentration of Go Taq DNA polymerase, dNTP mix and primer set were increased from 0.5 U to 1.25 U, 0.05 mM to 0.1 mM and 0.25 µM to 0.5 µM, respectively.	The annealing temperature was reduced from 60ºC to 56ºC (Liu *et al*., 2005).
K. pneumoniae	Total reaction volume was increased from 25 µl to 50 µl, primer concentration was decreased from 1 µM to 0.5 µM and the dNTP mix increased from 0.1 mM to 0.2 mM.	The annealing temperature was reduced from 57ºC to 55ºC (Liu *et al.,* 2008).
P. aeruginosa	Total reaction and template DNA volumes were increased from 25 µl to 50 µl and 2 µl to 5 µl, respectively. The concentration for the dNTP mix, decreased from 0.25 mM to 0.2 mM, while for primer set and Go Taq DNA polymerase were increased from 0.4 µM to 0.5 µM and 1 U to 1.25 U respectively.	Same cycling conditions were used as in the previous study by Spilker *et al*. (2004).
P. fluorescens	$MgCl_2$ and primer set concentrations were decreased from 2 mM to 1.5 mM and 0.5 µM to 0.1 µM, respectively.	Annealing temperature was decreased from 60ºC to 50ºC (Scarpellini *et al.,* 2004).
S. sonnei	Gelatin and Triton X-100 was not used as in the previous study by Hsu *et al.* (2007). Primer concentration was decreased from 0.8 µM to 0.5 µM, the Go Taq DNA polymerase concentration increased from 1 U to 1.25 U and the volume of template was increased from 1 µl to 5 µl.	Same cycling conditions were used as previously listed by Hsu *et al.* (2007).
V. cholerae	Total reaction and template DNA volumes were increased from 25 µl to 50 µl and 3 µl to 10 µl, respectively. The concentration for dNTP mix was reduced from 0.25 mM to 0.2 mM.	One cycle of the final extension at 72ºC for 10 minutes was added (Nandi *et al.,* 2000).
M. luteus 1	Performed in a total volume of 50 µl containing 10 µl template DNA, 1.25 U of Go Taq DNA polymerase, 0.2 mM dNTP mix, 1.5 mM of $MgCl_2$, 1X Buffer and each primer concentration of 0.5 µM	95°C for 2 minutes; 30 cycles- 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 10 minutes
M. luteus	Performed as previously done by Mukamolova *et al.* (2002).	Annealing temperature was increased from 55°C to 58°C (Mukamolova *et al*., 2002).
S. marcescens lux gene	Primer concentrations increased from 0.3 µM to 0.5 µM.	Annealing temperature was increased from 55°C to 60°C (Zhu *et al.,* 2008).
S. marcescens pfs gene	Performed as previously done by Zhu *et al.* (2008)	Annealing temperature was increased from 55°C to 58°C (Zhu *et al.,* 2008).
E. coli	The final volume was reduced from 100 µl to 50 µl and the primer concentrations were reduced from 0.4 µM to 0.2 µM.	Number of amplification cycles was reduced from 35 to 30 (Kong *et al.,* 1999).
S. typhimurium	Template DNA volume was increased from 1.0 µl to 5.0 µl.	Annealing temperature was increased from 56°C to 58°C (Yeh *et al.,* 2002).

Table 2.3.1 Conventional Polymerase Chain Reaction mixtures

Organism	Buffer 5X (µl)	$MgCl_2$ 25 mM (µl)	dNTP mix 10 mM (µl)	Primer Forward 10 µM (µl)	Primer Reverse 10 µM (µl)	Go *Taq* DNA Polymerase 5U/µl	Template DNA (µl)	Water (µl)
B. cereus	10	3	1	2.5	2.5	0.25	2	28.75
E. coli	10	3	1	1.0	1.0	0.25	2	31.75
E. faecalis	10	3	1	2.5	2.5	0.25	2	28.75
K. pneumoniae	10	3	1	2.5	2.5	0.25	5	25.75
M. luteus	10	3	1.5	6.0	6.0	0.5	10	13.0
P. aeruginosa	10	3	1	2.5	2.5	0.25	5	25.75
P. fluorescens	10	3	1	0.5	0.5	0.25	2	32.75
S. marcescens (lux gene*)*	10	3	1	2.5	2.5	0.25	5	25.75
S. sonnei	10	3	1	2.5	2.5	0.25	5	25.75
S. typhimurium	10	3	1	2.5	2.5	0.25	5	25.75
V. cholerae	10	3	1	5.0	5.0	0.25	10	15.75

Table 2.3.2 Conventional Polymerase Chain Reaction cycling conditions

Organism	Polymerase Chain Reaction cycling conditions	Product size (bp)
B. cereus	Initial denaturation at 95°C for 5 minutes; followed by 35 cycles of amplification (1 minute at 95°C, 1 minute at 54°C, 1 minute at 72°C). Final extension at 72°C for 7 minutes.	374
E. coli	Initial denaturation at 94°C for 2 minutes; followed by 35 cycles of amplification (1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C). Final extension was performed at 72°C for 10 minutes.	903
E. faecalis	Initial denaturation at 94°C for 2 minutes; followed by 30 cycles of amplification (20 seconds at 94°C, 20 seconds at 56°C and 45 seconds at 72°C). The final extension was performed at 72°C for 2 minutes.	518
K. pneumoniae	Initial denaturation at 94°C for 10 minutes; followed by 35 cycles of amplification (30 seconds at 94°C, 20 seconds at 55°C and 20 seconds at 72°C). The final extension step was performed at 72°C for 10 minutes.	130
M. luteus	Initial denaturation at 95 for 5 minutes; followed by 30 cycles of amplification (30 seconds at 95°C, 30 seconds at 58°C, 1 minute at 72°C). Final extension at 72°C for 7 minutes.	610
P. aeruginosa	Initial denaturation at 95°C for 2 minutes; followed by 25 cycles of amplification (20 seconds at 94°C, 20 seconds at 58°C, 40 seconds at 72°C). The final extension was performed at 72°C for 1 minute.	956
P. fluorescens	Initial denaturation at 94°C for 2 minutes; followed by 5 cycles of amplification (45 seconds at 92°C, 1 minute at 52°C, 2 minutes at 72°C); followed by 35 cycles of amplification (45 seconds at 92°C, 45 seconds at 50°C, 2 minutes at 72°C). The final extension was performed at 72°C for 2 minutes.	850
S. marcescens (*lux* gene)	Initial denaturation at 94°C for 5 minutes; followed by 30 cycles of amplification (45 seconds at 94°C, 30 seconds at 58°C, 15 seconds at 72°C). The final amplification was performed at 72°C for 10 minutes.	102
S. sonnei	Initial denaturation at 94°C for 5 minutes; followed by 30 cycles of amplification (1 minute at 95°C, 30 seconds at 50°C, 2 minutes at 72°C). The final amplification was performed at 72°C for 5 minutes.	369
S. typhimurium	Initial denaturation at 94°C for 2 minutes; followed by 30 cycles of amplification (1 minute at 94°C, 30 seconds at 58°C, 30 seconds at 72°C). Final extension was performed at 72°C for 10 minutes.	526
V. cholerae	Initial denaturation at 94°C for 5 minutes; followed 30 cycles of amplification (30 seconds at 94°C, 30 seconds at 64°C, 30 seconds at 72°C). Final extension was performed at 72°C for 10 minutes.	588

2.5.3.4 Extraction of DNA from *Escherichia coli* Control Strains

Escherichia coli strains that were used as positive controls in the multiplex PCR were obtained from the National Institute for Communicable Diseases (Johannesburg, SA). Strain O157:H7 was used as a positive control for Enterohaemorrhagic *Escherichia coli* (EHEC), B170 for Enteropathogenic *Escherichia coli* (EPEC), ATCC 3591-87 for Enteroaggregative *Escherichia coli* (EAEC), H10407 for Enterotoxigenic *Escherichia coli* (ETEC) and ATCC 43892 for Enteroinvasive *Escherichia coli* (EIEC). Deoxyribonucleic acid from the five strains of *E. coli* was extracted using the boiling method as described in **Section 2.5.3.1**.

2.5.3.5 Optimisation of Multiplex Polymerase Chain Reaction

For the optimisation of ETEC and an explanation of why ETEC was excluded from the optimisation of control strains, please refer to **Section 2.5.3.6**. The remaining four pathogenic *E. coli* reference strains were checked by singleplex PCR and optimised for multiplex PCR. **Table 2.4** shows the primer names and sequences, which were used during

the study for multiplex PCR analysis to detect genes specific for each pathogenic *E. coli* strain and their respective expected product sizes. For the single PCR reaction (for each of the 4 strains of *E. coli*), 10 µl of each strain was added to the respective tubes. Template DNA for the multiplex reaction was first prepared by mixing equal volumes of DNA of the 4 pathogenic strains of *E. coli* (EHEC, EPEC, EAEC and EIEC).

Table 2.4 Primer sequence and predicted size of amplicons in Multiplex Polymerase Chain Reaction (adopted from Toma *et al.,* 2003)

Organism	Primer name and Sequence (5'-3')	Target gene	Product size(bp)
EAEC	AggRKs1- GTATACACAAAAGAAGGAAGC AggRkas2- ACAGAATCGTCAGCATCAGC	*aggR*	254
EHEC	VTcomU- GAGCGAAATAATTTATATGTG Vtcomd- TGATGATGGCAATTCAGTAT	*stx*	518
EIEC	IpaIII- GTTCCTTGACCGCCTTTCCGATACCGTC IpaIV- GCCGGTCAGCCACCCTCTGAGAGTAC	*ipaH*	619
EPEC	SK1- CCCGAATTCGGCACAAGCATAAGC SK2- CCCGGATCCGTCTCGCCAGTATTCG	*eae*	881

Ten microlitres of this mixed DNA was used as the template for the multiplex reaction. Singleplex and multiplex PCR with primers targeting genes in EHEC, EAEC, EPEC and EIEC was first performed as previously described by Toma *et al.* (2003), but was subsequently optimised before it was used to identify the *E. coli* strains in the river water samples. The total PCR reaction volume was increased from 50 µl to 60 µl, while the concentration of $MgCl_2$, the VTcom primer set and Go Taq DNA polymerase were increased from 1.5 mM to 2.5 mM, 0.25 µM to 1.5 µM and 2.5 U to 5 U, respectively. The concentration of the *AggR* and the *Ipa* primer sets were decreased from 0.25 µM to 0.2 µM and 0.125 µM to 0.1 µM, respectively. The multiplex was also completed with and without the addition of the PCR additives [(Tris-HCl pH8.3), KCl and Triton X 100], which were included in the Toma *et al*. (2003) study. Singleplex PCR for the four pathogenic strains was done without the addition of the PCR additives. For the optimised PCR reaction volumes see **Table 2.4.1**. The final multiplex PCR cycling conditions included an initial denaturation cycle of 95ºC for 2 minutes, 30 cycles of (95ºC for 1 minute, 48ºC for 1 minute and 72ºC for 1 minute), and a final extension step of 72ºC for 7 minutes. The PCR products were eletrophoresed on a 2.0% agarose gel in 1X TAE buffer instead of the 2.5% agarose gel in 1X TBE buffer which was used in the previous study conducted by Toma *et al*. (2003). The optimised multiplex PCR conditions were used for the singleplex PCR reaction too.

Table 2.4.1 Multiplex Polymerase Chain Reaction mixture

		Multiplex (MP) PCR		Singleplex (SP) PCR				
Reagent	**[Stock]**	**Multiplex Without added Buffers (µl)**	**Multiplex With added Buffers (µl)**	**EPEC (µl)**	**EIEC (µl)**	**EHEC (µl)**	**EAEC (µl)**	**[Final]**
Tris-HCl pH (8.3)	0.5 M	-	1.2	-	-	-	-	10 mM
KCl	1 M	-	3.0	-	-	-	-	50 mM
Triton X 100	1%	-	6.0	-	-	-	-	0.1%
$MgCl_2$	25 mM	6.0	6.0	6.0	6.0	6.0	6.0	2.5 mM
PCR Buffer	5X	12	12	12	12	12	12	1X
dNTPs	10 mM	1.5	1.5	1.2	1.2	1.2	1.2	MP-0.25 mM SP-0.2 mM
SK Forward primer	10 µM	0.75	0.75	0.75	-	-	-	0.125 µM
SK Reverse primer	10 µM	0.75	0.75	0.75	-	-	-	0.125 µM
Ipa Forward primer	10 µM	0.6	0.6	-	0.6	-	-	0.1 µM
Ipa Reverse primer	10 µM	0.6	0.6	-	0.6	-	-	0.1 µM
Vt Forward primer	10 µM	1.5	1.5	-	-	1.5	-	0.25 µM
Vt Reverse primer	10 µM	1.5	1.5	-	-	1.5	-	0.25 µM
Ag Forward primer	10 µM	1.0	1.0	-	-	-	1.0	0.16 µM
Ag Reverse primer	10 µM	1.0	1.0	-	-	-	1.0	0.16 µM
Water	-	21.8	11.6	28.8	29.1	27.3	28.3	
Template DNA	100 ng	10.0	10	10.0	10.0	10.0	10.0	(100 ng each template)
Go Taq polymerase	5 U/µl	1.0	1.0	0.5	0.5	0.5	0.5	MP – 5 U SP – 2.5 U
Final Volume		60	60	60	60	60	60	

2.5.3.6 Optimisation of Enterotoxigenic *Escherichia coli*

2.5.3.6.1 Deoxyribonucleic Acid Extraction from Enterotoxigenic *E. coli*

The Enterotoxigenic *E. coli* strain (H10407) [assumed to contain DNA with both the heat-labile (*elt*) and heat-stable (*est*) enterotoxin genes] was obtained from the NICD (Johannesburg, South Africa). According to the NICD, they confirmed the presence of the plasmids containing the 2 genes within the H10407 strain before sending the sample to the client. Deoxyribonucleic acid was extracted using the boiling method as described in **Section 2.5.3.1** and was found to be successful after visualisation on a 1.5% electrophoresis gel. The total singleplex PCR reaction (targeting the *est* and *elt* genes in ETEC) volumes were 60 µl, while the final concentrations of PCR buffer, $MgCl_2$, dNTPs and Go Taq DNA polymerase were 1X, 2.5 mM, 0.2 mM and 2.5 U, respectively. The volume of template DNA used was 10 µl, while the final concentration of the *est* and *elt* primer sets were 0.5 µM and 0.25 µM, respectively. Double distilled water was used to top up the total reaction volume to 60 µl. The PCR conditions were the same as above for the optimised multiplex PCR reaction (**Section 2.5.3.5**). The PCR reaction (used for singleplex and multiplex, **Section 2.5.3.5**) using this extracted DNA was negative when using the primer sets of Toma *et al.* (2003) targeting the *elt* and *est* genes (**Table 2.5**). Alternative DNA extraction methods and other primer sets (listed in **Table 2.5**), which target the *elt* gene were then utilised.

Extraction of plasmid DNA was performed as per manufacturer's instructions using the Zippy™ plasmid miniprep kit (Zymo Research), High Pure Plasmid isolation kit (Roche diagnostics) and Pure Yield ™ plasmid miniprep kit (Promega, USA).

Table 2.5 Primer sequence and predicted size of amplicons for target genes in the ETEC strain

Primer name and Sequence (5'-3')	Target gene	Product size(bp)	Reference
LT-F- TCTCTATGTGCATACGGAGC LT-R- CCATACTGATTGCCGCAAT	*elt*	322	Toma *et al.,* 2003
AL65- TTAATAGCACCCGTACAAGGCAGG AL125- CCTGACTCTTCAAAAGAGAAAATTAC	*est*	147	Toma *et al.,* 2003
Lt A1- TACTTTTTATATTGAAAG Lt A2- CTAGTTTTCCATACTGATTG	*elt*	1 148	Current study
LT1- TTACGGCGTTACTATCCTCTCTA LT2- GGTCTCGGTCAGATATGTGATTC	*elt*	275	O'Meara *et al*., 1995
LT1-A1- AAACAAAACAAGTGGCG LT1-B2- GTTGTTATATAGGTTCCTAGC	*elt*	1 257	Lasaro *et al*., 2008

Extraction of DNA from the ETEC strain H10407 was then performed as indicated by Llop *et al.* (1999), where 500 µl of broth (ETEC overnight culture) was centrifuged at 10 000 x g for 10 minutes. The pellet was suspended in 500 µl extraction buffer [200 mM TrisHCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS] and left at room temperature with continuous shaking (100 rpm) for 1 hour. The mixture was then centrifuged at 13 000 x g for 10 minutes, the supernatant was discarded and the pellet was dried under a vacuum. The pellet was finally resuspended in 100 µl of sterile distilled water and used as template DNA during conventional PCR.

Genomic DNA was also extracted according to O'Meara *et al*. (1995). Firstly, a lysis buffer [10 mM TrisHCl (pH 8.3), 50 mM KCl, 0.1% Tween 20] was prepared. Two colonies (18-24 hours on NA) were suspended in 10 µl of the lysis buffer and incubated in a 99°C Accublock™ digital dry bath (Whitehead scientific, South Africa) for 5 minutes. The mixture was then centrifuged at 20 000 x g for 2 minutes and subsequently the supernatant was used as template DNA in the PCR reactions.

2.5.3.6.2 Amplification of the Enterotoxigenic *Escherichia coli* in Control DNA

Amplification was first performed using the LT and AL primer sets (**Table 2.5**) adopted from Toma *et al*. (2003) in separate reaction tubes as illustrated in PCR 1 (**Table 2.6)**. Optimisation was done in the sequence as shown in **Table 2.6** using the template DNA extracted by different methods. Amplification of the *est* (147 bp) and *elt* (322 bp) genes found in the H10407 strain failed to yield the expected amplicons with agarose gel electrophoresis after performing PCR 1 to 4 (**Table 2.6**), and led to the use of DNA extracted

using other methods and primer sets adopted from previous studies as illustrated in **Table 2.6** for PCR 5 to 9.

Table 2.6 Optimisation of Polymerase Chain Reaction for the genes found in ETEC strain

PCR	Template DNA used	PCR reaction mixture	Cycling conditions
1	Boiling method	10 µl of 5X PCR reaction buffer, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 3.0 mM $MgCl_2$, 0.25 mM dNTP mix, 10 µl template DNA, 1.5 µM LT-F and LT-R primers, 0.5 µM AL65 and AL125 primers, 2.5 U of Go Taq DNA polymerase and double distilled water was used to adjust to the final volume of 50 µl for each mixture.	Initial denaturation step at 95°C for 2 minutes followed by 30 cycles of amplification (denaturing at 95°C for 1 minute, annealing at 48°C for 1 minute, extension at 72°C for 1 minute) and the final extension at 72°C for 7 minutes.
2	Boiling method	10 µl of 5X PCR reaction buffer, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 3.0 mM $MgCl_2$, 0.25 mM dNTP mix, 10 µl template DNA, 1.5 µM LT-F and LT-R primers, 0.5 µM AL65 and AL125 primers, 2.5 U of Go Taq DNA polymerase and double distilled water was used to adjust to the final volume of 50 µl for each mixture.	Initial denaturation step at 95°C for 2 minutes followed by 30 cycles of amplification (denaturing at 95°C for 1 minute, annealing at 45-55°C for 1 minute, extension at 72°C for 1 minute) and the final extension at 72°C for 7 minutes.
3	Boiling method	Total reaction volume was increased to 100 µl and various concentrations of LT-F and LT-R primer set ranging from 1.5 µM to 10 µM were used with other reagents similar to PCR 1.	The annealing temperature was then increased to 55°C for 30 seconds and final extension at 72°C for 10 minutes.
4	Boiling method	Various concentrations of $MgCl_2$ (1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM) using the LT-F and LT-R primer set.	Same cycling conditions as listed in PCR 1.
5	DNA extracted according to Llop *et al.* (1999)	Same reaction mixture as listed in PCR 1.	Same cycling conditions as listed in PCR 2
6	DNA extracted according to O'Meara *et al*. (1995)	10 µl of 5X PCR reaction buffer, 3.0 mM $MgCl_2$, 0.25 mM dNTP mix, 10 µl template DNA, 1.5 µM LT-F and LT-R primers, 0.5 µM AL65 and AL125 primers, 2.5 U of Go Taq DNA polymerase and double distilled water was used to adjust to the final volume of 50 µl for each mixture.	Initial denaturation step at 94°C for 4 minutes followed by 30 cycles of amplification (denaturing at 94°C for 45 seconds, annealing at 51.7°C for 90 seconds, extension at 72°C for 60 seconds) and the final extension at 72°C for 5 minutes.
7	DNA extracted according to O'Meara *et al*. (1995)	Same reaction mixture as illustrated in PCR 6, but 0.5 µM Lt A1 and Lt A2 primers were used, also in a separate reaction with 0.5 µM LT1-A1 and LT1-B2 primers was conducted.	Same cycling conditions were used as listed in PCR 6, but the annealing temperature was reduced to 38°C for 30 seconds.
8	Plasmid DNA extracted using the Zippy™ plasmid miniprep kit	Used the same volumes as used in PCR 1. New set of primers previously used by O'Meara *et al.,* 1995, Lasaro *et al.,* 2008 and the ones designed from the current study.	Used the same conditions as used in PCR 1.
9	Plasmid DNA extracted using the High Pure Plasmid isolation kit	Used the same volumes as used in PCR 1. New set of primers previously used by O'Meara *et al*., 1995, Lasaro *et a*l., 2008 and the ones designed from the current study.	Used the same conditions as used in PCR 1.
10	Colony PCR	10 µl of 5X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1.5 µM PhoF and PhoR primers (**Table 2.2**), 2.5 U of Go Taq DNA polymerase and double distilled water was used to adjust to the final volume of 50 µl for each mixture.	Initial denaturation step at 94°C for 5 minutes followed by 30 cycles of amplification (denaturing at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute) and the final extension at 72°C for 10 minutes.

Unsuccessful amplification of the 2 genes (PCR 1- 9) in ETEC led to the performance of colony PCR using the *PhoA* primer set targeting the alkaline phosphatase gene found in all *E. coli* for verification analysis. The reaction mixture and cycling program was performed as shown in **Table 2.6** (PCR 10) using 5 different isolated colonies grown overnight on NA. For all PCR reactions, amplicons were analysed on a 2.0% agarose gel and no DNA bands corresponding to the expected sizes were observed for *est* and *elt* genes, respectively. During the extensive trouble-shooting process, NICD was contacted to confirm that the correct strain was sent and that it still contained the plasmids with the *elt* and *est* genes. The NICD assured the researchers of the intergrity of the strain. A new H10407 strain was then requested from NICD. The NICD then sent a correspondence to the researchers in which they confirmed that their H10407 strain had lost the plasmids carrying the *elt* and *est* genes of ETEC. The ETEC strain was thus not included in the optimisation process of the multiplex PCR. Both ETEC primer sets (*est* and *elt*) were however used in a conventional singleplex PCR for testing the river water samples. The PCR volumes and concentrations applied to the river water samples were that of the optimised multiplex PCR as listed in **Table 2.6.1**, while the cycling conditions were performed as shown in **Section 2.5.3.5**. All positive PCR amplicons from river water samples were DNA sequenced.

Table 2.6.1 Conventional Multiplex Polymerase Chain Reaction mixture for ETEC strain

Reagent	**[Stock]**	***est* gene (µl)**	***elt* gene (µl)**	**[Final]**
$MgCl_2$	25 mM	6.0	6.0	2.5 mM
PCR Buffer	5X	12	12	1X
dNTPs	10 mM	1.2	1.2	0.2 mM
Lt Forward primer	10 µM	-	1.5	0.25 µM
Lt Reverse primer	10 µM	-	1.5	0.25 µM
AL Forward primer	10 µM	3	-	0.5 µM
AL Reverse primer	10 µM	3	-	0.5 µM
DNA	100 ng	10	10	(100 ng each template)
Go Taq DNA polymerase	5 U/µl	0.5	0.5	2.5 U
Water		24.3	27.3	
Total Volume		60	60	

2.5.3.7 Optimisation of Real-time Polymerase Chain Reaction Using Control DNA

Real-time PCR was firstly performed on the pathogenic *E. coli* strains (single and multiplex) using the optimised cycling conditions that were used during multiplex PCR as described in **Section 2.5.3.5**. For the multiplex real-time PCR, equal volumes of the template DNA of EHEC, EAEC, EIEC and EPEC were used. The cycling program for the multiplex PCR was then changed from the program described in **Section 2.5.3.5** to an initial enzyme activation step of 98°C for 3 minutes followed by 35 cycles of amplification (98°C for 5 seconds, 48°C for 5 seconds and 72°C for 30 seconds) and a final extension at 72°C for 2 minutes. The

Sybr® green PCR master mix (Qiagen, USA) was also compared to the iQ™ Sybr® green PCR master mix (Bio-Rad, USA), containing all reagents listed in **Table 2.7**.

Table 2.7 Real-time Polymerase Chain Reaction mixture

Reagent	[Stock]	Multiplex	EPEC	EIEC	EHEC	EAEC	[Final]
Master mix	2X	15.0	15.0	15.0	15.0	15.0	0.75X
SK Forward primer	10 µM	2.0	2.0	0	0	0	0.5 µM
SK Reverse primer	10 µM	2.0	2.0	0	0	0	0.5 µM
Ipa Forward primer	10 µM	0.5	0	0.5	0	0	0.125 µM
Ipa Reverse primer	10 µM	0.5	0	0.5	0	0	0.125 µM
Vt Forward primer	10 µM	1.0	0	0	1.0	0	0.25 µM
Vt Reverse primer	10 µM	1.0	0	0	1.0	0	0.25 µM
Ag Forward primer	10 µM	1.0	0	0	0	1.0	0.25 µM
Ag Reverse primer	10 µM	1.0	0	0	0	1.0	0.25 µM
Water		6.0	11.0	14.0	13.0	13.0	-
Template DNA	100 ng	10.0	10.0	10.0	10.0	10.0	25 ng
Total Volume		40	40	40	40	40	-

The reaction volume used with the Sybr® green PCR master mix (Qiagen, USA) and iQ™ Sybr® green PCR master mix (Bio-Rad, USA) were changed from the manufacturers' recommended volume of 25 µl and 50 µl, respectively to 40 µl. No added buffers (Tris, KCl and Triton X 100) were included in the multiplex real-time PCR reaction as they were found to inhibit the PCR process. The multiplex PCR reaction was optimised after using the MJ Mini Opticon real-time thermal cycler (Bio-Rad, USA) to amplify each gene in each strain of *E. coli*. The on-line fluorescent detection system was observed for the earliest significant product peaks, and the number of cycles for the amplification of the control *E. coli* strains was reduced from 30 to 20 cycles, with the other conditions remaining unchanged (as shown in **Sections 2.5.3.7** and **2.5.3.10**). This reduced number of cycles was then applied to the conventional-multiplex PCR system when testing the control strains.

2.5.3.8 Conventional Singleplex Polymerase Chain Reaction for River Water Samples for 11 *Enterobacteriaceae* species

The DNA from water samples was extracted as previously indicated in **Section 2.5.3.2**. Positive control strains were used in all reactions, while double sterile distilled water was used as negative control to monitor for non-specific amplification. Optimised conventional singleplex PCR reactions (Table 2.3.1 and Table 2.3.1) were used to detect the presence of genes specific to the *Enterobacteriaceae* species (as listed in **Table 2.2)** from contaminated river water. The concentrations of conventional PCR reaction mixtures for each microorganism were adapted from previous studies (**Table 2.2**) as shown in **Table 2.3.1**. The PCR reaction mixture of all organisms identified contained 1X PCR buffer, 1.5 mM $MgCl_2$, and 1.25 U of Go *Taq* DNA polymerase. Varying primer concentrations, dNTP mix

and template DNA were used for each microorganism and the final volumes of the reactions were adjusted with sterile distilled water to 50 µl. The cycling conditions to amplify each gene in each microorganism were adapted from previous studies and the optimised amplification programs were performed as outlined in **Table 2.3.2** using species specific primers for each PCR assay, while distilled water was used as a negative control. The reagents were mixed in clean sterile, nuclease free 0.2 ml PCR tubes (Axygen, USA).

Fifteen microlitres of each PCR amplicon was analysed on a 1.5% molecular grade agarose gel stained with ethidium bromide (0.5 µg/ml) in 1X TAE electrophoresis buffer, and run at 90 volts for 50 minutes. A Generuler^{TM} 1 kb plus DNA ladder (Fermentas) was used as a molecular marker to compare the amplicon size. The amplified PCR products were purified as per manufacturer's instructions using the High Pure PCR Product Purification kit (Roche Diagnostics, Germany) or Wizard® SV Gel and PCR clean up system (Promega, USA) and sent for DNA sequencing to the DNA Sequencing Unit at the University of Stellenbosch. Sequencing was done in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems).

2.5.3.9 Detection of Pathogenic *E. coli* Strains in Water Samples Using the Multiplex Polymerase Chain Reaction

The DNA from water samples was extracted as previously indicated in **Section 2.5.3.2**. Positive control strains were used in all reactions, while double sterile distilled water was used as negative control to monitor for non-specific amplification. The optimised reaction mixture (**Table 2.4.1**) comprised of 10 µl 5X PCR reaction buffer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 0.1 µM Ipa IV and Ipa III primers, 0.125 µM of SK1 and SK2 primers, 1.5 µM VTcom-U, VTcom-d, 0.2 µM AggRks1 and AggRkas2, 5 U of Go Taq DNA polymerase,10 µl of template DNA and double sterile distilled water to adjust the final volume to 60 µl.

The PCR reaction program was adapted from Toma *et al.* (2003) and was performed using the My cycler^{TM} thermal cycler (Biorad). The optimised program was performed using the initial template denaturation step at 95°C for 2 minutes followed by 30 cycles of amplification (denaturing at 95°C for 1 minute, annealing at 48°C for 1 minute, extension at 72°C for 1 minute) and the final extension at 72°C for 7 minutes. Twenty microliltres of each PCR amplicon was electrophoresed on a 2.0% agarose gel with 1X TAE buffer. The gels were stained with ethidium bromide (0.5 µg/ml) and run at 90 volts for 50 minutes. The amplified PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics, Germany) or Wizard® SV Gel and PCR clean up system (Promega, USA) and were sent for DNA sequencing to the DNA Sequencing Unit at Stellenbosch

University. Sequencing was done in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems).

Detection of the ETEC strain was perfomed in water samples by the Conventional singleplex PCR to detect the *elt* and *est* genes as described in **Sections 2.5.3.5 and 2.5.3.6**.

2.5.3.10 Detection of Pathogenic *E. coli* Strains from Water Samples Using the Real-time Polymerase Chain Reaction

The DNA from water samples was extracted as previously indicated in **Section 2.5.3.2**. Positive control strains were used in all reactions, while double sterile distilled water was used as negative control to monitor for non-specific amplification. The real-time multiplex PCR reaction mixture as outlined in **Table 2.7** was used to analyse the water samples and control organisms. The final optimised real-time PCR conditions used on the water samples, involved an initial enzyme activation and denaturation step at 98°C for 3 minutes followed by 35 cycles of amplification (98°C for 5 seconds, 48°C for 5 seconds and 72°C for 30 seconds) and a final extension at 72°C for 2 minutes [adapted from the Sybr® green protocol (Qiagen, USA)]. Amplifications for each gene in each microorganism were performed using the species specific primers [for 4 strains of *E. coli* adopted from Toma *et al.* (2003)] as listed in **Table 2.4**, using the MJ MiniOpticon real-time thermal cycler (Bio-Rad, USA). The reagents were mixed in a clean sterile, nuclease free 0.2 ml PCR tubes (Axygen, USA). Twenty microlitres of each PCR amplicon was visualised on a 2.0% agarose gel stained with ethidium bromide (0.5 µg/ml) in 1X TAE buffer and run at 90 volts for 50 minutes. The amplified PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics, Germany) or Wizard® SV Gel and PCR clean up system (Promega, USA) and were sent to the DNA Sequencing Unit at Stellenbosch University for sequencing. Sequencing was done in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems).

3. RESULTS AND DISCUSSION

3.1 Measured Physical Parameters of River Water

During the sampling period (July 2010 to January 2011), the water temperature and pH values were recorded at the sites situated closest to the informal settlements of Mbekweni and Kayamandi, along the Berg- and Plankenburg River systems, respectively, as indicated in **Table 3.1**. Temperatures recorded in the Berg River ranged from 12.9°C (week 1) to 21.8°C (week 28), with the lowest and highest temperatures recorded in week 5 (12.7°C) and weeks 15 and 26 (23.5°C), respectively. The pH values for the Berg River water samples ranged from 7.07 (week 1) to 6.84 (week 28), with the lowest and highest values of 6.73 and 7.51 recorded in weeks 13 and 3, respectively.

Temperatures recorded in the Plankenburg River ranged from 14.9°C (week 1) to 19.6°C (week 28), with the lowest and highest temperatures recorded in week 3 (11.9°C) and week 26 (20.5°C), respectively. The pH values for the Plankenburg River water samples ranged from 6.99 (week 1) to 7.21 (week 28), with the lowest and highest values of 6.97 and 8.93 recorded in weeks 17 and 9, respectively. As illustrated in **Table 3.1**, the highest and lowest temperatures for both river systems, were recorded in the months of January (week 26) and August (weeks 3 and 5), respectively.

Table 3.1 Physical parameters recorded in the Berg- and Plankenburg River water samples

Sampling Week	Berg River		Plankenburg River	
	Temperature (ºC)	pH	Temperature (ºC)	pH
1	12.9	7.07	14.9	6.99
3	13.4	7.51	11.9	7.84
5	12.7	7.25	12.3	7.23
7	15.7	7.40	14.0	7.64
9	16.9	7.17	14.9	8.93
11	21.6	7.18	18.6	7.27
13	19.1	6.73	16.8	7.42
15	23.5	6.96	18.6	7.48
17	23.3	7.23	19.5	6.97
19	22.3	6.98	18.3	7.06
21	21.3	7.13	18.5	6.98
26	23.5	6.93	20.5	7.48
28	21.8	6.84	19.6	7.21

3.2 Enumeration of Coliforms and *Escherichia coli* in River Water

The South African Bureau of Standards (SABS, 1984) and the Department of Water Affairs and Forestry (DWAF, 1996) have stipulated guidelines for the acceptable levels of coliforms and *E. coli* in natural water sources to ensure overall safety to humans and to protect the water sources (**Section 1.3**). The Multiple Tube Fermentation (MTF) and Membrane Filtration (MF) techniques and the Colilert 18® system were used to detect and quantify coliforms and *E. coli* in water samples collected from the Berg- and Plankenburg River systems. Repeated measures ANOVA was then used to analyse and compare results obtained for the enumeration techniques.

3.2.1 Total Coliform and *Escherichia coli* Counts Obtained for the Berg River

The enumeration techniques enabled the quantification of total coliforms and *E. coli*, which are usually associated with faecal pollution, in samples collected from the site closest to the informal settlement of Mbekweni in Paarl along the Berg River. The results obtained for the MTF technique are depicted in **Figure 3.1**, while the results obtained using the MF technique are depicted in **Figure 3.2**. The Colilert 18® system results could not be graphically depicted using the Statistica™ program (**Section 2.4**, Chapter 2) due to a lack of variance between *E. coli* and coliform counts for most of the sampling period.

On average the most probable number (MPN) counts, representing all possible gas-producing organisms detected using the MTF technique ranged from 5.4×10^3 microorganisms/100 ml in the first week of sampling, to 7.9×10^4 microorganisms/100 ml in week 28. The count obtained in week 1 was also the lowest MPN count recorded during the study period, while the highest count of 9.2×10^6 microorganisms/100 ml was recorded in week 9. The faecal coliforms enumerated using the MTF technique ranged from 1.1×10^3 microorganisms/100 ml in week 1, to 1.4×10^4 microorganisms/100 ml in week 28. The highest faecal coliform count of 1.4×10^6 microorganisms/100 ml was obtained in weeks 19 and 21 as illustrated in **Figure 3.1**, while the lowest faecal coliform count of 1.1×10^3 microorganisms/100 ml was obtained in week 1. The total *E. coli* counts obtained using the MTF technique ranged from 5.4×10^3 microorganisms/100 ml in week 1 to 3.5×10^4 microorganisms/100 ml in week 28. The highest *E. coli* counts of 1.7×10^6 microorganisms/100 ml were observed in weeks 9, 19 and 21, while the lowest *E. coli* count of 2.4×10^3 microorganisms/100 ml was observed in week 5.

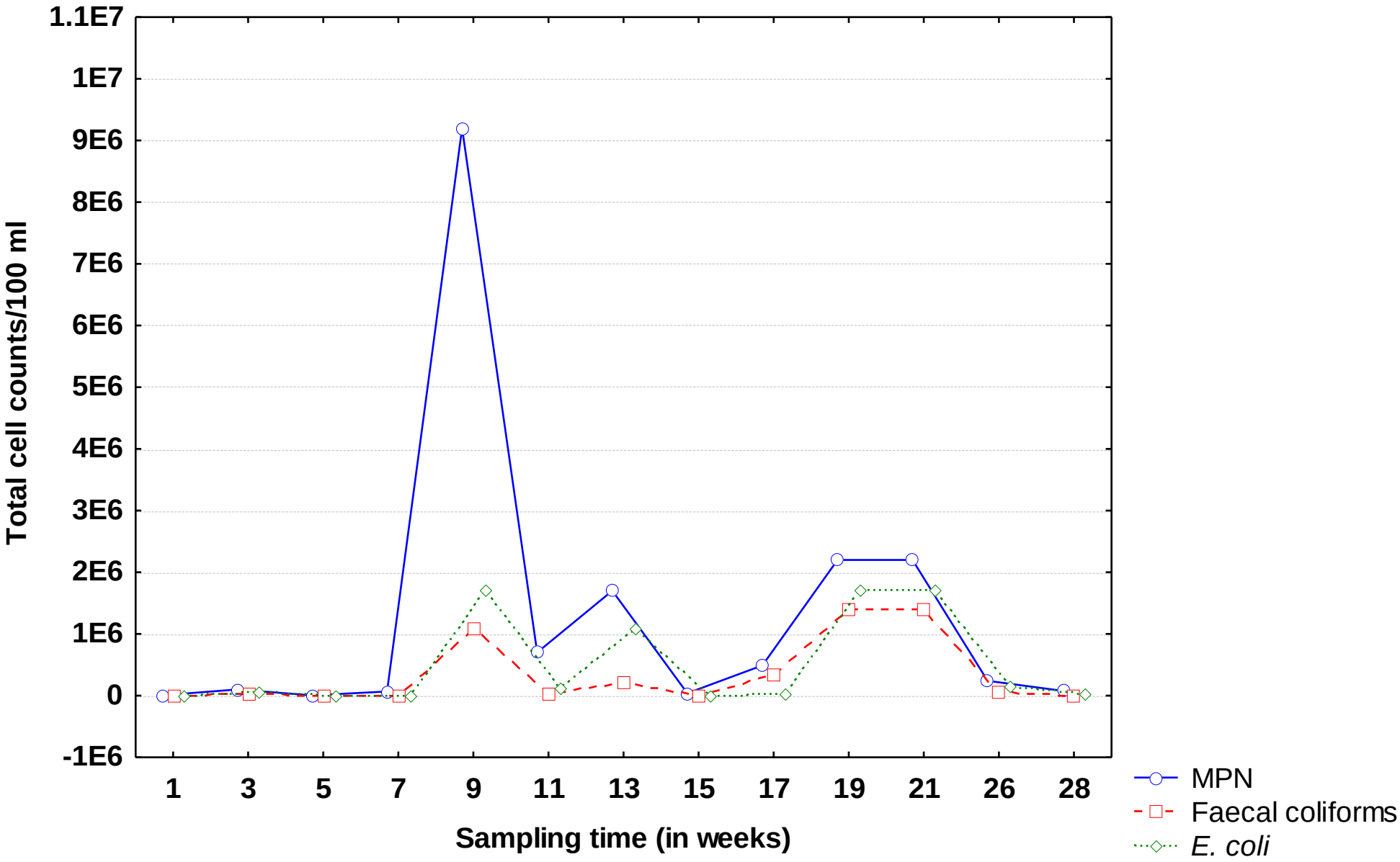

Figure 3.1 Enumeration of Most Probable Number (MPN), representing all possible gas-producing organisms, faecal coliforms and *E. coli* per 100 ml of river water sample by means of Multiple Tube Fermentation technique at Site A2 (Berg River) for weeks 1 to week 28.

The total coliform counts obtained using the MF technique ranged from 1.0×10^4 microorganisms/100 ml in week 1 to 7.8×10^3 microorganisms/100 ml in week 28 as illustrated in **Figure 3.2**. The highest total coliform count of 1.83×10^6 microorganisms/100 ml was obtained in week 9, while the lowest count of 2.2×10^3 microorganisms/100 ml was obtained in week 11. The *E. coli* counts obtained by the MF technique ranged from 6.9×10^3 microorganisms/100 ml in week 1 to 5.6×10^3 microorganisms/100 ml in week 28. The highest *E. coli* count of 9.9×10^5 microorganisms/100 ml was obtained in week 9, while the lowest count of 1.7×10^3 microorganisms/100 ml was observed in week 11.

The Colilert 18® system yielded the same total coliform counts of 2.42×10^3 microorganisms/100 ml during the first seven weeks of sampling, while in the last 6 weeks a count of 2.42×10^4 microorganisms/100 ml were recorded. During the first seven weeks, analysis of river water samples, using the Colilert 18® system, was performed according to the Paarl municipality protocol (no dilution of river water samples before analysis), which resulted in the same total coliform counts being recorded. When undiluted water samples were analysed by the Colilert 18® system, all the 97 wells in the Quanti-Tray/2000™ were positive, which corresponded to the highest count on the De Mans tables, and which yielded the 2.42×10^3 microorganisms/100 ml count for *E. coli* (weeks 2 to 13) and coliforms (weeks 1 to 13). However, as the water samples in weeks 15 to 28 were diluted to 10^{-2}, the dilution factor was taken into account in the calculation of the final coliform and *E. coli* count. *Escherichia coli* counts thus ranged from 1.1×10^3 microorganisms/100 ml in week 1 to 2.42×10^4 microorganisms/100 ml in week 28. The lowest *E. coli* count was obtained in the first week of sampling. The highest *E. coli* count of 1.9×10^5 microorganisms/100 ml was observed in week 26, when the samples were diluted before analysis.

For the MTF technique (**Figure 3.1**) the highest faecal coliform and *E. coli* counts were obtained in weeks 19 and 21 and weeks 9, 19 and 21, respectively, while for the MF technique (**Figure 3.2**) the highest coliform and *E. coli* counts were obtained in week 9. These results correspond to the results obtained for MPN (total number of gas-producing organisms) using the MTF technique, where the highest count was also detected in week 9. This coincided with a significantly higher faecal coliform count (MTF technique) of 1.1×10^6 microorganisms/100 ml recorded in week 9. The significantly high total coliform and *E. coli* counts detected by the MTF technique in weeks 19 and 21, were however, not detected using the MF technique. In comparison, as indicated above, the results for total coliforms and *E. coli* for the Colilert 18® system did not vary in weeks 1 to 13, when the undiluted samples were analysed but varied in weeks 15 to 28, when the samples were diluted to obtain more accurate results.

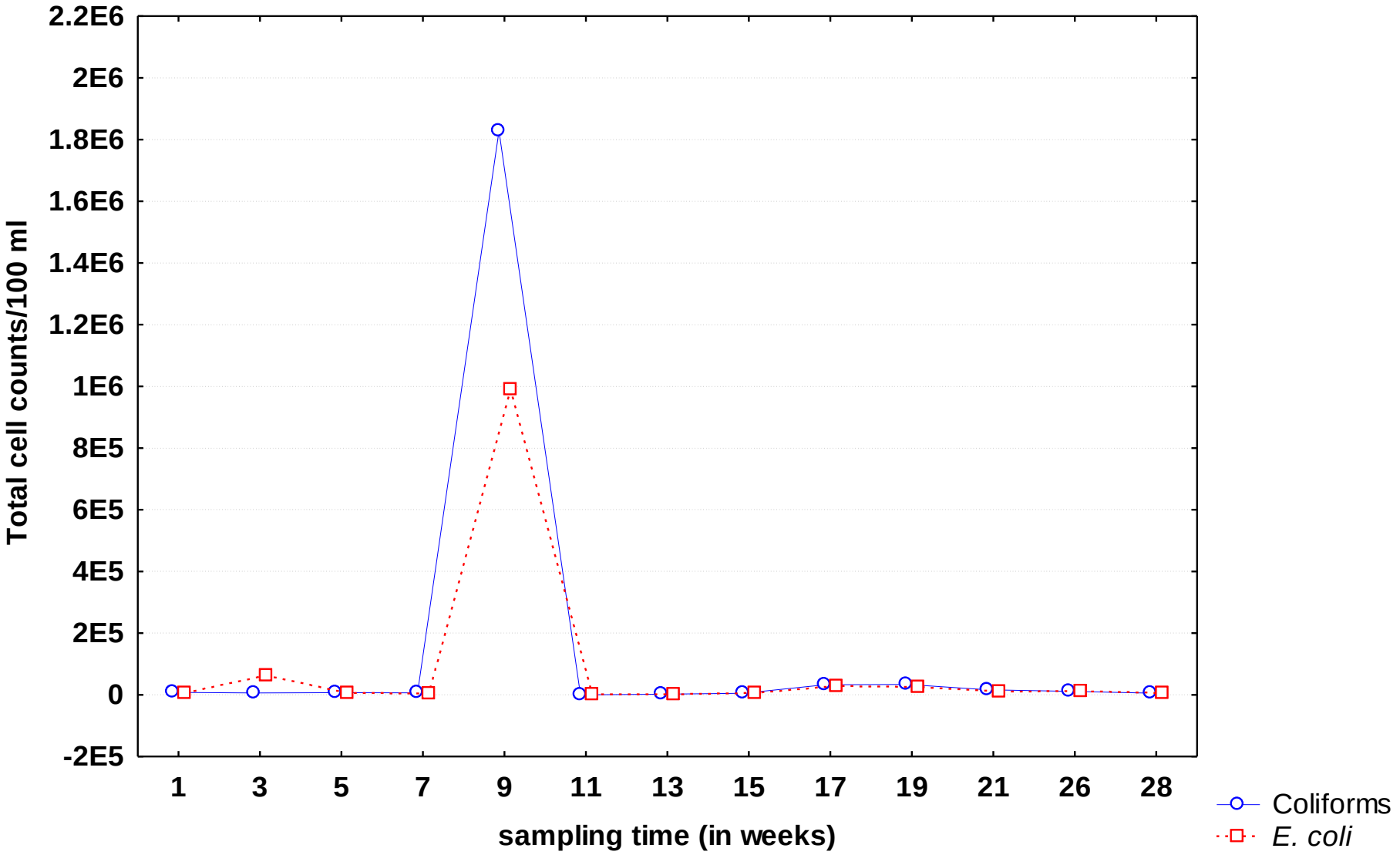

Figure 3.2 Enumeration of total coliforms and *E. coli* per 100 ml of river water sample at Site A2 (Berg River) by means of the Membrane Filtration technique for weeks 1 to 28.

In addition, the total coliform and *E. coli* counts for the MTF technique were also significantly higher ($p < 0.05$) than the results obtained using the MF technique and Colilert 18® system for most of the sampling period.

3.2.2 Total Coliform and *Escherichia coli* Counts Obtained for the Plankenburg River

The microbiological water quality in the Plankenburg River was determined using the *E. coli* and total coliform counts, which are the recommended indicators used by the SABS (1984) and DWAF (1996) for faecal contamination assessment. As indicated in **Section 3.2** above, the total coliform and *E. coli* counts were analysed using the Colilert 18® system, MTF and MF techniques at the point closest to the informal settlement of Kayamandi in Stellenbosch, South Africa. **Figure 3.3** illustrates the microbial counts obtained for the Plankenburg River using the MTF technique, while the MF technique results are presented in **Figure 3.4**. The Colilert 18® system results could not be presented graphically as the number of total coliforms and *E. coli* had no distinct variance due to similar counts obtained in most of the sampling weeks.

On average the most probable number (MPN) detected using the MTF technique ranged from 2.2×10^4 microorganisms/100 ml in week 1 to 1.6×10^7 microorganisms/100 ml in week 28 of the sampling period (**Figure 3.3**). The highest MPN count of 1.6×10^7 microorganisms/100 ml was obtained in weeks 21 and 28, while the lowest count of 2.2×10^4 microorganisms/100 ml was obtained in weeks 1 and 5. The faecal coliforms enumerated by the MTF technique ranged from 1.1×10^3 microorganisms/100 ml in week 1 to 9.2×10^6 microorganisms/100 ml in week 28. The highest faecal coliform count of 9.2×10^6 microorganisms/100 ml was obtained in weeks 21 and 28, while the lowest count of 1.1×10^3 microorganisms/100 ml was obtained in week 1, as illustrated in **Figure 3.3**. The total *E. coli* counts ranged from 2.6×10^3 microorganisms/100 ml in week 1 to 1.6×10^7 microorganisms/100 ml in week 28. The highest *E. coli* count of 1.6×10^7 microorganisms/100 ml was obtained in weeks 21 and 28, while the lowest count of 1.7×10^3 microorganisms/100 ml was obtained in week 5.

The total coliform counts obtained using the MF technique, for the Plankenburg River system, ranged from 1.45×10^3 microorganisms/100 ml in week 1, to 1.82×10^4 microorganisms/100 ml in week 28 (**Figure 3.4**). The highest coliform count of 1.56×10^6 microorganisms/100 ml was obtained in week 9, while the lowest count of 1.45×10^3 microorganisms/100 ml was obtained in week 1.

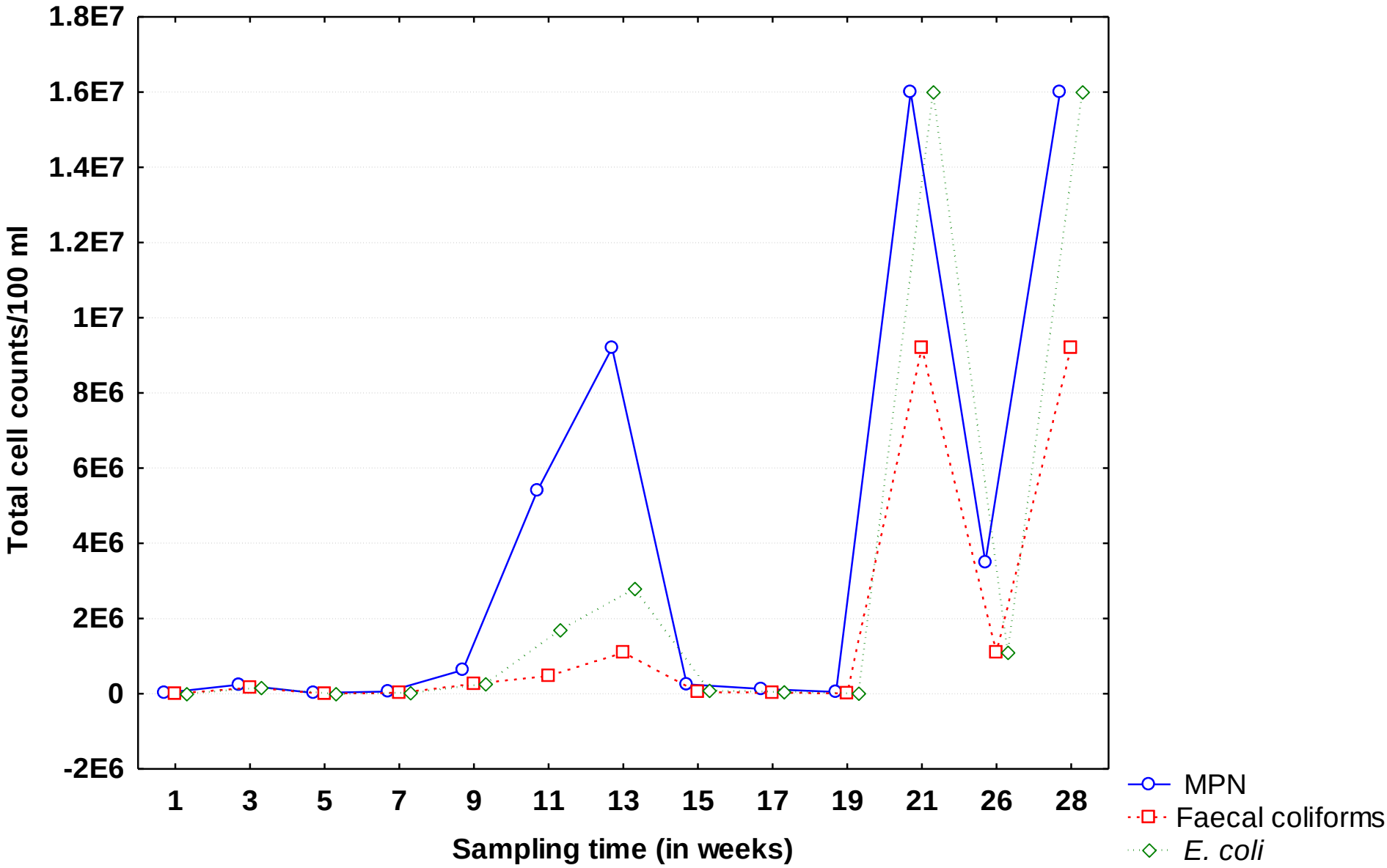

Figure 3.3 Enumeration of Most Probable Number (MPN), representing all possible gas-producing organisms, faecal coliforms and *E. coli* per 100 ml of river water sample by means of Multiple Tube Fermentation technique at Site A1 (Plankenburg River) for weeks 1 to 28.

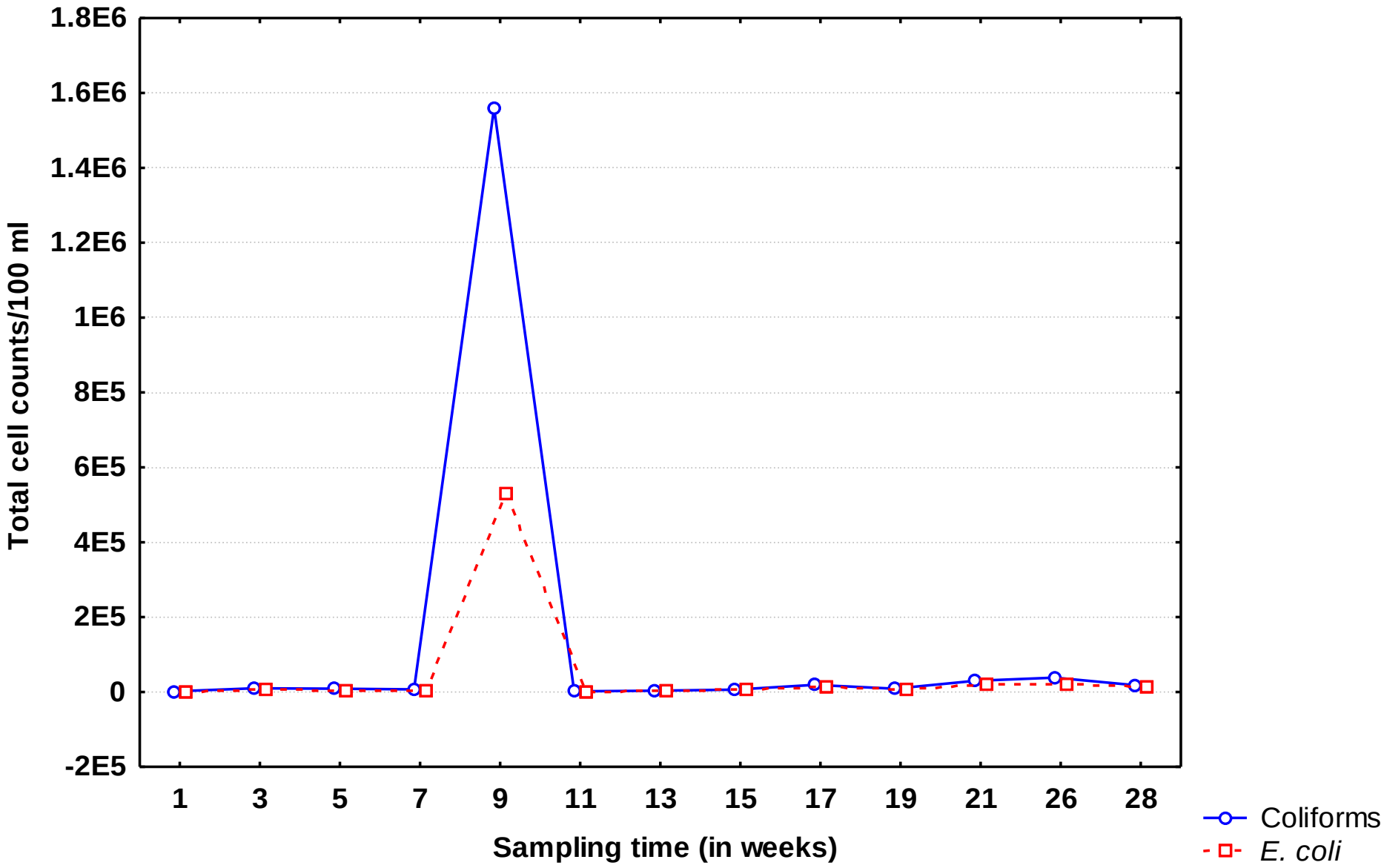

Figure 3.4 Enumeration of total coliforms and *E. coli* per 100 ml of river water sample at Site A1 (Plankenburg River) by means of the Membrane Filtration technique for weeks 1 to 28.

The *E. coli* counts ranged from 3.6×10^2 microorganisms/100 ml in week 1 to 1.41×10^4 microorganisms/100 ml in week 28. The highest *E. coli* count of 5.3×10^5 microorganisms/100 ml was obtained in week 9, while the lowest count of 3.6×10^2 microorganisms/100 ml was recorded in week 1.

The total coliform counts obtained using the Colilert 18® system ranged from 2.42×10^3 microorganisms/100 ml in week 1 to 2.42×10^4 microorganisms/100 ml in week 28. No variance in the total coliform count was detected during the first seven weeks of sampling (undiluted samples) using the Colilert 18® system, while in the last six weeks counts of 2.42×10^4 microorganisms/100 ml were detected. As discussed in **Section 3.2.2**, no variation in the *E. coli* and coliform counts (weeks 1 to 13) were obtained as the samples were analysed undiluted. However, after the samples were diluted and the dilution factor was considered, the *E. coli* and coliform counts increased accordingly.

For the MTF technique (**Figure 3.3**) the highest faecal coliform and *E. coli* counts were obtained in weeks 21 and 28, while for the MF technique (**Figure 3.4**) the highest coliform and *E. coli* counts were obtained in week 9. These results obtained for the MTF technique correspond to the results obtained for MPN (total number of gas-producing organisms) using the MTF technique, where the highest counts were also detected in weeks 21 and 28. However, the significantly high total coliform and *E. coli* counts detected by the MTF technique in weeks 21 and 28, were not detected using the MF technique. In comparison, as indicated above, the results for total coliforms and *E. coli* for the Colilert 18® system did not vary between undiluted samples (weeks 3-13) and the diluted samples (weeks 21 to 28). In addition, the total coliform and *E. coli* counts for the MTF technique were also significantly higher ($p < 0.05$) than the results obtained using the MF technique and Colilert 18® system for most of the sampling period.

3.2.3. Comparison of Multiple Tube Fermentation Technique, Membrane Filtration Technique and the Colilert 18® System

Significantly high ($p < 0.05$) coliform and *E. coli* counts were obtained for both river systems throughout the sampling period, by means of the enumeration techniques (MTF, MF and Colilert 18® system) employed. These results correlate with the coliform and *E. coli* counts observed in previous studies conducted where the water in the Berg and Plankenburg River systems were monitored (Barnes *et al*., 2004; Paulse *et al*., 2007; Paulse *et al*., 2009 and Ackermann, 2010). According to the study conducted by Barnes *et al*. (2004), high coliform and *E. coli* counts of 3.29×10^6 and 1.1×10^6 microorganisms/100 ml, respectively, were detected along the Plankenburg River system, while Ackermann (2010) detected coliform counts of up to 1.6×10^5 microorganisms/100 ml in the same river system. In addition, high

faecal coliform counts of 3.5×10^7 and 1.7×10^6 microorganisms/100 ml in the Berg- and Plankenburg River systems, respectively, were reported using the MTF technique (Paulse *et al.,* 2007; Paulse *et al.,* 2009). All the counts obtained over the one year study period in the Berg and Plankenburg River systems exceeded the stipulated guideline of 130 microorganisms/100 ml set by DWAF (1996) for full contact recreational purposes.

Coliform counts obtained for the Berg River system during the study period exceeded the stipulated guideline of 2 000 microorganisms/100 ml set by SABS (1984) for water used for recreational purposes, except in week 1 where a count of 1.1×10^3 microorganisms/100 ml was obtained using the MTF technique. In the Plankenburg River system, coliform counts also exceeded the stipulated guidelines except in weeks 1 and 11, where the counts of 1.5×10^3 microorganisms/100 ml and 1.8×10^3 microorganisms/100 ml were obtained respectively, using the MF technique.

In the Berg and Plankenburg River systems, all *E. coli* counts obtained by the Colilert 18® system, MF and MTF techniques exceeded the stipulated guidelines of 1 and 130 microorganisms/100 ml set by DWAF (1996) for water used in irrigational and recreational purposes, respectively. Furthermore, during the entire sampling period, the faecal coliform counts recorded using the MTF technique in the Berg River fell within the accepted range of less than 10 000 microorganisms/100 ml (DWAF, 1996) for water used for irrigational purposes in weeks 1, 5, 7 and 15, while in the Plankenburg River it only fell within the range in weeks 1 and 5. Irrespective of the low water temperature of 15.7°C (Plankenburg River) and 14.0°C (Berg River) measured in week 9, high coliform and *E. coli* counts were recorded in the two river systems using the MF and MTF techniques. The significantly high ($p < 0.05$) coliform and *E. coli* counts obtained in week 9 for both river systems using the three techniques could be ascribed to the fact that it was raining [with an average rainfall of 25 and 21 mm in the month of November in Stellenbosch and Paarl, respectively (South African Weather Services, 2010)] and resulted in high water flows through storm drainage pipes from the informal settlements directly into the river systems at the sampling sites (Winter and Mgese, 2011). In weeks 26 and 28, the significantly high ($p < 0.05$) coliform and *E. coli* counts obtained in the Plankenburg River could be ascribed to the high influx of wastewater from the surrounding areas of Kayamandi informal settlement and the industrial park into the storm drainage systems, which flows directly into the river system at the time of sampling, which was also noted during a previous study conducted by Paulse *et al*. (2009).

The MTF technique was the only method, where the total number of possible gas-producing organisms [all organisms that ferment lactose at 35°C in 48 hours (including faecal coliforms and *E. coli*)] in the two river systems could be detected. The coliform and *E. coli* results obtained by the Colilert 18® system could not be compared to the MTF and MF techniques, as similar results were obtained for most of the sampling weeks as outlined in

Sections 3.2.2 and **3.2.3**. The coliform counts obtained by the MTF and MF techniques for the Plankenburg River system were comparable in weeks 1 and 17, while in weeks 5 and 9, coliform and *E. coli* counts recorded by the MF technique were higher than the MTF technique. However, the MTF technique obtained higher coliform and *E. coli* counts than the MF technique for the remainder of the sampling weeks in the Plankenburg River. In the Berg River system, the MTF and MF results were similar for *E. coli* counts obtained in weeks 1, 15 and 17, while the coliform counts were similar in weeks 7 and 15. Higher coliform and *E. coli* counts were obtained in weeks 1, 5 and 9 and weeks 3 and 5, respectively, when compared to the MTF technique for the Berg River system. However, the MTF technique recorded higher coliforms and *E. coli* for the remainder of the sampling weeks in the Berg River system, when compared to results obtained by the MF technique. The overall results for coliforms and *E. coli* obtained for most of the study period showed that higher counts were obtained using the MTF technique in comparison to the MF technique and the Colilert 18® system.

The results of this study indicated that the MTF and MF techniques were comparable in weeks 1, 3, 5, 7, 9, 15 and 17 in the detection and enumeration of coliforms and *E. coli* in river water, and were not comparable to the Colilert 18® system for the entire sampling period. The results obtained during this study were not in agreement with previous studies, where classical methods (MF and MTF techniques) were compared to enzymatic methods (including the Colilert method) in the detection of coliforms and *E. coli* in various water sources (Edberg *et al*., 1990; Jagals *et al.,* 2001; Schets *et al.,* 2002; Noble *et al*., 2004; Buckalew *et al.,* 2006; Nikaaen *et al*., 2009). The MF technique yielded lower *E. coli* and coliforms for most of the sampling weeks, possibly due to clumping of colonies on the culture media. A study conducted by Noble *et al*. (2003) corroborated the results obtained in the current study, as the MTF technique yielded higher coliform and *E. coli* counts when compared to the MF technique in natural seawater samples and seawater samples spiked with wastewater effluents. These results were comparable with previous studies, where the MTF technique was effective in the detection and enumeration of coliforms from various water samples (Eckner, 1998; Rompre *et al.,* 2002).

For the coliforms and *E. coli* counts obtained using the three enumeration techniques, it was noted that the MTF method was more sensitive and obtained higher counts for most of the sampling weeks. It was however comparable to the MF technique in certain weeks of the sampling in the Plankenburg (weeks 1 and 17) and Berg (weeks 1, 3, 7, 15 and 17) River systems. Low sensitivity of the Colilert 18® system was also observed for the entire sampling period in both river systems. It has previously been shown that dilutions (up to 10^{-3}) of highly polluted waters increase the accuracy of the Colilert 18® system to enumerate colifoms and *E. coli* in marine waters (Pisciotta *et al.,* 2002). Eckner (1998) stated that the Swedish standard methods (MTF and MF techniques) and the Colilert 18® methods were equally

sensitive in detecting coliforms and *E. coli* in drinking and bathing water samples. The sensitivity of the Colilert 18® system has also been previously evaluated for the detection of coliforms and *E. coli* in fresh water (Chao *et al.,* 2004; Chao, 2006), drinking water (DeSarno *et al.,* 2008), sewage effluent (Fricker *et al.,* 2008), river water (Buckalew *et al.,* 2006) and it proved to be reliable and equally sensitive to the MTF technique.

The types of media used in each technique have been found to contribute to the efficiency of each technique in detecting and enumerating coliforms and *E. coli*. The MLGA was used in the MF technique to culture coliforms and *E. coli* from water samples. Previous studies found that MLGA, when used in the MF technique, showed low recovery of coliforms and *E. coli* and poor sensitivity in the detection of β-D-glucuronidase activity in *E. coli* isolated from contaminated water (DeSarno *et al.*, 2008; Fricker *et al.*, 2008). The β-D-glucuronidase is pH sensitive and produces acid when *E. coli* ferments the lactose present in MLGA media (it is found to have a higher source of fermentable lactose than other chromogenic media, resulting in significant amount of acids during fermentation thereby reducing the pH) (Fricker *et al.*, 2010a). Surface waters also have high non-coliform organism counts, which could influence the enumeration of target organisms on MLGA plates (DeSarno *et al.*, 2008). Fricker *et al*. (1994) also showed that the MLGA yielded lower recovery and produced smaller target colonies on prepared media stored for more than two weeks. However, the use of selective media such as CCA, on which coliforms and *E. coli* have been successfully cultured (in various water samples), could increase the sensitivity and selectivity of the MF technique (Jagals *et al.,* 2001; Wang and Fiessel, 2008; Hannan *et al.,* 2010).

The media used in the Colilert 18® system can simultaneously detect coliforms and *E. coli* in various water samples. Coliform bacteria maintain their enzyme activity even though they are viable but non-culturable (Eckner, 1998; Fricker *et al.,* 2008), which means that these organisms can be detected by the Colilert 18® system (as they utilise the activity of enzymes produced by coliforms and *E. coli* on chromogenic substrates) and not by the MTF technique. In addition, the use of enzyme activity in this technique to break down specific substrates (*E. coli* and coliforms produce β-glucuronidase, which breaks down the 4-methyl-umbellifryl-β-glucuronide substrate and changes colour from clear to yellow after incubation), reduces cost in re-sampling due to false positives and there is no need for confirmation tests (Sundram *et al.,* 2000; Chao *et al.,* 2004; Chao, 2006; DeSarno *et al.,* 2008; Fricker *et al.,* 2008).

Previous studies have shown that the media used in the Colilert method and MF technique influence the recovery of environmentally stressed microbial cells (maintain their viability and are still able to undergo metabolic activities), which are unable to grow in media used in the MTF technique (Fricker *et al.,* 1997; Eckner, 1998; Olstadt *et al.*, 2007; Nikaeen *et al.,* 2009; Fricker *et al*., 2010b). However, the media used in the

Colilert 18® system and the MF technique are more expensive for routine sampling compared to the media used in the MTF technique.

In the present study, the Colilert 18® system proved to be the most time-effective technique as results were obtained after 19 hours. In comparison, the MF technique yielded results after 24 hours, while the MTF technique results were obtained after 75 hours. The MF and Colilert 18® system are highly reproducible, meaning they can be used to test relatively large sample volumes and yield results more rapidly than the MTF technique (Eckner, 1998; Geissler *et al*., 2000; Chao, 2006; Sadowsky and Whitman, 2011). This is in agreement with previous studies, where these techniques have been used to detect coliforms and *E. coli* in food and water samples (Eckner, 1998; Rompre *et al.,* 2002; Barnes, 2003; Barnes *et al.,* 2004; Buckalew *et al.,* 2006; Paulse*,* 2009). If the coliforms or *E. coli* counts are above the recommended levels in various water types, the analysis and response time implies that the Colilert 18® system can be employed (Schets *et al*., 2002; Niemela *et al*., 2003; Chao *et al.,* 2004; Fricker *et al.,* 2010a) as an indicator of pollution. Based on the results obtained in the recent study, it is thus recommended that polluted water samples be diluted to increase the accuracy of the Colilert 18® system.

The Colilert 18® system and the MF technique are easy to perform, requires less interpretation and laboratory equipment for analysis. The MTF technique employs cost-effective materials (media and reusable glassware), which makes it affordable for routine analysis and basic microbiological training is also required (Buckalew *et al.,* 2006). However, the use of many tubes in the MTF technique for the analysis of one sample could lead to human error (Eckner, 1998; Rompre *et al.,* 2002). Compared to the MTF technique and the Colilert 18® system, the MF technique is also considered more effective as it isolates discrete microbial colonies, which are easily enumerated instead of an approximation by the use of statistical tables (Rompre *et al.,* 2002; Sadowsky and Whitman, 2011).

The MF technique also proved valuable for the routine enumeration of *E. coli* and coliforms in terms of sensitivity, cost- and time-effectiveness (obtaining results in just over 24 hours from sampling time) in this study. The Colilert 18® system could be an alternative technique to provide better and more rapid information for the assessment of coliforms and *E. coli* in river water as it has a low turn-around time to obtain final results (within 24 hours from sampling), less handling of samples is required, which implies less contamination, and it is reproducible and sensitive in detecting coliforms and *E. coli* from all types of water samples. Dilution of highly polluted water samples is however crucial when employing the Colilert 18® system as an enumeration tool.

Compared to the guidelines stipulated by DWAF (1996) and SABS (1984), water from these two river systems poses health risks for irrigation and recreation purposes as the *E. coli* counts obtained during the entire study period using the three techniques (MTF, MF and the Colilert 18® system) exceeded the guidelines stipulated by the South African

authorities. In addition, high indicator organism counts from these river systems is of great concern to fresh produce farmers, consumers and aquatic life, as most farmers draw water from these rivers for irrigation without any treatment and quality analysis due to the high cost of routine analysis (Lotter, 2010; Gemmell and Schmidt, 2010). Monitoring of water quality in these rivers is thus crucial in order to prevent waterborne outbreaks when used for irrigational and recreational purposes, and to prevent the destruction of the aquatic ecosystem.

3.3 identification of *Enterobacteriaceae* species from River water

Three different selective media [Chromocult Coliform Agar (CCA), MacConkey and Violet Red Bile (VRB) agars] were used to isolate coliforms and *E. coli* from river water samples. Water samples were diluted from 10^{-1} to 10^{-2} and spread plated onto the different media. After incubation the colonies typical of *Enterobacteriaceae,* were identified based on colour and morphology as stated in **Sections 1.6.2.1**, **1.6.2.2** and **1.6.2.3.1** for the MacConkey, VRB and CCA agars, respectively. Isolated colonies were then re-streaked onto nutrient agar (NA) and incubated for 18-24 hours before they were identified by the RapID ONE and API 20 E identification systems.

3.3.1. Selective Media Utilised for Water Samples from the Berg- and Plankenburg Rivers

Samples collected at Site A1 along the Plankenburg River and Site A2 along the Berg River systems were cultured on all the selective media used in this study. On MacConkey agar, coliforms ferment lactose thereby producing gas, which produces a neutral red to pink colour, while the non-coliforms do not ferment lactose and produce no gas therefore they appear colourless on the agar medium. *Enterobacteriaceae* species that ferment lactose in the Violet Red Bile agar (VRB) results in the acidification of medium, which is indicated by the changing colour of the neutral red and precipitation of bile acids around colonies to pink and non-fermenters remain colourless. Chromocult coliform agar (CCA) contains two chromogenic substrates namely the salmon-GAL, which is cleaved by β-galactosidase produced by coliforms to yield a red colour on the media and X-glucuronide that is cleaved by β-glucuronidase to yield a blue/violet colour (Manafi, 2000; Finney *et al.,* 2003).

Water samples were cultured on all selective media within six hours from collection and well isolated colonies were re-streaked after 20 to 24 hours of incubation. For both river systems, only a few blue/violet colonies representing *E. coli* were observed on chromocult coliform agar (CCA) for the entire sampling period, which could be due to the presence of non-culturable cells in water (Prescott *et al.,* 2005). Several red colonies, representing the coliform group on CCA, were observed which varied in shape and size. Coliforms are a

large group of organisms with some occurring naturally in the environment and other members of the group finding their way into the environment through discharges of intestinal flora from warm blooded animals (Leclerc *et al.,* 2001; Prescott *et al.,* 2005). **Figure 3.5** illustrates the typical coliform (pink/red) and *E. coli* (blue/violet) colony morphology on CCA when cultured from a water sample collected from the Berg River in week 19.

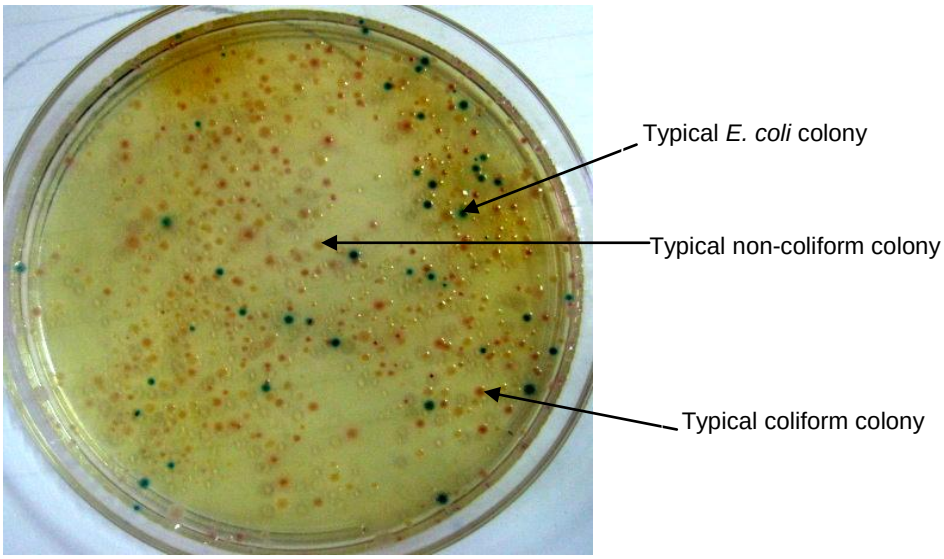

Figure 3.5 Colonies cultured on Chromocult coliform agar from a Berg River water sample collected in week 19.

Non-coliforms were also observed during the sampling period on each CCA plate, and appeared yellow to white in colour. The presence of chromogenic enzyme substrate made it easy to distinguish between non-coliform, coliform and *E. coli* colonies. The specificity of CCA media was also assessed in a previous study conducted by Finney *et al*. (2003) in the identification of *E. coli* and coliforms from faeces. Using this chromogenic media, colonies resembling *E. coli* from the CCA plate were successfully identified, which were then confirmed using the Vitex identification system (BioMérieux, France).

Figure 3.6 (Berg River, week 19) illustrates the growth of typical coliforms on VRB, which distinguishes between lactose fermenting and non-lactose fermenting colonies in water samples. The presence of crystal violet and bile salts inhibits the growth of Gram-positive microorganisms and therefore, many morphologically different violet red (typical lactose positive *Enterobacteriaceae* including coliforms and *E. coli*) and colourless (lactose negative *Enterobacteriaceae,* possible *Klebsiella* species) colonies were observed during the entire sampling period in water samples collected from both river systems.

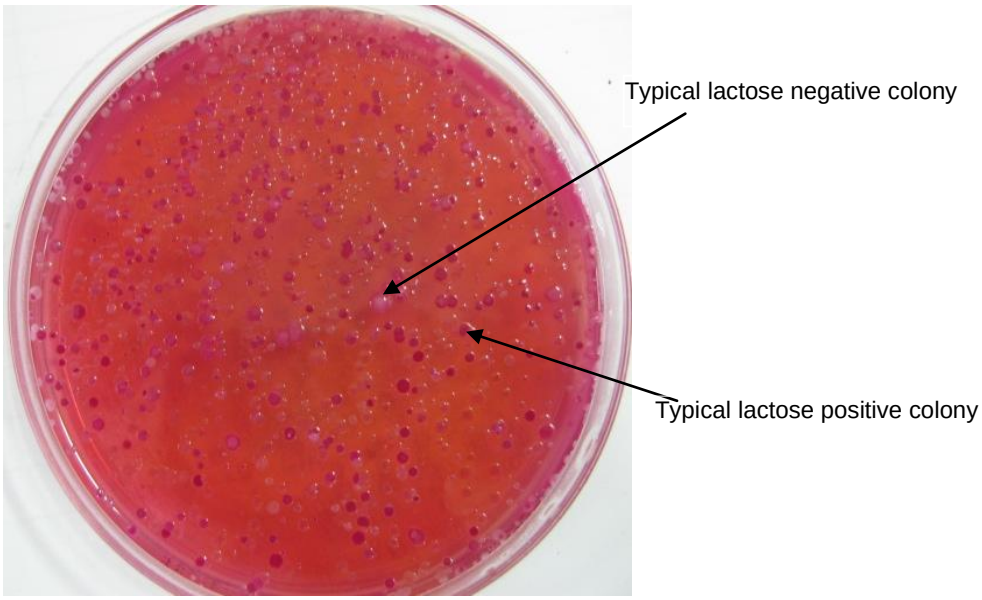

Figure 3.6 Colonies cultured on Violet Red Bile agar from a Berg River water sample collected in week 19.

In general, for both river systems, more lactose fermenting (appearing pink) colonies were detected on MacConkey agar, in comparison to the non-lactose fermenting (appearing colourless) *Enterobacteriaceae* species during the sampling period. **Figure 3.7** illustrates typical lactose and non-lactose fermenting colonies grown on MacConkey agar from water samples collected from the Plankenburg River in week 19.

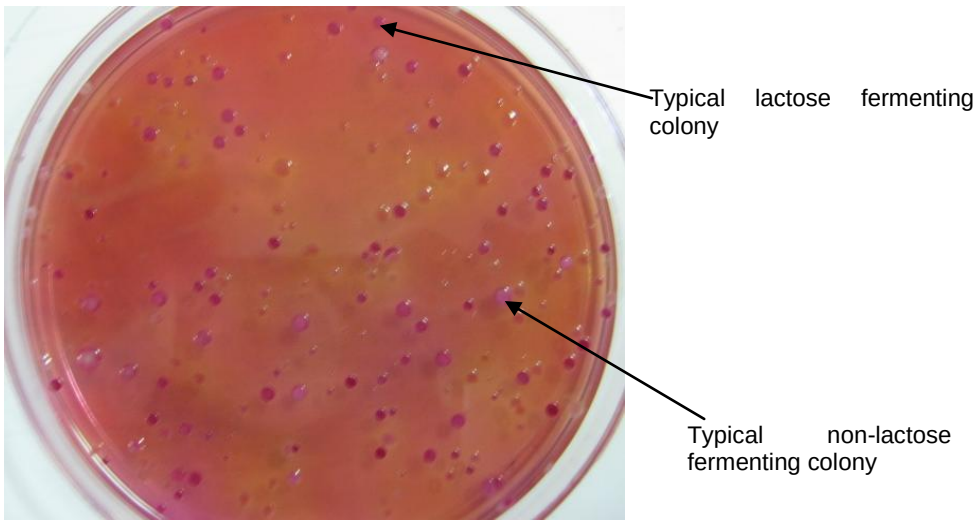

Figure 3.7 Colonies cultured on MacConkey agar from Plankenburg River water sample collected in week 19.

The use of selective media reduces the growth of non-target microorganisms, thereby increasing the recovery of the targeted isolates. Many viable microbial species can be easily cultured using selective and differential media, however difficulties in their identification on isolation media have been experienced. MacConkey and the VRB agars were successfully employed for the isolation of lactose negative and positive *Enterobacteriaceae* species from

water samples for both river systems. However, it was not possible to distinguish between coliforms and *E. coli* using these two selective media. Based on phenotypical differences, the CCA media proved to be the most effective in distinguishing *E. coli* from other coliforms and non-coliform colonies. In addition, CCA proved to be more sensitive than MacConkey and VRB agar for the culturing of *E. coli* and coliforms as more colonies, representing the typical growth of these organisms, were observed. Many of the colonies identified as *E. coli* (based on morphology and colour) on the CCA were then also preliminary identified as *E. coli* using the API 20 E system. Less *E. coli* colonies were identified from the MacConkey and VRB agar plates due to the fact that the colonies on the plates could not be clearly distinguished from other coliforms. Gonzalez *et al*. (2003) conducted a study where CCA was compared to VRB for the culturing of total coliforms and *E. coli* in ready-to-eat meals. Their results also showed that CCA was the more sensitive and accurate media for the cultivation of coliforms and *E. coli*.

3.3.2 Identification of *Enterobacteriaceae* in River Water Samples Using RapID ONE and API 20 E Identification Systems

3.3.2.1 Berg River

The presumptive positive colonies that were identified from each medium (dark-blue/violet and salmon/red colour on CCA plates; violet red and colourless on VRB agar plates; and pink and colourless on MacConkey agar plates) were selected based on their morphology and colour typically representative of *Enterobacteriaceae* strains. The API 20 E and RapID ONE identification systems are used to successfully identify Oxidase negative colonies, which is an additional test required to complete a series of biochemical tests in each system (Kitch *et al*., 1994; Sabae and Rabeh, 2007). The Oxidase activity of colonies was therefore noted prior to identification. **Table 3.2** represents *Enterobacteriaceae* species that were isolated throughout the sampling period using both identification systems in the Berg River water samples. One hundred and sixty one colonies grown on the three selective media from water samples collected throughout the study period were randomly isolated. However, only 132 and 140 colonies could be identified using the RapID ONE and API 20 E systems, respectively, due to insufficient biochemical information with no codes recorded.

The *Enterobacteriaceae* species isolated most often by the API 20 E system, for the entire sampling period, included *K. pneumoniae (15%)*, *K. oxytoca* (14%), *E. coli* (14%) and *Enterobacter cloacae* (11%). The RapID ONE system mostly identified *E. cloacae* (14%), *E. coli* (14%) and *K. oxytoca* (8%) in the Berg River system. *Rahnella aquatilis*, *Cedecea neteri* and *Leminorella richardii* were only identified during weeks 5, 9 and 17, respectively by the RapID ONE system, while the API 20 E system identified *Pantoea* species, *Serratia*

odorifera and *Enterobacter intermedius* during weeks 3, 9 and 19, respectively. Seemingly, more *Enterobacter* species were identified throughout the sampling period using the RapID ONE system, which included *E. aerogenes*, *E. cancerogenous*, *E. cloacae*, *E. sakazakii*, *E. asburiae* and *E. gergoviae*. For the entire sampling period, the RapID ONE system did not detect any *Vibrio* species, *Aeromonas hydrophila*, *Proteus* species, *Serratia odorifera*, while the API 20 E system could not identify *S. sonnei* and *L. richardii*. This indicated that there was more genera diversity in organisms identified by the API 20 E system when compared to the RapID ONE system for the entire study period.

Table 3.2 *Enterobacteriaceae* species isolated from Berg River water samples (Weeks 1-28)

RapID ONE identification system	API 20 E identification system
Acinetobacter calcoaceticus	*Acinetobacter baumannii 1*
Cedecea neteri	*Aeromonas hydrophila*
Citrobacter freundii	*Citrobacter freundii*
Citrobacter species	*Citrobacter koseri*
Enterobacter asburiae	*Enterobacter asburiae*
Enterobacter cancerogenus	*Enterobacter cloacae*
Enterobacter cloacae	*Enterobacter gergoviae*
Enterobacter gergoviae	*Enterobacter intermedius*
Enterobacter sakazakii	*Enterobacter sakazakii*
Enterobacter species	*Esherichia coli*
Esherichia coli	*Hafnia alvei*
Hafnia alvei	*Klebsiella oxytoca*
Klebsiella ornithinolytica	*Klebsiella pneumoniae*
Klebsiella oxytoca	*Kluyvera* species
Klebsiella pneumoniae	*Pantoea* species 3
Kluyvera ascobarta	*Proteus vulgaris*
Kluyvera cryocrescens	*Providencia alcalifaciens*
Leminorella richardii	*Pseudomonas fluorescens/putida*
Proteus mirabilis	*Roultella orthinolytica*
Proteus vulgaris	*Roultella terrigena*
Rahnella aquatilitis	*Salmonella* species
Salmonella 3	*Serratia liquefaciens*
Salmonella choleraesius	*Serratia odorifera*
Salmonella species	*Shewanella putrefaciens*
Shigella sonnei	*Vibrio fluvialis*
Strenotophomonas maltophila	

The detailed list of organisms identified in each sampling week from the Berg River, using the RapID ONE and API 20 E identification systems, are presented in **Appendices C** and **D**, respectively. *Escherichia coli* was identified throughout the sampling period except in week 15, while *K. pneumoniae* was the most isolated organism in the first week of sampling using both identification systems. *Klebsiella oxytoca* was identified during the entire sampling period using the API 20 E system, except in week 1 and 13, while the RapID ONE system did not detect the organism in weeks 5, 17, 21 and 26. Two morphologically different colonies selected from MacConkey agar in week 1 were identified as *Vibrio fluvialis* using the API 20 E system, while in week 9, two colonies with varying morphology (selected from CCA) were both identified as *E. coli*. Most of the individual dark blue/violet coloured colonies that resembled *E. coli* on CCA were however, preliminary identified as *E. coli* using the RapID ONE and API 20 E identification systems. *Shigella sonnei* was only identified using the

RapID ONE system in weeks 1 and in week 19 the *Shigella* isolate identified required more biochemical tests for identification to the species level.

3.3.2.2 Plankenburg River

A total of 152 colonies were randomly isolated from the Plankenburg River water samples during the sampling period, of which 114 and 129 colonies were identified using the RapID ONE and the API 20 E identification systems, respectively. A detailed table presenting all microorganisms identified from the isolated colonies on different media using the RapID ONE and API 20 E identification systems is depicted in **Appendices E** and **F**, respectively. Certain colonies could not be identified as they required additional biochemical tests to complete the identification process. Each identification system required an Oxidase test to complete the biochemical tests required, the RapID ONE system only identifies Oxidase negative bacteria (Kitch *et al.,* 1994), while the API 20 E system requires the Oxidase test to complete the set of biochemical tests (Sabae and Rabeh, 2007). **Table 3.3** represents the *Enterobacteriaceae* species that were isolated throughout the sampling period using both identification systems in the Plankenburg River water samples.

Table 3.3 *Enterobacteriaceae* species isolated from the Plankenburg River water samples (Weeks 1-28)

RapID ONE identification system	API 20 E identification system
Acinetobacter calcoaceticus	*Acinetobacter baumannii*
Cedecea species	*Aeromonas hydrophila* 1 *and 2*
Citrobacter freundii	*Citrobacter freundii*
Citrobacter koseri	*Citrobacter koseri*
Citrobacter species	*Citrobacter youngae*
Enterobacter aerogenes	*Enterobacter cloacae*
Enterobacter asburiae	*Enterobacter sakazakii*
Enterobacter cancerogenus	*Enterobacter* species
Enterobacter cloacae	*Erwina* species
Enterobacter sakazakii	*Esherichia coli*
Enterobacter species	*Hafnia alvei*
Esherichia coli	*Klebsiella oxytoca*
Hafnia alvei	*Klebsiella pneumoniae*
Klebsiella ornithinolytica	*Kluyvera* species
Klebsiella oxytoca	*Lecleria adecarboxylata*
Klebsiella pneumoniae	*Pantoea* species
Klyuvera ascobarta	*Providencia alcalifaciens*
Klyuvera cryocrescens	*Providencia stuartii*
Leminorella richardii	*Pseudomonas aeruginosa*
Proteus mirabilis	*Pseudomonas fluorescens*
Proteus vulgaris	*Pseudomonas luteola*
Providencia alcalifaciens	*Roultella orthinolytica*
Salmonella 1	*Roultella terrigena*
Salmonella choleraesius	*Salmonella* species
Salmonella species	*Serratia fonticola*
Serratia marscecens	*Serratia liquifaciens*
Serratia odorifera	*Serratia marscecens*
Shigella sonnei	*Shewanella putrifaciens*
Shigella species	*Vibrio fluvialis*

In the first week of sampling, *E. coli* was only identified by the RapID ONE system from the MacConkey plates, while in week 13 it was detected on all the selective media used. *Klebsiella oxytoca* (17%), *K. pneumoniae* (16%) and *E. coli* (15%) were identified most often in the Plankenburg River system by the API 20 E system, while *Citrobacter freundii (17%)*, *E. cloacae* (14%) and *K. oxytoca* (18%) were identified more frequently using the RapID ONE system during the study period. *Enterobacteriaceae* species identified during this study period is in agreement with bacterial species previously identified by Barnes *et al*. (2004), using various selective media (MacConkey, Tryptose blood agar, *Salmonella* and *Shigella* agar, Xylose lysine desoxycholate agar and Brain heart infusion Tetrathionate broths) from the Plankenburg River system.

The majority of the isolates identified using the API 20 E system and the RapID ONE test belonged to the *Enterobacteriaceae* group. The RapID ONE system identified *K. oxytoca (*a normal microbiota of the large intestine) during the entire sampling period, indicating faecal contamination (Prescott *et al.,* 2005). *Salmonella* could not be identified to the species level using the API 20 E system, while the RapID ONE could identify *Salmonella choleraesuis* in week 1 of sampling. The RapID ONE system identified *Shigella* species in weeks 1 and 9, from colonies isolated from the CCA plates, while this species could not be identified using the API 20 E system. *Shigella* and *Amalonaticus* species were only identified by the RapID ONE system, while *Lecleria adecarboxylata*, *Raoultella terrigena* and the *Pseudomonas* species were only identified by the API 20 E system during the sampling period. Lotter (2010) isolated indicator organisms using culture-specific media, and then proceeded to identify these organisms up to the species level using the API identification systems (including API 20 E system).

The RapID ONE and API 20 E systems were used for the preliminary identification of colonies isolated from water samples after culturing on selective media to the species level. Some of the *Enterobacteriaceae* species isolated from the two river systems could not be identified to species level using the API 20 E and RapID ONE systems, as additional biochemical tests were required to accurately identify each microorganism (Juang and Morgan, 2001). Most of the individual violet coloured colonies, that resembled *E. coli* on CCA, were preliminary identified as *E. coli* using the RapID ONE and API 20 E identification systems, while it was difficult to distinguish *E. coli* colonies from other coliforms using the MacConkey and VRB agars. The API 20 E system proved to be a better identification system, as more genera diversity in the results were obtained when compared to the RapID ONE system. This showed that the API 20 E system is more sensitive than the RapID ONE system, as previously observed in a study conducted on clinical samples (Overman *et al*., 1985).

3.3.2.3 Comparison of API 20 E and RapID ONE Systems

The majority of the isolates identified using the API 20 E system and the RapID ONE test belonged to the *Enterobacteriaceae* group. It is much easier to read the API 20 E results than the RapID ONE system results. This is in agreement with the previous studies, where the API 20 E was used for the identification of *Enterobacteriaceae* species from activated sludge and river water and it was concluded that this system was highly sensitive for the identification of these species (Juang and Morgan, 2001; Sabae and Rabeh, 2007). The RapID ONE system was also previously found to yield results comparable to the API 20 E system (Kitch *et al.*, 1994). However, some *Enterobacteriaceae* species were not completely identified using the API 20 E and RapID ONE systems because of errors when reading the strips (colour reactions). In addition, certain microorganisms are not listed in the databases. This was highlighted in a study conducted by Popovic *et al.* (2007), who identified bacterial species in fish. The API identification systems were also used to assist in the assessment of pathogen-transfer from contaminated water onto vegetables and fruits where this water (without treatment) was used for irrigation (Ackermann, 2010; Lotter, 2010).

Approximately 48 hours is required from the culturing of the respective colonies, to the incubation of test organisms in their respective biochemical wells in both identification systems. The RapID ONE system has a turn-around time of approximately five hours to obtain the final results, while for the API 20 E system it is 18-24 hours. This concurs with the previous study performed by Kitch *et al.* (1994), where the two identification systems were compared for the identification of *Enterobacteriaceae* species from clinical samples. The API 20 E system is more expensive compared to the RapID ONE system, which implies that it could not be used in routine analysis.

The presence of *Enterobacteriaceae* species, as shown in **Table 3.2** and **Table 3.3**, indicates that the Berg- and Plankenburg Rivers were faecally contaminated at Site A2 and Site A1, respectively. Malfunctioning sewage systems and general run off from the informal settlements can transport microorganisms into the river systems (Winter and Mgese, 2011). Human waste, which generally contains high levels of possible pathogenic microorganisms, also commonly finds its way into rivers due to a lack of proper sanitation facilities (Barnes and Taylor, 2004; Gemmell and Schmidt, 2010). In addition, many of the organisms identified are commensals of warm blooded animals, belonging to the coliform group and are generally used as indicators to evaluate faecal pollution in environmental samples (Jagals *et al.,* 2001; Prescott *et al.*, 2005). The river systems were also found to have a large variety of *Enterobacteriaceae* species present throughout the sampling period, indicating the possible presence of highly pathogenic parasites, bacteria and viruses, which can lead to major health disorders of humans, animals and aquatic organisms if the contaminated water is used without treatment (Prescott *et al*., 2005; Ackermann, 2010; Gemmell and

Schmidt, 2010). The presence of *Enterobacteriaceae* strains such as *Klebsiella*, *Enterobacter* and *Salmonella* species constitutes a potential health hazard to the elderly, children and the immunosuppressed individuals, as these strains are classified as opportunistic pathogens, which may cause gastroenteritis, diarrhoea and urinary tract infections (Prescott et al., 1993; Obasohan et al., 2010).

3.4 Polymerase Chain Reaction

3.4.1 Optimisation of DNA Extraction from Control Strains

To optimise a method for the rapid extraction of bacterial deoxyribonucleic acid (DNA) from reference strains and water samples, two methods namely the boiling method and High Pure PCR Template Preparation kit were used (**Section 2.5.3.1**). The boiling method was performed on the reference bacterial strains as previously outlined by Watterworth *et al.* (2005), DNA was analysed on a 1.0% agarose gel but did not show significant yields of DNA. The boiling method was then modified by the addition of a cooling step on ice for 10 minutes, which showed a significant increase in the yields of DNA from some strains after analysis on a 1.0% agarose gel as illustrated in **Figure 3.8**.

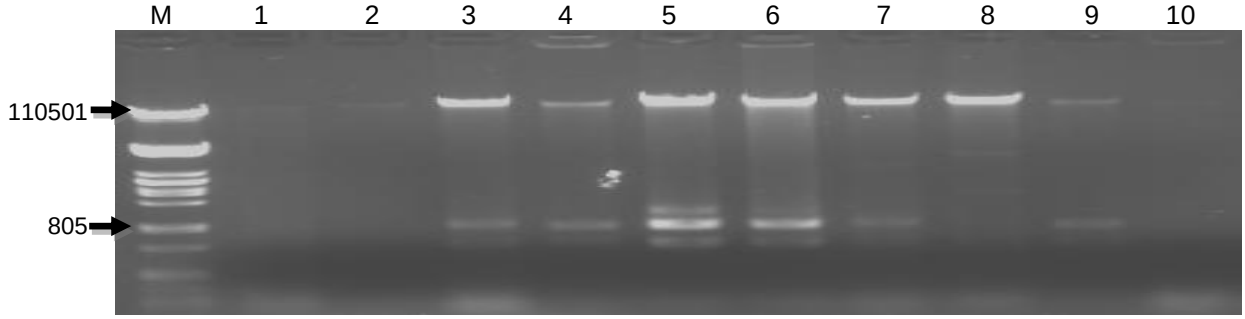

Figure 3.8 Agarose gel electrophoresis showing uncut genomic DNA extracted from reference strains using the boiling method. Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *Bacillus cereus*; lane 2: *Micrococcus luteus*; lane 3: *Klebsiella pneumoniae*; lane 4: *Enterococcus faecalis*; lane 5: *Pseudomonas aeruginosa;* lane 6: *Serratia marcescens*; lane 7: *Shigella sonnei*; lane 8: *Salmonella typhimurium*; lane 9: *Pseudomonas fluorescens* and lane 10: *Vibrio cholerae.*

Visible DNA bands were observed in lane 3 to lane 9 and represented DNA extracted from *K. pneumoniae*, *E. faecalis*, *P. aeruginosa*, *S. marcescens*, *S. sonnei*, *S. typhimurium* and *P. fluorescens,* respectively. Lane 1 (*B. cereus*), lane 2 *(M. luteus)* and lane 10 (*V. cholerae)* showed light bands. One microlitre of template DNA for each control strain was used in the Qubit fluorometer (Invitrogen, USA), to detect the concentration.

Figure 3.9 illustrates DNA samples extracted from the *Escherichia coli* strain using the boiling method and High Pure PCR template preparation kit. The *E. coli* was obtained just after the extraction of DNA using the boiling method from all other *Enterobacteriaceae* species (except *V. cholerae*) and electrophoresis had been performed, and it led to the

analysis of DNA in a separate agarose gel (**Figure 3.9**). The DNA extracted using the boiling method (lane 1) showed a thicker DNA band than the kit (lane 2). The DNA concentration obtained using both methods was measured by the Qubit™ fluorometer and recorded in **Table 3.4.**

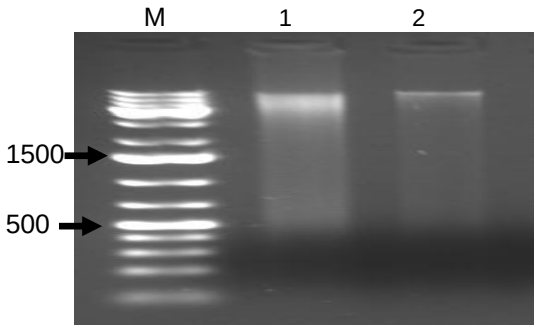

Figure 3.9 Agarose gel electrophoresis showing uncut genomic DNA extracted from *Escherichia coli* reference strain*:* Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: boiling method; lane 2: Kit

The measurements of DNA concentrations for all the *Enterobacteriaceae* reference strains using the Qubit™ fluorometer are shown in **Table 3.4**. The DNA concentrations ranged from 1.19 µg/ml (*M. luteus*) to 29.4 µg/ml (*Serratia marcescens)* as shown in **Table 3.4**. The concentration for DNA bands representing *B. cereus*, *M. luteus* and *V. cholerae* (not visible on agarose gel) were 4.19, 1.19 and 5.17 µg/ml, respectively. Even though some organisms DNA was not visible on the agarose gel, the DNA obtained using the boiling method from all organisms were still used for PCR to detect specific genes for each strain.

Table 3.4 Deoxyribonucleic acid concentrations from *Enterobacteriaceae* species used as reference strains

Organism	**Boiling method (µg/ml)**	**High Pure Template Preparation Kit (µg/ml)**
Bacillus cereus	4.29	3.77
Enterococcus faecalis	2.18	1.72
Escherichia coli	19.2	14.3
Klebsiella pneumoniae	16.0	5.18
Micrococcus luteus	1.19	0.6
Pseudomonas aeruginosa	16.0	13.3
Pseudomonas fluorescens	12.59	7.6
Salmonella typhimurium	18.8	5.29
Serratia marcescens	29.4	15.6
Shigella sonnei	18.9	5.14
Vibrio cholerae	5.17	Not used

Previous studies have shown that as little as 10 ng/µl DNA was sufficient to be amplified by PCR (Manzano *et al*., 2003; Liu *et al*., 2008). Furthermore, Watterworth *et al*. (2005) used the boiling method to extract DNA from pathogenic *E. coli* strains (enteric and some environmental strains). The authors showed that the boiling method yielded quality DNA, which could be used to amplify specific genes by PCR.

The High Pure PCR Template preparation kit involved many steps in the extraction of DNA and was more time consuming and costly compared to the boiling method. **Figure 3.10** illustrates results of DNA analysis (on 1.0% agarose gel) obtained using the High Pure PCR Template Preparation kit. As observed in **Figure 3.10**, lanes 3 (*K. pneumoniae*), 5 (*P. aeruginosa*) and 9 (*P. fluorescens*) produced visible DNA bands on the agarose gel while the other lanes, which represented the rest of the DNA extracted from other strains, were not visible on the gel. Deoxyribonucleic acid concentration (1 µl) was measured and recorded as shown in **Table 3.4**. The Qubit™ fluorometer measured the DNA on all *Enterobacteriaceae* strains and it ranged from 0.6 µg/ml (*M. luteus*) to 15.6 µg/ml (*S. marcescens*). Lanes 1, 2, 4, 6, 7 and 8 were not visible on agarose gel but after the measurement of DNA concentration, it was thought to be sufficient for PCR.

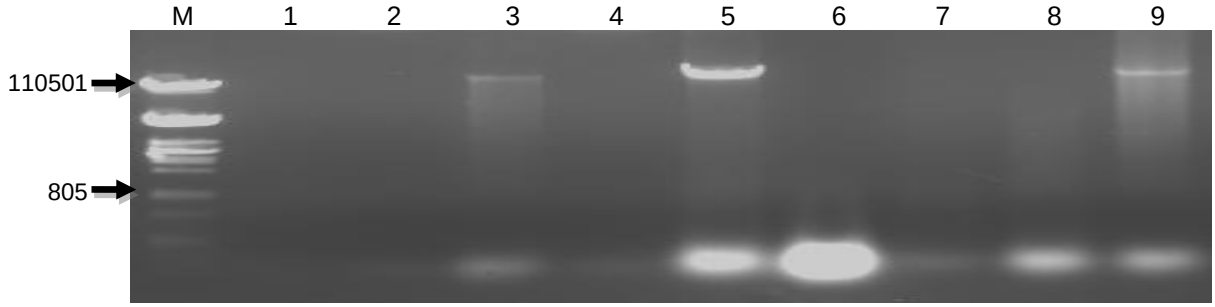

Figure 3.10 Agarose gel electrophoresis representing uncut genomic DNA extracted from reference strains using the High Pure PCR Template Preparation kit. Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *B. cereus*; lane 2: *M. luteus*; lane 3: *K. pneumoniae;* lane 4: *E. faecalis;* lane 5: *P. aeruginosa;* lane 6: *S. marcescens;* lane 7: *S. sonnei*; lane 8*: S. typhimurium*; lane 9: *P. fluorescens*.

The *V. cholerae* reference strain was received late within the project and since the boiling method proved to be successful for all other strains, was cost-effective and less time-consuming, it was the only method employed for extraction of DNA from the *V. cholerae* strain (Table 3.4). The Qubit fluorometer showed that both methods successfully extracted DNA from all the *Enterobacteriaceae* species (except *V. cholerae* as explained above) used as reference strains as illustrated by the DNA concentrations recorded in **Table 3.4**. The noticable difference between DNA extracted using the two methods (**Table 3.4**) was that the High Pure Template Preparation kit extracted lower concentration of DNA, while the boiling method extracted higher concentrations. It is assumed that the quality of DNA yielded using the High Pure PCR Template Preparation kit was good, since the boiling method did not have a step to remove inhibitors such as humic acids, organic and inorganic substances,

which influences the quality of DNA. It is proposed that these inhibitors can interrupt the amplification of specific genes when compared to the boiling method, which does not have inhibitor removal steps. However, literature has shown that DNA extracted from various samples using the boiling method could successfully be used as template DNA to amplify any gene of interest using PCR (Millar *et al.,* 2000; Watterworth *et al.,* 2005; Chaudhuri *et al*., 2006; HwangBo *et al.,* 2010). The boiling method was found to be cost- and time-effective, and less tedious when compared to the High Pure PCR Template Preparation kit, which involved many steps and required more time. After extraction of DNA using both methods (except for *V. cholerae*) and the measurements of DNA concentration, optimisation of the conventional PCR was then done on all DNA samples extracted from reference strains used throughout the study.

3.4.1.1 Optimisation of Conventional PCR Using Control Strains

Deoxyribonucleic acid extracted from reference strains obtained from the boiling method (adapted from Watterworth *et al*., 2005) and High Pure PCR Template Preparation kit was used as the DNA templates for the optimisation of conventional PCR. Previously published species specific primers were used to amplify specific genes in each *Enterobacteriaceae* species used in the study (**Table 2.2**). The negative control used for all PCR reactions contained all reagents used within the PCR for specific organism amplification, excluding the template DNA, which was substituted with sterile water. **Figure 3.11** illustrates the PCR products obtained using the template DNA from *K. pneumoniae* and *E. faecalis.* Specificity of the primers targeting the 16S-23S rRNA internal transcribed spacer (ITS) in *K. pneumoniae* was previously evaluated by Liu *et al*. (2008), when the same primers were used on 31 different strains of *K. pneumoniae* and 79 non-*K. pneumoniae* bacterial strains.

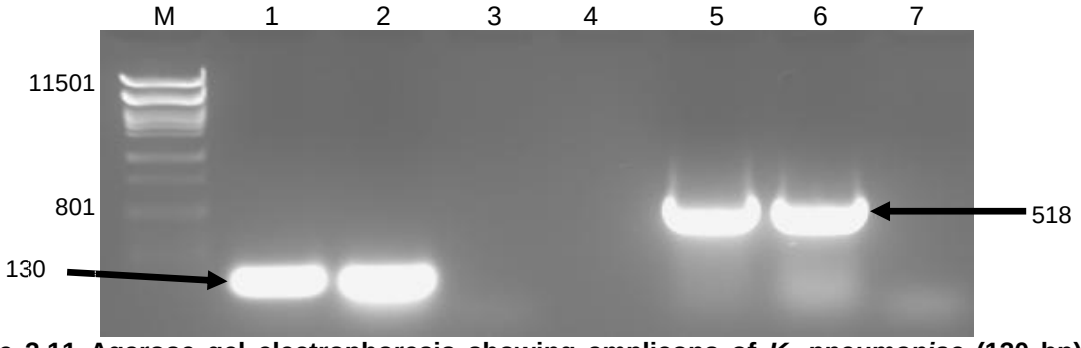

Figure 3.11 Agarose gel electrophoresis showing amplicons of *K. pneumoniae* (130 bp) and *E. faecalis* (518 bp). Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *K. pneumonia* (boiling method); lane 2: *K. pneumoniae* (kit); lane 3: *K. pneumoniae* negative control; lane 4: No sample loaded; lane 5: *E. faecalis* (boiling method); lane 6: *E. faecalis* (kit); lane 7: *E. faecalis* negative control.

The primers were shown to be specific for *K. pneumoniae*. The 16S-23S rDNA internal transcribed spacer (ITS) gene specific for *K. pneumoniae* was successfully amplified from

DNA templates extracted using both methods as shown (**Figure 3.11**) in lane 1 (boiling) and lane 2 (kit) by producing a 130 bp product size on agarose gel. Lane 3 represented the negative control, where double distilled water was used instead of template DNA with the species specific primers targeting the internal transcribed spacer for the *K. pneumoniae*. It was noted that only the expected 130 bp DNA band was observed on agarose gel for *K. pneumoniae* from DNA extracted using the two methods. A slightly thicker DNA band was observed in lane 2 (kit), even though lower DNA concentrations were observed using the kit (5.18 µg/ml) than the boiling method (16.0 µg/ml) (Table 3.4). A possible reason could be that the DNA extracted by the kit (lane 2) was purer when compared to the DNA obtained by the boiling method (lane 1). Both amplicons were sent for sequencing and were confirmed positive for the ITS region by the online Basic Logarithm Search Tool (BLAST) program (Altschul *et al.,* 1997) (**Figure 3.11**).

The species specific primers successfully amplified the putative transcriptional gene (518 bp) in *E. faecalis* using template DNA extracted by the 2 methods [lane 5 (boiling) and lane 6 (kit)] as shown on the agarose gel (**Figure 3.11**). During the optimisation in this study, only a 518 bp DNA band was observed on the agarose gel and no DNA band was seen when sterile water was used as a template (lane 7). For *E. faecalis*, approximately the same amplicon intensity was observed for template DNA extracted using the boiling method (lane 5) and the kit (lane 6). Polymerase chain reaction amplicons were then sent for sequencing and confirmed positive by the online BLAST program for the putative transcriptional regulator gene from both DNA templates (Altschul *et al.,* 1997).

Figure 3.12 illustrates the amplicons of *P. aeruginosa* (956 bp) and *P. fluorescens* (850 bp) analysed on 1.5% agarose gel from DNA extracted using the two aforementioned methods. Lane M represented the molecular weight marker, while lane 1 (boiling) and lane 2 (kit) showed a 850 bp DNA band amplified in the 16S rRNA region specific for *P. flourescens*. A product of 956 bp was observed in lanes 5 (boiling) and 6 (kit), which represented the 16S rDNA region specific for *P. aeruginosa*. The 4 amplicons illustrated in **Figure 3.12** were sequenced and confirmed positive by the online BLAST program for the 16S rRNA and 16S rDNA regions specific for *P. fluorescens* and *P. aeruginosa*, respectively (Altschul *et al.,* 1997). As shown in **Figure 3.12**, more product was observed from the templates obtained using the kit [lane 2 (*P. fluorescens)* and lane 6 (*P. aeruginosa*)] compared to the boiling method [lane 1 (*P. fluorescens)* and lane 5 (*P. aeruginosa*)]. As mentioned before, the reason for the differences could be due to the purity of DNA extracted using the kit and presence of inhibitors in the template DNA extracted using the boiling method. Negative controls containing distilled water as a template were prepared for each organism and are represented in lane 3 (*P. fluorescens* negative control) and lane 7 (*P. aeruginosa* negative control).

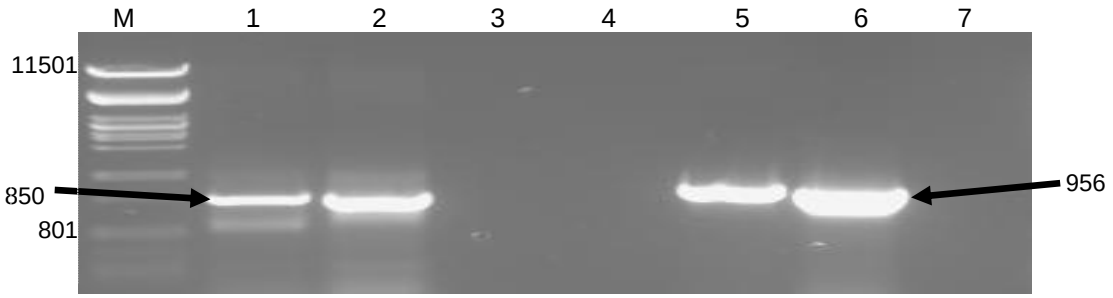

Figure 3.12 Agarose gel electrophoresis showing PCR amplification results of the *P. fluorescens* (850 bp) and *P. aeruginosa* (956 bp). Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *P. fluorescens* (boiling); lane 2: *P. fluorescens* (kit); lane 3: *P. fluorescens* negative control; lane 4: Empty; lane 5: *P. aeruginosa* (boiling); lane 6: *P. aeruginosa* (kit); lane 7: *P. aeruginosa* negative control.

The species specific primers targeting 16S rRNA region (*M. luteus 1*, **Table 2.2***)* and *fimY* gene (*S. typhimurium*) generated 1600 bp and 526 bp amplicons, respectively as illustrated in **Figure 3.13** from template DNAs extracted using both the boiling method and High Pure PCR Template Preparation kit. The 16S rRNA region specific primers for *M. luteus* 1 produced an expected product size of 1600 bp as observed on an agarose gel (**Figure 3.13**), with a thicker band produced on template DNA extracted using the boiling method (lane 1). The 1600 bp DNA bands were then sent for sequencing but were negative for the 16S rRNA region specific for *M. luteus* after searching the online BLAST (Altschul *et al.,* 1997).

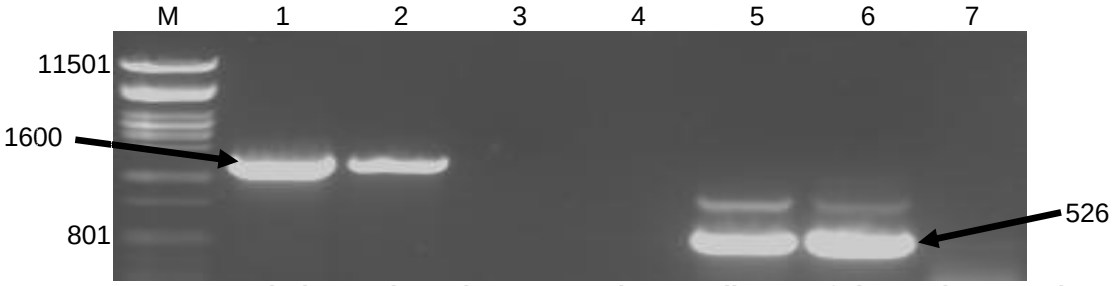

Figure 3.13 Agarose gel electrophoresis representing amplicons of the *M. luteus 1* (1600 bp) and *S. typhimurium* (526 bp). Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *M. luteus* (boiling); lane 2: *M. luteus* (kit); lane 3: *M. luteus* negative control; lane 4: Empty; lane 5: *S. typhimurium* (kit); lane 6: *S. typhimurium* (boiling); lane 7: *S. typhimurium* negative control.

A new set of primers adopted from Mukamolova *et al*. (2002), which targeted the *rpf* gene (**Table 2.2**) in *M. luteus* were then used. This set of species specific primers produced a 610 bp product as shown in **Figure 3.14**. It was only applied using the template DNA extracted using the boiling method, as the primers were received later in the project and also the amplicons from the previous primer sets produced a thicker band on the template DNA extracted using the boiling method (**Figure 3.13**). After sequencing, the *rpf* gene in *M. luteus* was confirmed using the online BLAST program (Altschul *et al.,* 1997). This primer set was then applied to DNA collected from river water.

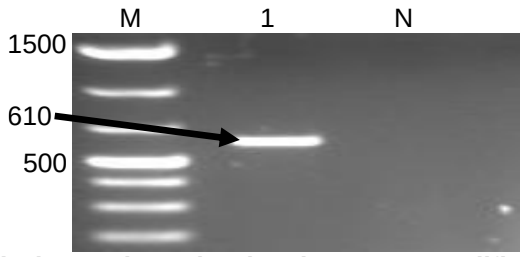

Figure 3.14 Agarose gel electrophoresis showing PCR amplification results of the *rpf* gene (610bp) in *M. luteus* reference strains. Lane M: Molecular weight marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane 1: *M. luteus* (boiling); lane N: *M. luteus* negative control.

Non-specific bands above the 526 bp DNA band were observed for the *fimY* gene in *S. typhimurium* [lane 5 (kit), lane 6 (boiling)] (**Figure 3.13**), which led to a further increase of the annealing temperature, which yielded a single 526 bp DNA band from template DNA extracted using both methods (**Figure 3.15**). Similar amounts of amplicon products (**Table 3.4**) were observed from template DNA extracted by the boiling method (lane 1) and the kit (lane 2). The 526 bp DNA bands representing the *fimY* gene in *S. typhimurium* illustrated in **Figure 3.15** were then sequenced and confirmed positive by the online BLAST program (Altschul *et al.,* 1997).

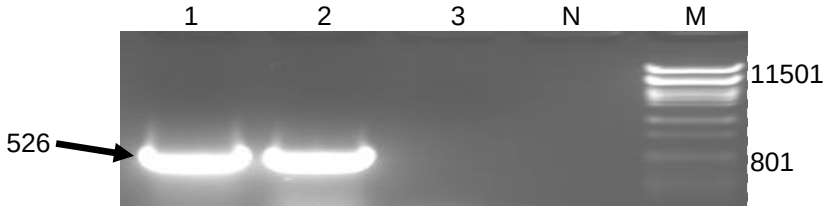

Figure 3.15 Agarose gel electrophoresis representing amplicons of *S. typhimurium* (526 bp). Lane 1: *S. typhimurium* (boiling); lane 2: *S. typhimurium* (kit); lane 3: Empty; lane N: *S. typhimurium* negative control and lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas).

With the aid of species specific primers targeting the *gyrB* gene of *B. cereus*, a 374 bp amplicon was observed on an agarose gel (**Figure 3.16**). The amplicons produced from template DNAs extracted using the boiling method (lane 1) and the kit (lane 2) were approximately of the same intensity. It was sequenced and confirmed positive for the *gyrB* gene found in *B. cereus* by the online BLAST program (Altschul *et al.,* 1997) obtained from the National Centre for Biotechnology Information website.

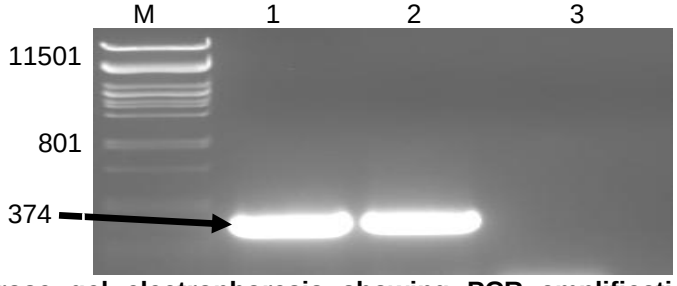

Figure 3.16 Agarose gel electrophoresis showing PCR amplification results of the *gyrB* (374 bp) gene specific for *B. cereus*. Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *B. cereus* (boiling); lane 2: *B. cereus* (kit); lane 3: *B. cereus* negative control.

Figure 3.17 shows the PCR products (369 bp) obtained after the amplification of the insertion sequence 1 region for *S. sonnei* from DNA templates extracted using the boiling method (lane 1) and the kit (lane 2). Similar DNA band intensity was observed for both the products for boiling method (lane 1) and kit (lane 2) DNA templates.

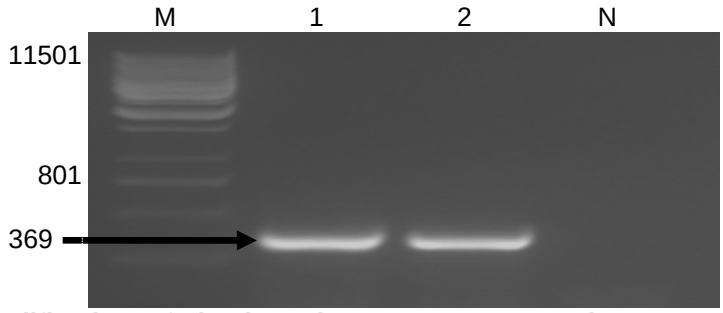

Figure 3.17 Amplification of the insertion sequence gene in *S. sonnei* (369 bp). Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *S. sonnei* (Boiling); lane 2: *S. sonnei* (kit); lane N: *S. sonnei* negative control.

The amplification of the insertion sequence 1 region specific for the *S. sonnei* was successful after sequencing and using the online BLAST program (Altschul *et al.,* 1997) obtained from the National Centre for Biotechnology Information website.

The *ompW* gene of *V. cholerae* was amplified using the species specific primers to produce a 588 bp DNA band as shown in **Figure 3.18**. As indicated earlier (**Section 3.4.1**), only the template DNA extracted using the boiling method was used, and the *ompW* gene was confirmed positive after sequencing and using the online BLAST program (Altschul *et al.,* 1997).

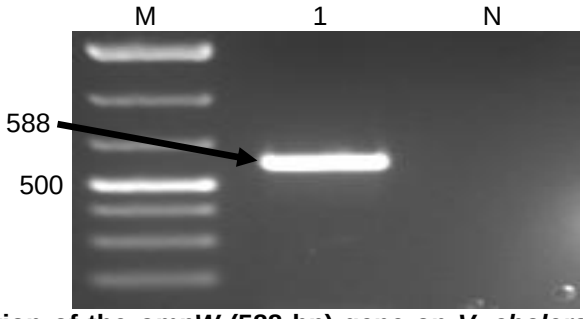

Figure 3.18 Amplification of the *ompW* (588 bp) gene on *V. cholerae.* Lane M: Molecular weight marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane 1: *V. cholerae* (boiling); lane N: *V. cholerae* negative control.

Zhu *et al.* (2008) used the *pfs* and *luxS* genes responsible for quorum-sensing molecules as diagnostic markers for *S. marcescens*. During this study amplification of the *S. marcescens* was first performed using these two sets of primers targeting the *pfs* (lanes 1 and 2) and the *luxS* (lanes 3 and 4) genes, which yielded DNA bands of 193 bp and 102 bp, respectively on an agarose gel as shown in **Figure 3.19**. For the *pfs* gene in *S. marcescens*, specific primers were used and non-specific bands were observed on template DNA extracted using

the boiling method (lane 1) and kit (lane 2). The *pfs* negative control (lane 6) also produced a DNA band on the agarose (**Figure 3.19**), which proved to be non-specific and most likely resulted from the formation of primer dimers or contamination. A higher annealing temperature (**Table 2.3**) was employed on template DNA extracted using the boiling method and proved to be successful in eliminating non-specific bands for the *pfs* amplicon as illustrated in **Figure 3.20**. The negative control did not yield an amplicon at the elevated temperature (**Figure 3.20**).

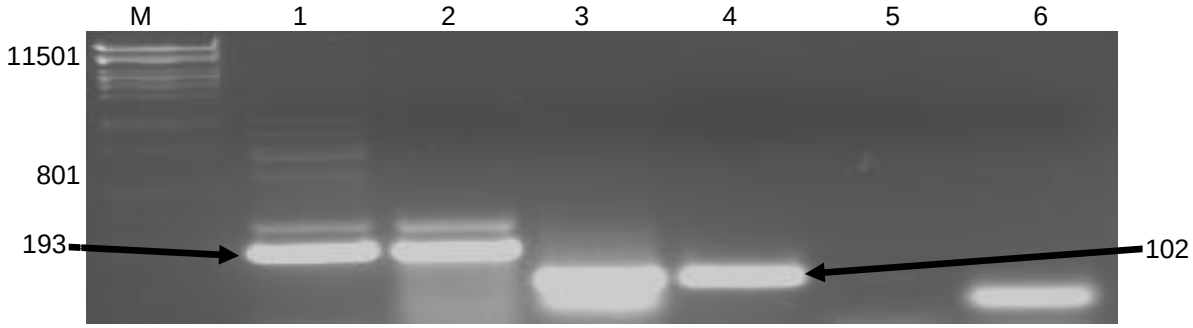

Figure 3.19 Agarose gel electrophoresis showing the PCR amplification results of the *S. marcescens* targeting the *pfs* (193 bp) and *luxS* (102 bp). Lane M: Molecular weight marker (Lambda DNA/PstI Marker, Fermentas); lane 1: *S. marcescens (pfs* gene) (boiling); lane 2: *S. marcescens (pfs* gene) (kit); lane 3: *S. marcescens* (*luxS* gene) (boiling); lane 4: *S. marcescens* (*luxS* gene) (kit); lane 5: *S. marcescens (luxS* gene) negative control; lane 6: *S. marcescens* (*pfs* gene) negative control.

The primer set used for the detection of the *luxS* gene yielded the 102 bp DNA band from template DNA extracted using the boiling method (lane 3) and the kit (lane 4) on agarose gel as shown in **Figure 3.19**. The *luxS* negative control (lane 5) with primer set targeting the *luxS* gene did not produce an amplicon.

The generated expected PCR products for the *pfs* (**Figure 3.20**) and *luxS* (**Figure 3.19**) genes were sent for sequencing and the *luxS* gene (102 bp) was confirmed positive, while the *pfs* was negative after sequencing and using the online BLAST program (Altschul *et al.,* 1997). The species specific primers targeting the *luxS* gene successfully amplified the *S. marcescens* strain; therefore this primer set was subsequently employed on water samples.

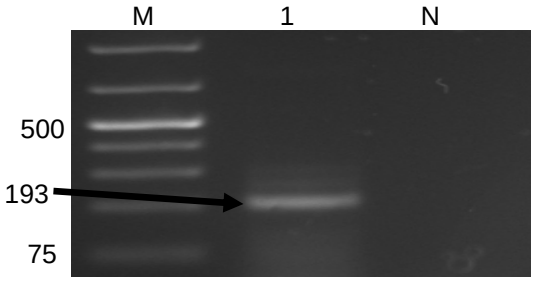

Figure 3.20 Agarose gei electrophoresis showing the PCR amplification results of *S. marcescens* targeting the *pfs* gene (193 bp). Lane M: Molecular weight marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane 1: *S. marcescens (pfs* gene*)* (Boiling); lane N: *S. marcescens (pfs gene)* negative control.

For the identification of *E. coli*, the *PhoA* gene specific for all *E. coli* strains, was used with an amplicon of 903 bp being produced after PCR amplification as shown in **Figure 3.21**. Template DNA used in the PCR, were obtained from the kit (lane 1) and the boiling method (lane 2), and they were all successfully amplified using the *E. coli* species specific primers, while no amplicon was generated in the negative control (lane 3). More PCR product was observed for template DNA extracted using the boiling method (lane 2) compared to the kit (lane 1) (**Figure 3.21**). The amplification of the *PhoA* gene was successful after sequencing and confirmation of sequence using the online BLAST program (Altschul *et al.,* 1997).

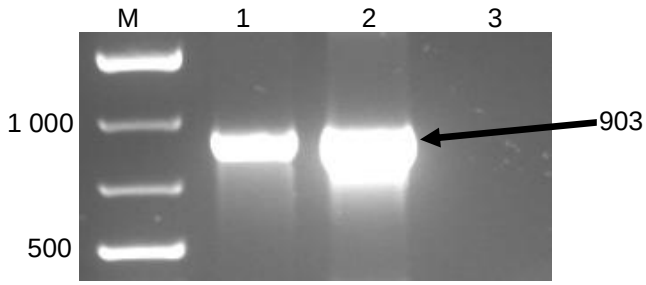

Figure 3.21 Agarose gel electrophoresis showing the PCR amplification results of the P*hoA* gene (903 bp) specific for *E. coli.* Lane M: Molecular weight marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane 1: *E. coli* (kit); lane 2: *E. coli* (boiling); lane 3: *E. coli* negative control.

Table 3.5 displays the successful amplification of individual organisms using species specific primers from reference strains using DNA templates extracted by the boiling method and the kit. The DNA templates from both methods (except *V. cholerae* and *M. luteus*, previously explained) were successfully amplified and detected each gene found in each organism.

Table 3.5 PCR using DNA templates extracted using different methods and sequencing of amplicons

Organism	Boiling method	High Pure PCR Template Preparation Kit	Sequencing
B. cereus	√	√	√
E. coli	√	√	√
E. faecalis	√	√	√
K. pneumoniae	√	√	√
M. luteus (rpf gene*)*	√	Not done	√
P. aeruginosa	√	√	√
P. fluorescens	√	√	√
S. marcescens (luxS gene*)*	√	√	√
S. sonnei	√	√	√
S. typhimurium	√	√	√
V. cholerae	√	Not done	√

Analysis on a 1.0% agarose gel, sequencing and using the BLAST system (Altschul *et al.,* 1997) obtained from the National Centre for Biotechnology Information website proved that both methods successfully extracted DNA from the *Enterobacteriaceae*

family. Even though the High Pure Template Preparation kit yields a better quality DNA, it involves many steps that are complicated, such as the enzymatic lysis and inhibitor removal steps. However, even though the boiling method does not have the necessary steps to remove all humic acids, metal content and debris of organic and inorganic content, it mostly yielded a higher concentration of DNA, and this could be ascribed to the high temperature (95ºC) that enable the complete lysis of microbial cells so that all the DNA will be released from cells. The boiling method is also inexpensive and time effective. Furthermore, PCR amplicons were obtained for all strains where the boiling DNA templates were used. The boiling method was subsequently employed to extract DNA from microbial cells in river water samples collected during the entire study period because of its cost effectiveness, taking less time to perform the procedure and extracting sufficient DNA for PCR reactions.

3.4.2. Extraction of DNA Directly from the River Water Samples

Water samples were collected from the two river systems as described in **Section 2.1.1** and genomic DNA was then extracted using the optimised boiling method (**Section 2.5.3.2**), which was performed within 24 hours from sampling time. Extracted genomic DNA was then analysed by 1.0% agarose gel electrophoresis and the results are shown in **Figure 3.22** and **Figure 3.23** for water samples collected from the Berg- and Plankenburg River systems, respectively for the entire sampling period. Deoxyribonucleic acid extracted from water samples could not be visualised on agarose gel for all samples collected in the two river systems. Similar results were observed for the DNA extracted from *B. cereus*, *M. luteus* and *V. cholerae* reference strains (**Figures 3.8 and 3.9**). However, after performing PCRs using these DNA templates it was confirmed successful as the amount of DNA was sufficient for PCR.

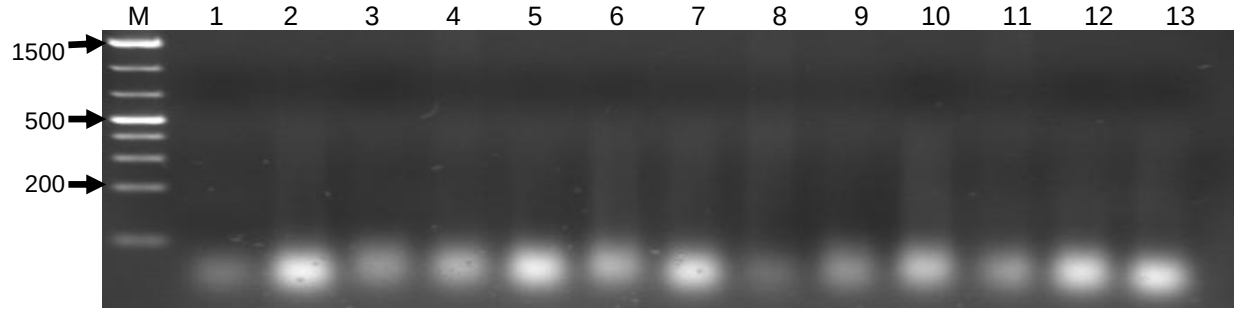

Figure 3.22 Agarose gel electrophoresis showing uncut genomic DNA extracted from water samples collected in the Berg River using the boiling method. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents DNA extracted from water samples collected fortnightly throughout the sampling period from the Berg River from week 1 (lane 1) to week 28 (lane 13).

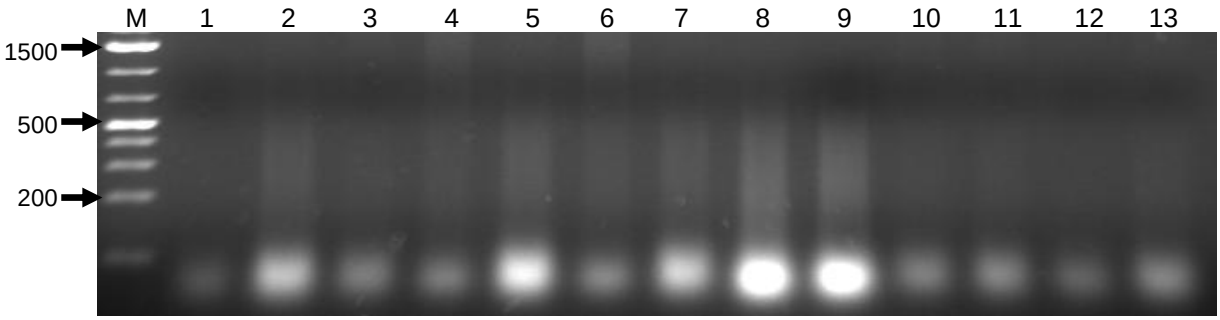

Figure 3.23 Agarose gel electrophoresis showing uncut genomic DNA extracted from water samples collected in the Plankenburg River using the boiling method. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents DNA extracted from the Plankenburg River water samples collected fortnightly during the entire sampling period from week 1 (lane 1) to the last week 28 (lane 13) of sampling.

The DNA obtained from water samples was then used as template DNA to amplify genes specific for each *Enterobacteriaceae* (**Table 2.5**) used in the study. Chaudhuri *et al.* (2006) showed that the extraction of microbial DNA by the boiling method could be successfully employed on soil and natural water samples with different organic and metal content.

3.4.3 Identification of *Enterobacteriaceae* Species from Berg River by Conventional Singleplex PCR

The optimised conventional PCR assays enabled the detection of *Enterobacteriaceae* in water samples collected from the Berg River. Samples were amplified by PCR along with a positive control for each *Enterobacteriaceae* (**Section 2.5.3.3**) under investigation. The *ompW*, *fimY* and *rpf* genes specific for *V. cholerae*, *S. typhimurium* and *M. luteus*, respectively, were not detected (results not shown) during the entire sampling period in the Berg River system. These microorganisms were not detected by the API 20 E and RapID ONE system used in this study either. Only the *Vibrio vulnificus* was detected by both identification systems, and although closely related to *V. cholerae,* it was not amplified by the high specificity of the primers used in of the PCR technique (Cabral, 2010).

The putative transcriptional regulator gene specific for *E. faecalis* was detected in week 3 (lane 2) as shown in **Figure 3.24** from the Berg River system. Faint DNA bands of 518 bp were also observed for weeks 5 (lane 3), 7 (lane 4), 9 (lane 5), 13 (lane 7), 15 (lane 8), 17 (lane 9), 19 (lane 10) and 21 (lane 11) of sampling. The positive control (lane C) and DNA sample amplicons were verified by sequencing and confirmed positive by the online BLAST program (Altschul *et al.,* 1997). The putative transcriptional regulator gene specific for *E. faecalis* was detected in 69% of water samples collected in the Berg River system. The habitat of *Enterococcus faecalis* is the intestinal tract of warm blooded animals, including humans. It is possible that feaces from stormwater drains and burst sewage systems from the informal settlement at Mbekweni in Paarl, Cape Town found its way into the river, which

runs through the informal settlement. Previous studies have also detected *E. faecalis* in river water samples using biochemical tests and PCR (Harwood *et al.*, 2004).

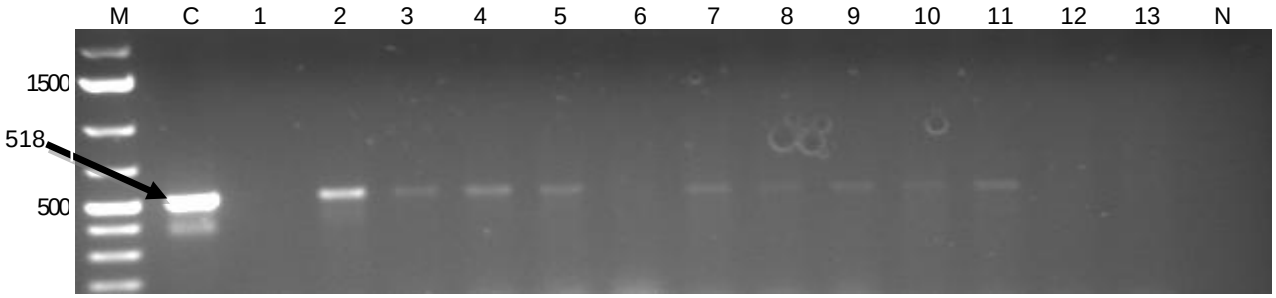

Figure 3.24 Agarose gel electrophoresis showing PCR amplification results (518 bp) using the *E. faecalis* species specific primers from DNA extracted in water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *E. faecalis* positive control; lanes 1-13: represents the Berg River water samples collected during the entire sampling period from week 1 (lane 1) to week 28 (lane 13) and lane N: *E. faecalis* negative control.

Bacillus cereus belonging to the genera of *Bacillus*, are commonly found in soil, water, air and food products (Manzano *et al.*, 2003). The unique *gyrB* gene found in *B. cereus* was detected in all samples except in week 13 (lane 7) of sampling in the Berg River system. As illustrated in **Figure 3.25**, a 374 bp DNA band was detected during the entire sampling period except in week 13 (lane 7) when no amplicon corresponding to the *gyrB* positive control was observed, with faint DNA bands in lane 1 (week 1) and lane 6 (week 15), respectively.

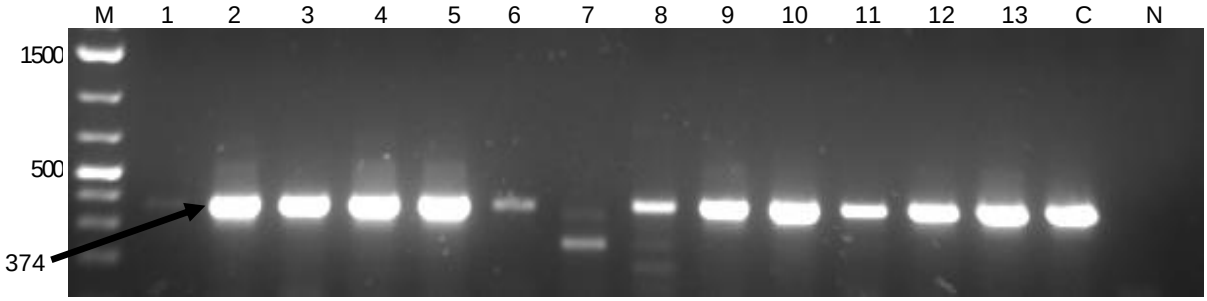

Figure 3.25 Agarose gel electrophoresis showing amplicons (374 bp) generated by *B. cereus* species specific primers from DNA extracted in water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents the Berg River samples collected fortnightly from week 1 (lane 1) through to week 28 (lane 13) of sampling period; lane C: *B. cereus* positive control and lane N: *B. cereus* negative control.

All positive DNA bands and the positive control were sequenced and confirmed positive for the *gyrB* gene in *B. cereus* by the online BLAST program (Altschul *et al.,* 1997). Ninety two percent of water samples collected in the Berg River system was confirmed positive for the *gyrB* gene indicating presence of *B. cereus*. Since *B. cereus* is found in soil and water, it was not suprising that most samples contained this microorganism. Previous studies were also successful in the identification of this organism in river water (Østensvik *et al.,* 2004; Kumar *et al.,* 2010).

After analysis on 1.5% agarose gel electrophoresis, the 16S-23S rRNA internal transcribed spacer gene (130 bp amplicon) specific for *K. pneumoniae* was detected throughout the sampling period except in week 3 (lane 2) as shown in **Figure 3.26**. There was a distinct difference in the intensity of DNA bands each week. Faint DNA bands were observed in lanes 1, 5, 7, 8, 9, 10 and 11, which could not be sequenced due to low amounts of amplicon. The positive control (lane C) and positive amplicons in lanes 3, 4, 6, 12 and 13 were verified by sequencing and were all confirmed positive using the BLAST online program (Altschul *et al.,* 1997). In all water samples collected from the Berg River, approximately 92% were regarded as positive for the ITS gene specific for *K. pneumoniae*. The natural habitat of *K. pneumoniae* is the intestinal tract of humans and warm blooded animals. Similar to *E. faecalis*, contaminated faeces could possibly have found its way into the Berg River through surface run off and burst sewage systems (Winter and Mgese, 2011). Previously, the use of PCR has been shown to be rapid and sensitive for the detection of *K. pneumoniae* in surface water sources (Siri *et al.,* 2011).

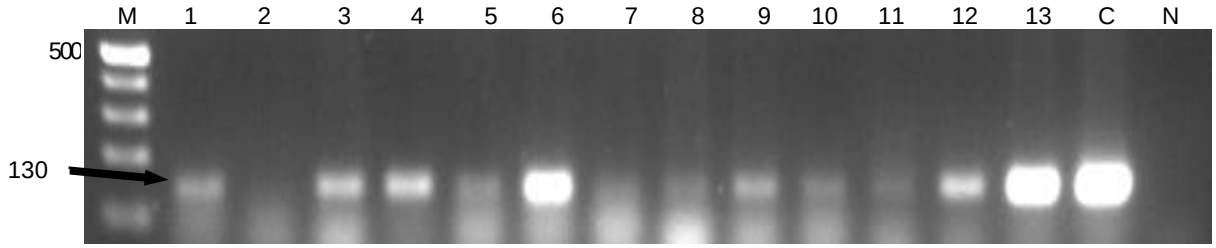

Figure 3.26 Agarose gel electrophoresis showing amplicons (130 bp) generated by *K. pneumoniae* species specific primers from water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents water samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) of the sampling period from the Berg River; lane C: *K. pneumoniae* positive control and lane N: *K. pneumoniae* negative control.

The distribution of 16S rRNA region specific for the *P. fluorescens* was investigated in the Berg River system for the entire sampling period. Specific DNA fragments with the molecular weight of 850 bp were amplified on samples collected in weeks 3 (lane 2), 5 (lane 3), 11 (lane 6) and 26 (lane 12) as shown in **Figure 3.27**. Samples collected in week 1 (lane 1) and 7 (lane 4) produced faint DNA bands which could not be sequenced due to low amount of amplicons generated. The 850 bp DNA bands in lanes 2, 3, 6, 12 and the positive control in lane C were sequenced and confirmed positive for the 16S rRNA region specific for *P. fluorescens*. It was concluded that the 16 rRNA region in *P. fluorescens* was present in 46% of water samples collected from the Berg River, which was also detected in previous studies in the same river by Barnes *et al.* (2004) and Lotter (2010) using biochemical tests, while Jackson *et al.* (2012) identified this organism using PCR.

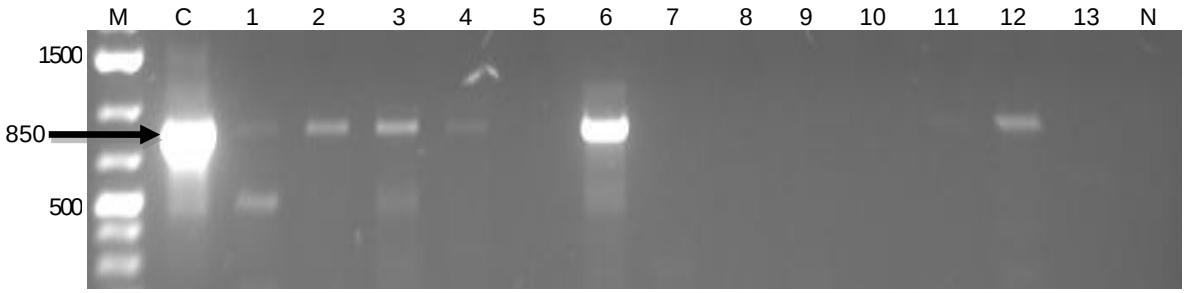

Figure 3.27 Agarose gel electrophoresis showing amplicons (850 bp) generated by the *P. fluorescens* species specific primers from river water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *P. fluorescens* positive control; lanes 1-13: represents the Berg River water samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) during the entire study and lane N: *P. flourescens* negative control.

Amplifying the 16S rDNA region produced a 956 bp PCR amplicon on agarose gel enabled the identification of *P. aeruginosa*. *Pseudomonas aeruginosa* was identified in weeks 9 (lane 5), 15 (lane 8), 21 (lane 11), 26 (lane 12) and 28 (lane 13) of sampling in the Berg River as represented by the agarose gel picture in **Figure 3.28**, using the primers specific for the 16S rDNA region in *P. aeruginosa*. Faint DNA bands were observed in lanes 5 (week 9) and 11 (week 21). The 956 bp amplicons produced in samples collected during these weeks could not be sequenced. The positive control in lane C and positive samples in lanes 8, 12 and 13 were sent for sequencing. The 16S rDNA region specific for *P. aeruginosa* was concluded present in approximately 38% of water samples collected from Berg River after sequencing and using the online BLAST program (Altschul *et al.,* 1997). Detection of *P. aeruginosa* was also detected in previous studies in river water samples (Barnes *et al.,* 2004; Mena and Gerba, 2009), and it is expected to be found in natural waters as it is regarded as a terrestrial and freshwater organism (Kimata *et al.,* 2004; Prescott *et al.,* 2005).

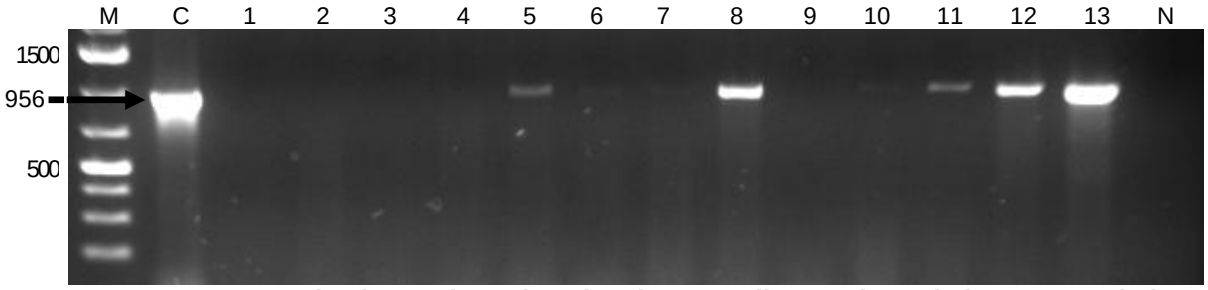

Figure 3.28 Agarose gel electrophoresis showing amplicons (956 bp) generated by *P. aeruginosa* species specific primers from river water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *P. aeruginosa* positive control*;* lanes 1-13: represents Berg River water samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) and lane N: *P. aeruginosa n*egative control.

The *luxS* gene uniquely found in *S. marcescens* was detected throughout the sampling period as illustrated by **Figure 3.29** showing the 102 bp DNA bands. All 102 bp amplicons were sent for sequencing, verified and confirmed positive for the *luxS* gene, which

is specific for the *S. marcescens.* It was concluded that *S. marcescens* was identified in 100% of water samples collected in the Berg River. *Serratia marcescens* is a ubiquitous bacterium in the environment, which is also present in sewage (Sadowsky and Whitman, 2011), therefore it was expected to be detected in river water samples. In addition, their abundance in the environment did not require selective enrichment media for its detection, and also the boiling method was sufficient to extract the template DNA.

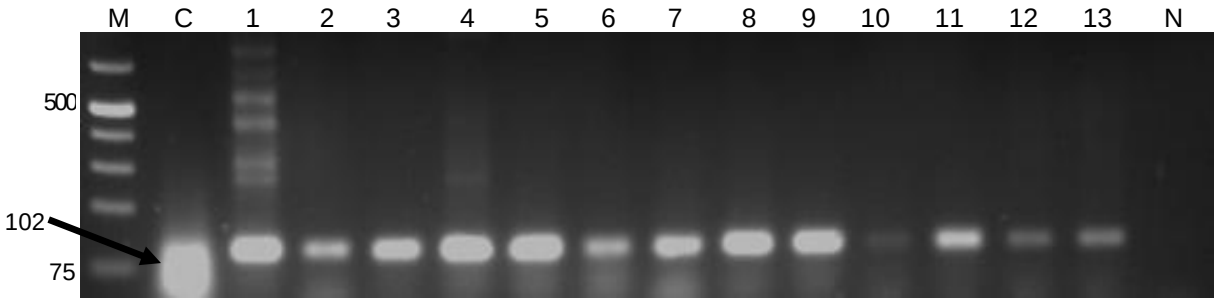

Figure 3.29 Agarose gel electrophoresis showing amplicons (102 bp) generated with *S. marcescens* species specific primers from DNA extracted from river water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *S. marcescens* positive control; lanes 1-13: represents the Berg River samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) and lane N: *S. marcescens* negative control.

The *PhoA* gene specific for *E. coli* generated a DNA band size of 903 bp, and was detected in 85% of all samples collected in the Berg River as illustrated on agarose gel (**Figure 3.30**). It was not detected in weeks 9 (lane 5) and 19 (lane 10). Light bands were observed in lane 7 (week 13) and lane 9 (week 17) and could not be verified by sequencing for the *PhoA* gene. The *PhoA* gene was confirmed by sequencing all positive samples (except weeks 13 and 17) and using of the BLAST program (Altschul *et al.,* 1997).

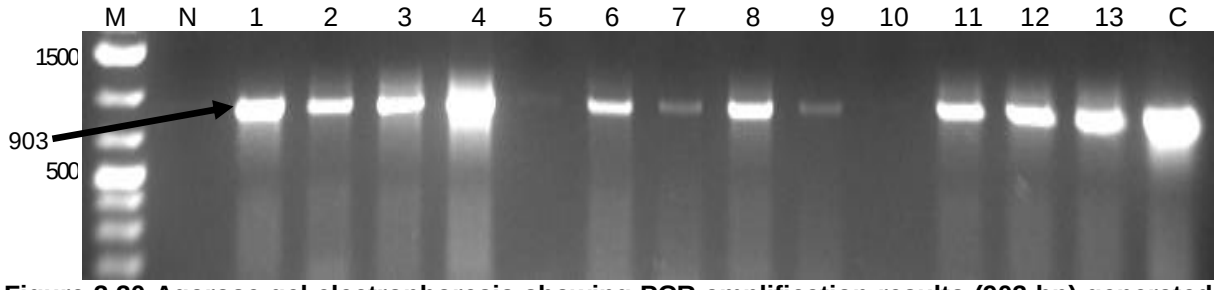

Figure 3.30 Agarose gel electrophoresis showing PCR amplification results (903 bp) generated with *E. coli* species specific primers from water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *E. coli* positive control*;* lanes 1-13: represents water samples collected from the Berg River fortnightly from week 1 (lane 1) to week 28 (lane 13) during the entire sampling period and lane N: *E. coli* negative control.

Since *E. coli* is widely distributed in the environment and in warm blooded animals (Kong *et al.,* 1999; Prescott *et al.,* 2005), it was expected that the *PhoA* gene would be detected throughout the sampling period since the MTF, MF techniques and the Colilert 18®

system detected the culturable *E. coli* cells (**Section 3.2.1** and **3.2.2**) throughout the sampling period. Inefficient removal of PCR inhibitors using the boiling method could possibly have affected the *E. coli* PCR for some sampling periods.

The 369 bp amplicon size (**Figure 3.31**) representing the insertion sequence gene specific for the *S. sonnei* was detected in weeks 3 (lane 2), 5 (lane 3), 7 (lane 4), 9 (lane 5), 13 (lane 7), 26 (lane 12) and 28 (lane 13). Non-specific bands were observed in samples obtained in lanes 1, 2, 3, 4, 5 and 7. Only the expected size of 369 bp was sent for sequencing and confirmed positive for the insertion sequence gene specific for *S. sonnei*. *Shigella sonnei* was successfully detected in approximately 54% of water samples collected in the Berg River system. *Shigella sonnei* is usually found to be associated with water contaminated with sewage. Hsu *et al*. (2007) used PCR to accurately detect the insertion sequence gene in *S. sonnei* from spiked water samples, and it detected very low numbers of up to 1.3 CFU/50 ml of water samples.

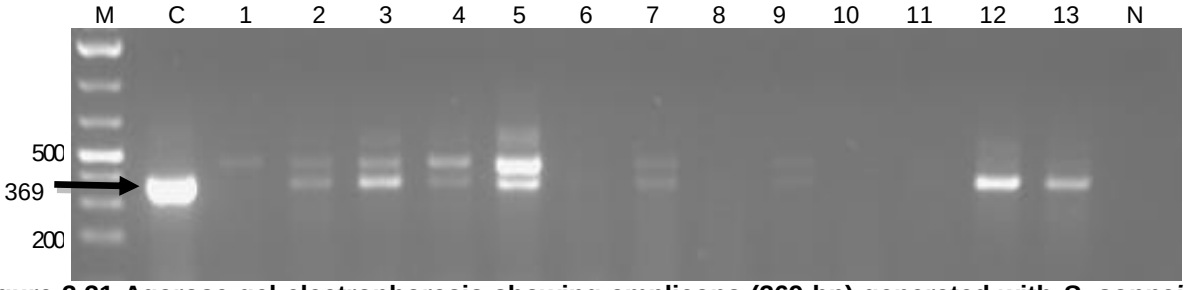

Figure 3.31 Agarose gel electrophoresis showing amplicons (369 bp) generated with *S. sonnei* species specific primers from water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *S. sonnei* positive control*;* lanes 1-13: represents the Berg River water samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) throughout the sampling period and lane N: *S. sonnei* negative control.

Results depicted in **Figure 3.24** to **Figure 3.31** shows the *Enterobacteriaceae* species detected along the Berg River. Pollution at the site of sampling was visible from the large volume flow of water washed from the informal settlement of Mbekweni in Paarl and the possibility of run off from burst sewages pipes into the river. In addition, rain on the day of sampling (weeks 9, 13, 26 and 28), could also have led to wastewater from the malfunctioning or burst sewage systems flowing into the river system. Rain flow could have carried all the wastes (possibilities of a variety of microorganisms) from the informal settlement and the river bank. This could account for the detection of faecal coliform bacteria during the sampling period. These microorganisms also survive for extended time periods in water (Campbell *et al*., 2011; Prescott *et al.,* 2005). The *ompW*, *fimY* and *rpf* genes specific for *V. cholerae*, *S. typhimurium* and *M. luteus*, respectively, were not detected (results not shown) during the entire sampling period in the Berg River system.

Detection of these strains by PCR has previously been shown to be difficult. Yam *et al.* (1999) could not detect *V. cholerae* and *S. typhimurium* in coastal waters by

means of PCR, while Faparusi *et al.* (2011) detected *M. luteus* in ground water using solid media (Plate count, Eosin methylene blue agars) and biochemical tests. *Salmonella typhimurium*, *V. cholerae* and *M. luteus* belong to the *Enterobacteriaceae* family, and these organisms are found in small numbers in freshwaters. Furthermore, environmental stresses force them to enter a dormant stage inside extrapolysaccharide biofilms containing nutrients, therefore increasing their survival (Yam *et al.,* 1999; Cabral, 2010). Since only water samples were collected to identify these organisms and not material with biofilm formation, this may account for low organism numbers present in water.

Salmonella typhimurium, found as a free living microorganism in soil, fresh and marine waters, and also in the waste of humans and other warm blooded animals (Prescott *et al.,* 2005; Sadowsky and Whitman, 2011) was not detected in the Berg River. The *fimY* gene specific for *S. typhimurium* could not be detected using PCR during the entire sampling period. This could be due to the low concentration of the extracted template DNA for this microorganisms or the possibility that this microorganisms was not present in the collected water samples. The latter is confirmed by the fact that the organisms could not be detected by the API 20 E and RapID ONE identification systems, either.

The *M. luteus* is also a free living Gram-positive bacteria in water, soil and human skin. It possesses the essential *rpf* gene (responsible for secreting the *rpf* protein to stimulate the bacterial growth from an extracytoplasmic location) required for its growth (Mukamolova *et al.,* 2002). *Micrococcus luteus* was not detected throughout the sampling period and the possible reason could be that the plasmid containing the *rpf* gene in environmental isolates are said to be more tightly bound to the bacterial cell wall unlike the positive control (which have a more relaxed cell wall structure to the maintenance in the laboratory environment) (Mukamolova *et al.,* 2006; Koltunov *et al.,* 2010). This could have affected the amount of the *rpf* gene that was extracted using the boiling method.

Vibrio cholerae a Gram-negative bacteria, which can be found in aquatic environments and also in infected individuals, was not detected in water samples collected from the Berg River. The reason could be due to their low numbers in the environment and that alkaline peptone water, which is the enrichment media of this microorganism (Tuteja *et al*., 2007; Goel *et al*., 2007) was not used.

Serratia marcescens and *B. cereus* are ubiquitous bacteria in the environment and are present in sewage, natural waters (Sadowsky and Whitman, 2011). They were detected in almost all water samples for the duration of the study period by PCR. Their abundance in the environment did not require selective enrichment media for its detection.

Pseudomonas aeruginosa and *P. fluorescens* are free living bacteria found in soil, water, sewage and other moist environments. Surprisingly however, they were not detected constantly throughout the sampling period. Since only water was tested, it can be assumed

that a large population of these organisms were present in biofilms, which are known to help in sustaining them in aquatic environments (Campbell *et al.,* 2011).

The presence of all these *Enterobacteriaceae* species indicates the presence of disease causing microorganisms, which can be released into water from skin, wounds, urine, faeces, etc. Use of this water for irrigational or recreational purposes may lead to water-related illnesses. Polymerase chain reaction allowed for the rapid identification of these microorganisms from river water, achieved by the use of species specific primers. The boiling method was employed in extracting DNA from water samples, and this reduced the time required to obtain the final results and also reduced the total cost for performing the procedure, compared to the use of specialised kits, which can be very expensive for routine monitoring.

3.4.4 Identification of *Enterobacteriaceae* Species from Plankenburg River by Conventional PCR

Figure 3.32 represents the agarose gel picture for amplified *E. faecalis* from the Plankenburg River system. The PCR amplicons were detected in lanes 2 (week 3), 3 (week 5), 4 (week 7), 11 (week 21), 12 (week 26) and 13 (week 28). The positive control along with the positive samples in lanes 3, 11, 12 and 13 were verified and confirmed positive by sequencing and using the online BLAST program online (Altschul *et al.,* 1997). The PCR products observed in weeks 3 (lane 2) and 7 (lane 4) were not verified as they showed faint bands on the agarose gel and could not be sequenced. Approximately 46% of water samples collected from the Plankenburg River were positive for the *E. faecalis*.

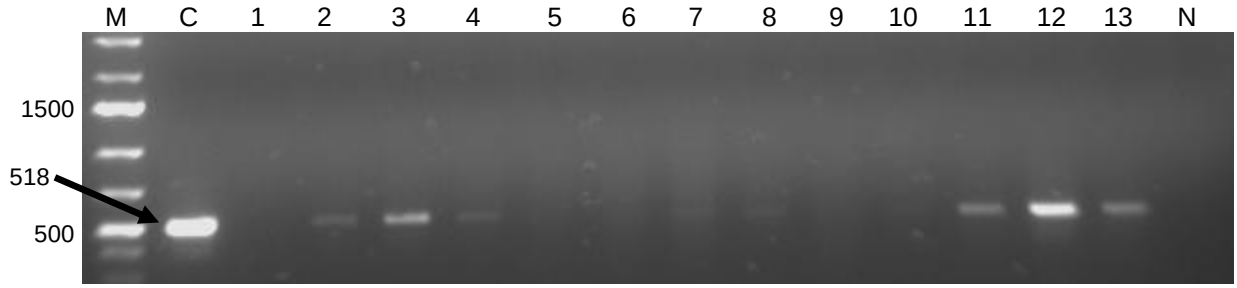

Figure 3.32 Agarose gel electrophoresis showing PCR amplification results (518 bp) using the *E. faecalis* species specific primers from DNA extracted in water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *E. faecalis* positive control; lanes 1-13: represents all the water samples collected from the Plankenburg River during the entire sampling period and lane N: *E. faecalis* negative control.

The *gyrB* gene (374 bp) specific for *B. cereus* was found to be present throughout the sampling period in water samples collected in the Plankenburg River except in week 11 (lane 7) as illustrated in **Figure 3.33**. In the first week, the PCR product was faint and also non-specific bands appeared in samples collected in weeks 5 (lane 3) and 21 (lane 11). The positive control and amplicons obtained from water samples were verified by sequencing and

confirmed positive for the *gyrB* gene in *B. cereus* by the online BLAST program (Altschul *et al.,* 1997). It was then concluded that *B. cereus* was detected in approximately 92% of water samples collected in the Plankenburg River.

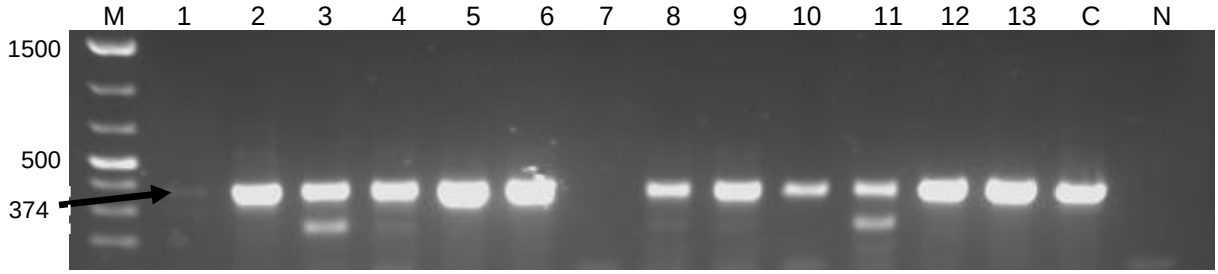

Figure 3.33 Agarose gel electrophoresis showing amplicons (374 bp) generated by *B. cereus* species specific primers from DNA extracted in water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler™ 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents the Plankenburg River samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13); lane C: *B. cereus* positive control and lane N: *B. cereus* negative control.

Figure 3.34 shows the 130 bp DNA bands generated by primers specific for the 16S-23S rRNA transcribed spacer in *K. pneumoniae.* The positive control (lane C) and positive samples (except DNA bands in lanes 1, 2, 6, 7 and 11, which were too faint) were verified by sequencing and confirmed positive for the 16S-23S rRNA internal transcribed spacer a region specific for the *K. pneumoniae.* Approximately 92% of water samples collected from the Plankenburg River were confirmed positive for the 16S-23S rRNA region in *K. pneumoniae*.

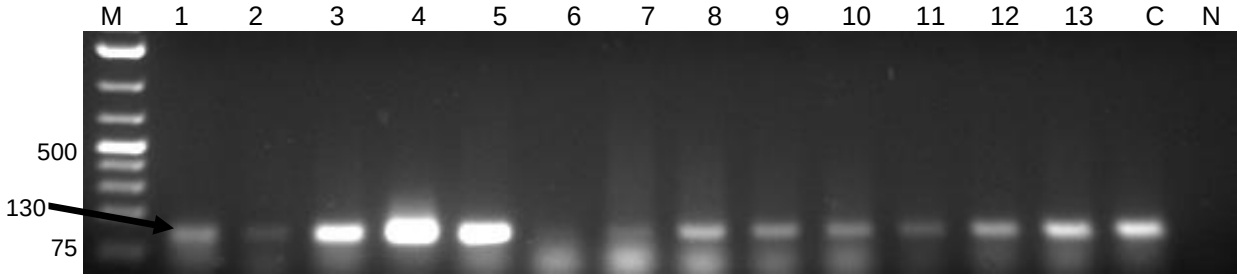

Figure 3.34 Agarose gel electrophoresis showing amplicons (130 bp) generated by *K. pneumoniae* species specific primers from water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler™ 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents water samples collected from the Plankenburg River fortnightly from week 1 (lane 1) to week 28 (lane 13); lane C: *K. pneumoniae* positive control and lane N: *K. pneumoniae* negative control.

Figure 3.35 shows the amplicons produced for the 16S rRNA region specific for *P. fluorescens* in water samples from the Plankenburg River. The distribution of 16S rRNA region specific for the *P. fluorescens* was investigated in the Plankenburg River system for the entire sampling period. The specific DNA fragments with a size of 850 bp were amplified in weeks 3 (lane 2), 5 (lane 3), 9 (lane 5), 11 (lane 6), 13 (lane 7) and 26 (lane 12). Faint bands were observed in lanes 5 and 6, and they could not be sequenced due to low intensity

on agarose gel. It was concluded that the 16S rRNA region specific for *P. fluorescens* was detected in approximately 46% of water samples by PCR.

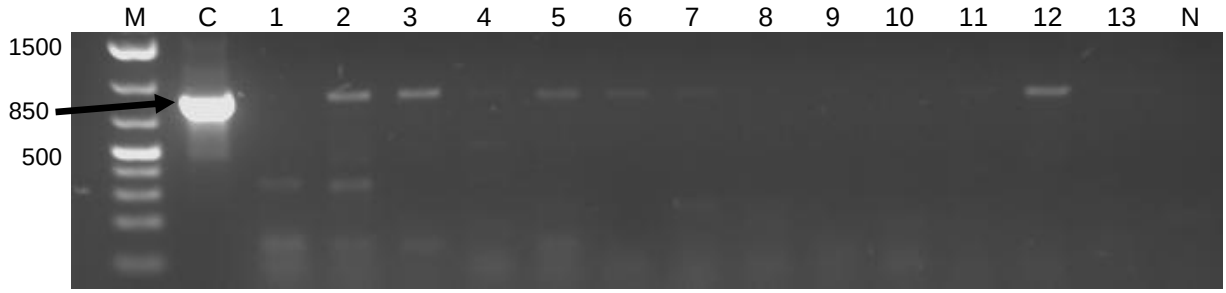

Figure 3.35 Agarose gel electrophoresis showing amplicons (850 bp) generated by *P. fluorescens* species specific primers from water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *P. flourescens* positive control; lanes 1-13: represents the Plankenburg River water samples collected throughout the sampling period and lane N: *P. flourescens* negative control.

A 956 bp amplicon representing the 16S rDNA region of *P. aeruginosa* was produced in lanes 7 (week 13), 12 (week 26) and 13 (week 28) as shown in **Figure 3.36**. The faint DNA band in lane 7 could not be sequenced due to low amount of amplicon. The 16S rDNA region specific for *P. aeruginosa* was then confirmed in water samples collected in weeks 26 and 28 after the positive control and these amplicons were sequenced. It was concluded that 23% of water samples were positive for *P. aeruginosa* by PCR.

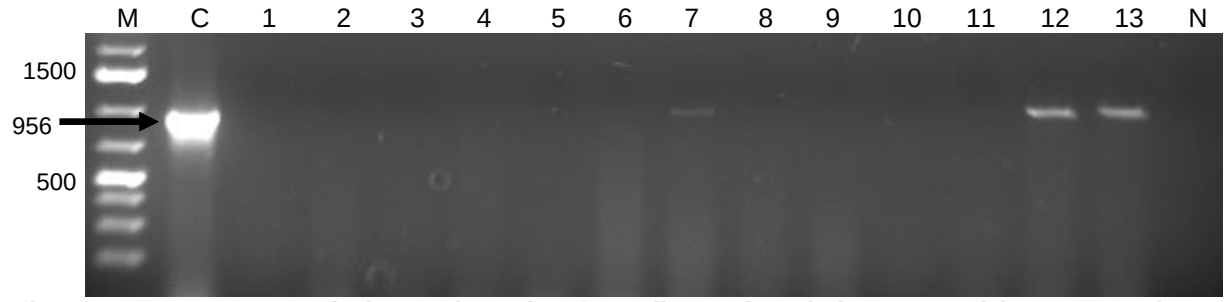

Figure 3.36 Agarose gel electrophoresis of amplicons (956 bp) generated by *P. aeruginosa* species specific primers from DNA extracted from river water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *P. aeruginosa* positive control*;* lanes 1-13: represents Plankenburg River water samples collected from week 1 to week 28 and lane N: *P. aeruginosa* negative control.

The *luxS* gene specific for the *S. marcescens* was detected throughout the sampling period as shown on a 1.5% agarose gel (**Figure 3.37**). The positive control and all amplicons were sequenced and confirmed positive for the *luxS* gene specific for *S. marcescens*. It was confirmed that *S. marcescens* was present in all water samples collected in the Plankenburg River system.

The *PhoA* gene specific for *E. coli* was amplified using the species specific primers and produced a 930 bp DNA band on agarose gel as shown in **Figure 3.38**, no bands were produced in lanes 9 (week 17) and 10 (week 19). The positive control and all 903 bp DNA

bands were sequenced and confirmed positive for the *PhoA* gene specific for *E. coli*. It was then concluded that approximately 85% of all samples collected from the Plankenburg River had the *PhoA* gene, which was used in the identification of *E. coli*.

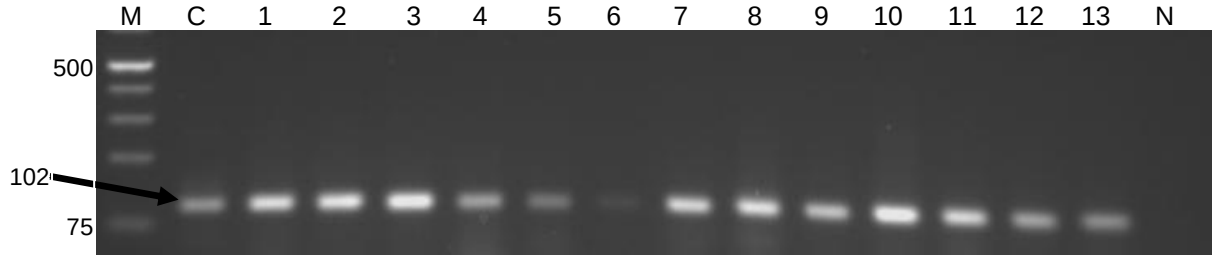

Figure 3.37 Agarose gel electrophoresis showing amplicons (102 bp) generated with *S. marcescens* species specific primers from water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane C: *S. marcescens* positive control; Lanes 1-13: represents the Plankenburg River water samples collected for the entire sampling period during the whole study period and Lane N: *S. marcescens* negative control.

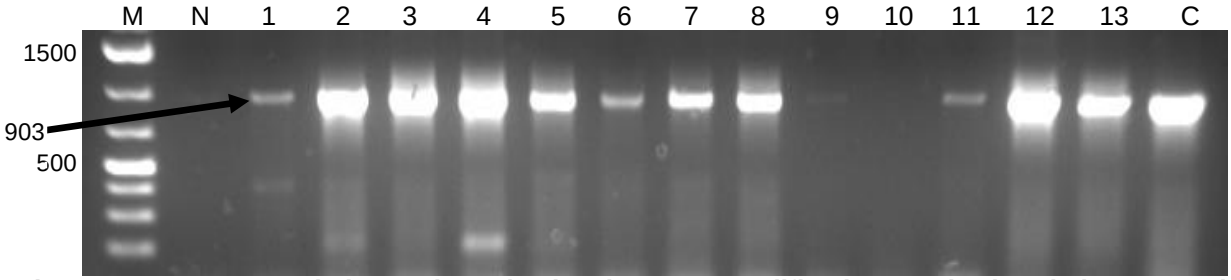

Figure 3.38 Agarose gel electrophoresis showing PCR amplification results (903 bp) generated by *E. coli* species specific primers from DNA extracted from water samples. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *E. coli* positive control*;* lanes 1-13: represents water samples collected fortnightly from week 1 (lane 1) to week 28 (lane 13) from the Plankenburg River system and lane N: *E. coli* negative control.

Figure 3.39 illustrates the 369 bp DNA bands representing the insertion sequence gene specific for the *S. sonnei* from river water samples collected along the Plankenburg River. Non-specific DNA bands (above the expected 369 bp DNA band) were observed along with the 369 bp bands in lanes 2, 3, 12 and 13, while in lane 1 only the non-specific DNA band was observed.

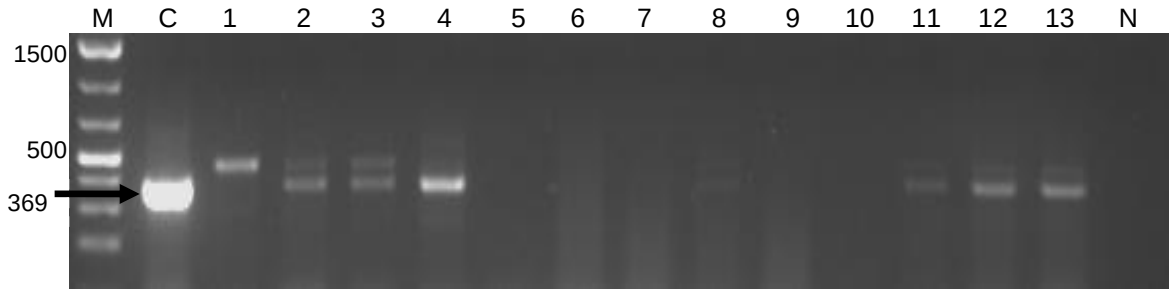

Figure 3.39 Agarose gel electrophoresis showing amplicons (369 bp) generated with *S. sonnei* species specific primers from DNA extracted from river water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: *S. sonnei* positive control*;* lanes 1-13: represents samples collected fortnightly from week 1(lane 1) to week 28 (lane 13) in the Plankenburg River and lane N: *S. sonnei* negative control.

All DNA bands at 369 bp were sequenced and confirmed positive for the insertion sequence gene specific for *S. sonnei*. It was detected in the Plankenburg river system in weeks 3 (lane 2), 5 (lane 3), 7 (lane 4), 21 (lane 11), 26 (lane 12) and 28 (lane 13). It was then concluded that 46% of water samples were *S. sonnei* positive by PCR.

Micrococcus luteus is a free living Gram-positive bacteria found in water, soil and human skin. It possesses the essential *rpf* gene (responsible for secreting the *rpf* protein to stimulate the bacterial growth from an extracytoplasmic location) required for its growth (Mukamolova *et al.,* 2002). It was not detected throughout the sampling period (results not shown) and this could be due to the complicated location of the plasmid containing the *rpf* gene in environmental bacterial cells. The plasmid is said to be more tightly bound to the bacterial cell wall unlike the positive control (which have a more relaxed cell wall structure to the maintenance in the laboratory environment) (Koltunov *et al.,* 2010). This could have led to very low amount of the *rpf* gene extracted using the boiling method. *Vibrio cholerae*, a Gram-negative bacterium, which can be found in the aquatic environment and also in infected individuals, was not detected in water samples collected from the Plankenburg River (results not shown). The reason could be due to their low numbers in the environment and that Luria Bertani broth was used for enrichment instead of the alkaline peptone water, which is selective for *V. cholerae* (Tuteja *et al*., 2007; Goel *et al*., 2007).

Salmonella typhimurium, a common bacterium isolated from the soil, fresh and marine waters, also in the waste of humans and other warm blooded animals (Prescott *et al.,* 2005; Sadowsky and Whitman, 2011) was only detected in week 13 (results not shown). This could be due to low concentration of the extracted template DNA as well as presence of inhibitors such humic acids, organic and inorganic debris, which could have interfered with the PCR procedure.

The Plankenburg River is mostly polluted through storm drainage pipes that are used to drain the waste effluents from the informal settlements and flow directly into the river system. This activity contributes to the introduction of *Enterobacteriaceae* species into the river. In addition farmers along the river also make use of fertilisers, which are washed away during the rainy season and deposited into the river system. Metals that may have leached from plumbing fittings, healthcare products and galvanised structures used by informal settlement dwellers, may also have a negative impact on the detection of microorganisms by PCR.

Bacillus cereus, E. coli, *K. pneumoniae* and *S. marcescens* were the most prevalent organisms in the Plankenburg River system. *Escherichia coli* and *K. pneumoniae* are largely found in sewage and also faecal matter of other warm blooded animals (Cabral, 2010; Obasohan *et al.,* 2010). Due to the abundance of *S. marcescens* and *B. cereus* in the environment and sewage they were detected in water samples throughout the sampling

period by PCR. Cultivation of these microorganisms did not require selective enrichment media and also the boiling method was sufficient for extraction of template DNA.

Pseudomonas aeruginosa and *P. fluorescens* are free living bacteria found in soil, water and other moist environments. Since only water was tested, it could be that a large population of these organisms were present in biofilms, which may help in sustaining these organisms in aquatic environments (Campbell *et al.,* 2011). It could therefore have led to very low amount of extracted template DNA.

Table 3.6 illustrates the overall percentage of *Enterobacteriaceae* species detected from the Berg- and Plankenburg River systems by PCR. *Serratia marcescens* was detected throughout (100%) the sampling period in both river systems, while *V. cholerae* and *M. luteus* were not detected. *Salmonella typhimurium* was not detected in the Berg River, while it was detected once (8%) in the Plankenburg River. Overall a lower *Enterobacteriaceae* species variety was observed in the Berg River system (eight out of 11) during the sampling period, than in the Plankenburg River (nine out of 11). *Enterobacteriaceae* species such as the *E. faecalis, P. aeruginosa* and *S. sonnei* occurred more frequently in the Berg River system than in the Plankenburg River system.

Table 3.6 Overall results of *Enterobacteriaceae* species identified by PCR in river water samples

Organism	Berg River	Plankenburg River
B. cereus	92%	92%
E. coli	85%	85%
E. faecalis	69%	46%
K. pneumoniae	92%	92%
M. luteus	0%	0%
P. aeruginosa	38%	23%
P. fluorescens	46%	46%
S. marcescens	100%	100%
S. sonnei	54%	46%
S. typhimurium	0%	8%
V. cholerae	0%	0%

For the identification of the *Enterobacteriaceae* species in both river systems, the PCR technique proved to be the most sensitive technique with the use of species specific primers. *Micrococcus luteus* and *V. cholerae* were not detected for the duration of the study by means of the three identification methods (PCR, API 20 E and RapID ONE) used in the study, while *E. coli* and *K. pneumoniae* were detected by all techniques. The PCR technique detected *E. faecalis*, *B. cereus* and *S. typhimurium* (detected once in the Plankenburg River), but these organisms were not detected using the API 20 E and RapID ONE system for both river systems. *Serratia marcescens* was detected in both river systems by the PCR method, but it

was not detected in the Berg River by the API 20 E and RapID ONE system, while it was detected in the Plankenburg River system using both systems. The API 20 E identification system and PCR detected *P. aeruginosa* and *P. fluorescens* in a few sampling weeks in both river systems. These organisms were not identified by the RapID ONE system, while *S. sonnei* was identified using PCR and the RapID ONE system in the Plankenburg River.

Since *Enterobacteriaceae* species were found in the water samples, it could suggest that the water is not suitable for irrigational and recreational purposes as it indicates faecal pollution and possibly the presence of pathogenic microorganisms. The presence of these organisms in the river water poses a threat to humans who ingest raw shellfish and fish, use the water for recreational purposes and consume raw fruits and vegetables irrigated with this water (without prior treatment) (Ackermann, 2010). Polymerase chain reaction proved to be the best technique to identify the *Enterobacteriaceae* to species level and obtained results rapidly. Results obtained using the conventional PCR to identify *Enterobacteriaceae* was done in less than 10 hours. All the detected organisms indicated faecal pollution (Leclerc *et al.,* 2001). The API 20 E and RapID ONE identification systems involve the use of many materials and equipment such as media, incubators, test tubes and many analytical steps, which are time consuming. These identification systems thus require more time and cost for analysis is high and less sensitive when compared to PCR.

3.4.5 Optimisation of Multiplex Polymerase Chain Reaction

Deoxyribonucleic acid extracted from control strains [Enteroinvasive *E. coli* (EIEC) ATCC 43892, Enteropathogenic *E. coli* (EPEC) B170, Enterohaermorrhagic *E. coli* (EHEC) O157:H7, Enteroaggregative *E. coli* (EAEC) ATCC 3591-87 and Enterotoxigenic *E. coli* (ETEC) H10407] were obtained by the boiling method (adapted from Watterworth *et al*., 2005). The DNA was then used as templates for the optimisation of multiplex PCR. Deoxyribonucleic acid from the pathogenic *E. coli* control strains were also analysed on 1.5% agarose gel as illustrated in **Figure 3.40** (lanes 8-12). Deoxyribonucleic acid extraction using the boiling method was successful for all the five strains of *E. coli* as illustrated in **Figure 3.40** [lanes 8 (EIEC), 9 (ETEC), 10 (EPEC), 11 (EHEC) and 12 (EAEC).

Initially the multiplex PCR was perfomed using the template DNAs from all five *E. coli* strains (EAEC, EIEC, EHEC, EPEC and ETEC). Six pairs of primers were used, whereby two primer sets were specific for the heat stable enterotoxin (*est)* and heat labile enterotoxin (*elt)* genes found in the ETEC (H10407) strain. Single PCRs were also performed simultaneously for each primer set on specific control strains under the same conditions to evaluate the specificity of the primers used.

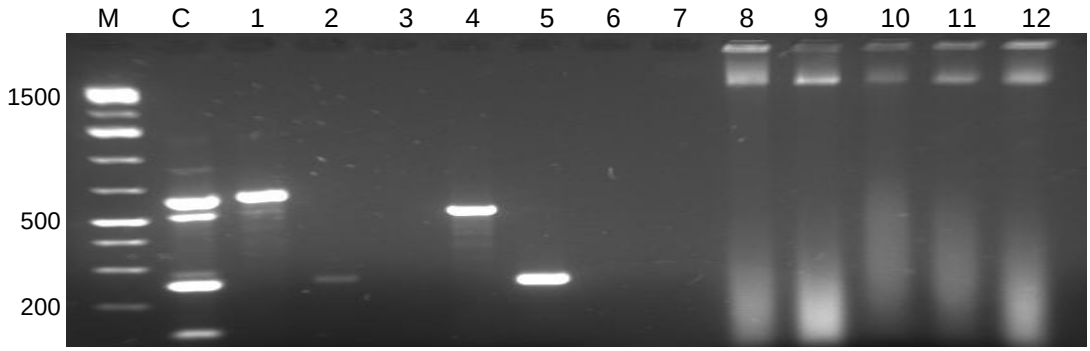

Figure 3.40 Agarose gel electrophoresis showing amplicons generated with *E. coli* strains specific primers in DNA extracted from control strains. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex PCR (EPEC, ETEC, EAEC, EIEC and EHEC); lane 1, EIEC (619 bp, *ipaH* gene); lane 2: ETEC (147 bp, *elt* gene); lane 3: ETEC (322 bp, *est* gene); lane 4: EHEC (518 bp, *stx* gene); lane 5: EAEC (254 bp, *aggR* gene); lane 6: EPEC (881 bp, *eae* gene); lane 7: Multiplex negative control. Lanes 8-12: DNA extracted using the boiling method from the control strains, lane 8: EIEC (ATCC 43892); lane 9: ETEC (H10407); lane 10: EPEC (B170); lane 11: EHEC (O157:H7); lane 12: EAEC (ATCC 3591-87).

As illustrated in **Figure 3.40**, lane C represents the multiplex PCR amplicons. Within the multiplex PCR, a faint band for EPEC (881 bp) amplicon was observed, while the EIEC (619 bp), EHEC (518 bp) and the EAEC (254 bp) DNA amplicons were more pronounced. A DNA band approximately 147 bp in size was also observed in lane C (expected amplicon size for *est* gene), but it was confirmed *est* negative after sequencing and using the online BLAST program (Altschul *et al.,* 1997). The results (**Figure 3.40**) after single amplification proved to be successful for the EIEC (lane 1), EHEC (lane 4) and EAEC (lane 5) after sequencing and using the online BLAST program (Altschul *et al.,* 1997). Expected DNA band sizes of 147 bp (*est*, ETEC) (lane 2), 322 bp (*elt*, ETEC) (lane 3) and 881 bp (*eae*, EPEC) (lane 6) were not produced in the single PCR assays as shown in **Figure 3.40**. A DNA band of approximately 260 bp was observed in lane 2 for the ETEC strain instead of the 147 bp representing the *est* gene, but it was confirmed negative for all *E. coli* strains after sequencing and using the online BLAST program (Altschul *et al.,* 1997). The negative control used for all multiplex and single PCR reactions, contained all the reagents used within a PCR against excluding the template DNAs.

Most ETEC strains are known to express one of the two genes (*elt* and *est*) or both (Toma *et al*., 2003; Reischl *et al.*, 2004; Lasaro *et al.,* 2008). These two genes could not be detected in the control strain (H10407), which is known to contain both genes (Toma *et al.,* 2003; Lasaro *et al.,* 2008) and it led to an extensive optimisation (see **Section 3.4.7**). Multiplex PCR was henceforth optimised using four strains of *E. coli*, excluding the ETEC strain.

The amount of EPEC template DNA used was increased after the first PCR reaction for single PCR and the *eae* gene was successfully amplified (**Figure 3.41**, lane 1). Multiplex PCR assay was further performed under the optimised PCR conditions listed in **Section 2.5.3.6** and yielded the expected sizes [EPEC (881 bp, *eae* gene), EIEC (619 bp,

ipaH gene), EHEC (518 bp, *stx* gene) and EAEC (254 bp, *aggR* gene)] in both multiplex and single assays.

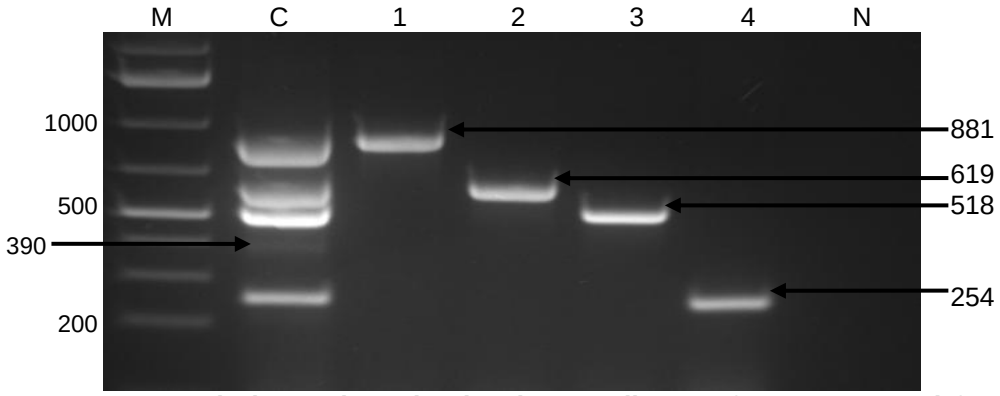

Figure 3.41 Agarose gel electrophoresis showing amplicons of DNA extracted from control strains of *E. coli* using strains specific primers. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex representing four strains of *E. coli* (EPEC, EIEC, EHEC, EAEC); lane 1: EPEC (881 bp); lane 2: EIEC (619 bp); lane 3: EHEC (518 bp); lane 4: EAEC (254 bp) and lane N: Multiplex negative control.

Figure 3.41 illustrates the successful multiplex PCR amplicons (lane C) and single reaction amplicons for EPEC (lane 1), EIEC (lane 2), EHEC (lane 3) and EAEC (lane 4). All DNA bands produced were further verified by sequencing and confirmed positive using the online Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.,* 1997). Multiplex PCR was further optimised after using a real-time cycler (MJ MiniOpticon, Bio-Rad, USA) to investigate if successful amplicons could be obtained using a reduced number of cycles. The numbers of cycles in the conventional PCR (as extrapolated from real-time PCR) were reduced from 30 to 20 (**Figure 3.42)** during amplification of the four strains of *E. coli* (EAEC, EIEC, EHEC and EPEC). The same cycling conditions on DNA extracted from river water samples were applied.

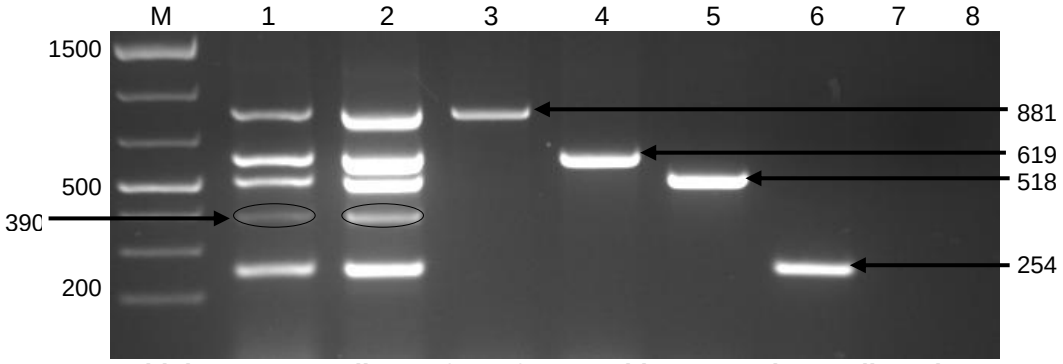

Figure 3.42 Multiplex PCR amplicons from four positive control *E. coli* strains. Lane M: DNA Ladder Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane 1: Multiplex (without KCl, Tris, Triton X-100) and four sets of primers; lane 2: Multiplex with added buffers and four sets of primers; lane 3: EPEC (881 bp); lane 4: EIEC (619 bp); lane 5: EHEC (518 bp); lane 6: EAEC (254 bp) and lane 7: Negative control [Multiplex (with KCl, Tris, Triton X-100)]; lane 8: Negative control [Multiplex (without KCl, Tris, Triton X-100)].

Figure 3.42 shows the amplicons produced from control organisms (EPEC, EIEC, EHEC and EAEC) with two different multiplex PCR assays (shown in lanes 1 and 2) and single assays of *E. coli* strains after analysis on 1.5% agarose gel. As illustrated in **Figure 3.42**, lane 1 represents multiplex PCR amplicons produced without added buffers (KCl, Tris and Triton X-100), while lane 2 represents multiplex PCR with added buffers. A faint non-specific DNA band (390 bp) produced in lane C was outside the expected size for any of the *E. coli* strains under investigation. The fragment was sequenced and while positive for *E. coli*, was not specific for any of the pathogenic strains. For single PCR assays, no added buffers were used. Thicker DNA bands were noted in lane 2 than those in lane 1 after analysis on agarose gel. Once again the non-specific DNA band (390 bp) was observed in both lanes of the multiplex PCR products (lanes 1 and 2), but the band was a different size from the expected amplicon sizes [881 bp (EPEC), 619 bp (EIEC), 518 bp (EHEC) and 254 bp (EAEC)]. The non-specific DNA band produced in lanes 1 and 2 was once again sequenced and confirmed negative for the *stx*, *eae, ipaH* and *aggR* genes after using the online BLAST program (Altschul *et al.,* 1997). The fragment was positive for non pathogenic *E. coli* strain. The optimised reaction mixture comprising of the added reaction buffers and 30 cycles of amplification, which produced amplicons in lane 2 (**Figure 3.42**) was then applied to water samples.

3.4.6 Identification of *Escherichia coli* Strains in Water Samples Using Multiplex PCR

3.4.6.1 Berg River

Simultaneous multiplex PCR amplification of the EHEC (*stx* gene), EIEC (*ipaH* gene), EPEC (*eae* gene) and EAEC (*aggR* gene) strains was applied to water samples collected from the Berg River. The *eae* (881 bp) gene for EPEC was not detected after electrophoresis, throughout the sampling period. Previous studies using multiplex PCR was able to detect the EPEC strain in sewage polluted seawater samples in Hong Kong (Kong *et al.,* 1999). In a different study perfomed by Omar and Barnard (2010), the use of multiplex PCR also enabled the detection of EPEC in sewage at different treatment stages, including the final stage of treatment to be released into the environment. This shows that it can be detected in river water samples when sewage water flows into river systems. The possibility exists that EPEC was not present in the river water samples collected. As illustrated in **Figure 3.43**, faint DNA bands of 619 bp for the *IpaH* gene (EIEC) were detected in weeks 1 (lane 1), 5 (lane 3), 7 (lane 4) and 13 (lane 13). In a previous study, performed on environmental water samples, PCR enabled the identification of the *ipaH* gene specific for the EIEC, which was present in 11 water samples (Hsu *et al.,* 2010).

As illustrated in **Figure 3.43**, faint DNA bands (518 bp) representing the shiga toxin (*stx*) gene of the EHEC strain were seen in lanes 1 (week 1) and 13 (week 28). A study

conducted by Doughari *et al.* (2012) detected the verotoxins 1 and 2, which are found in the EHEC strain in the Berg River system using the antibody-based rapid slide agglutination assay with a Duopath Kit (Merck, Johannesburg, South Africa). There were also non-specific DNA bands produced in water samples having the same size observed in lane C, in lanes 3 (week 5), 4 (week 7), 5 (week 9) and 13 (week 28). These non-specific DNA bands fell outside the size of the four *E. coli* strains under investigation. The strains were sequenced and confirmed negative for the *stx*, *eae, ipaH* and *aggR* genes after using the online BLAST program (Altschul *et al.,* 1997).

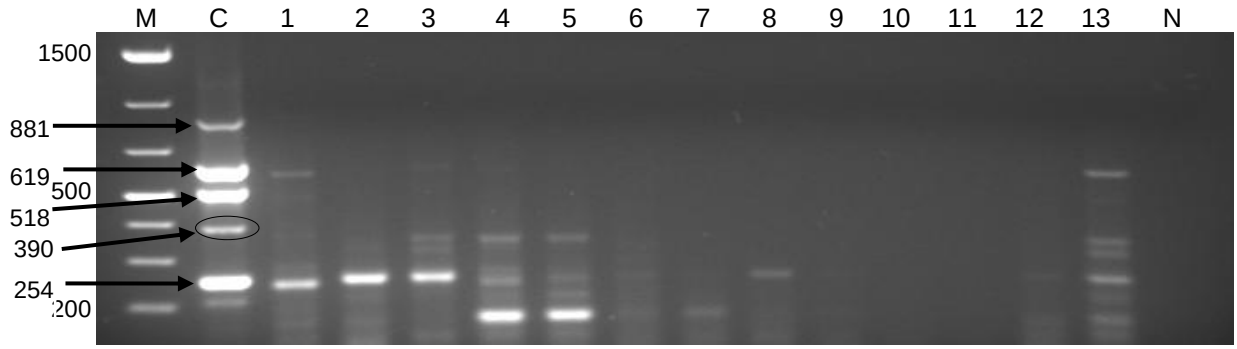

Figure 3.43 Agarose gel electrophoresis showing multiplex amplicons generated by *E. coli* strains specific primers from water samples collected along the Berg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex representing four strains of *E. coli;* lanes 1-13: represent water samples collected from week 1 to week 28 in the Berg River system and lane N: Negative control.

The *aggR* (254 bp) for EAEC was detected in weeks 1 (lane 1), 3 (lane 2), 5 (lane 3), 28 (lane 13) and faint DNA bands were observed in lanes 4 (week 7), 5 (week 9), 6 (week 9), 8 (week 11) and 12 (week 26). The EAEC was confirmed present in water samples after sequencing DNA bands in lanes 1 (week 1), 2 (week 3), 3 (week 5) and 13 (week 28). It can be concluded that 69% of water samples collected in Berg River had the EAEC strain. A study performed by Omar and Barnard (2010), detected EAEC in all untreated sewage samples and in approximately 57% of treated sewage water samples ready to be released into the environment. Kalnauwakul *et al*. (2007) also identified EAEC, ETEC, EPEC and EIEC in stool samples collected from patients with diarrhoea.

The reduced number of cycles in the multiplex PCR (as performed in **Figure 3.42**, lane 2) was then applied to water samples collected in the Berg River system as illustrated in **Figure 3.44**. These results were only positive for the reference strains and negative for all water samples tested.

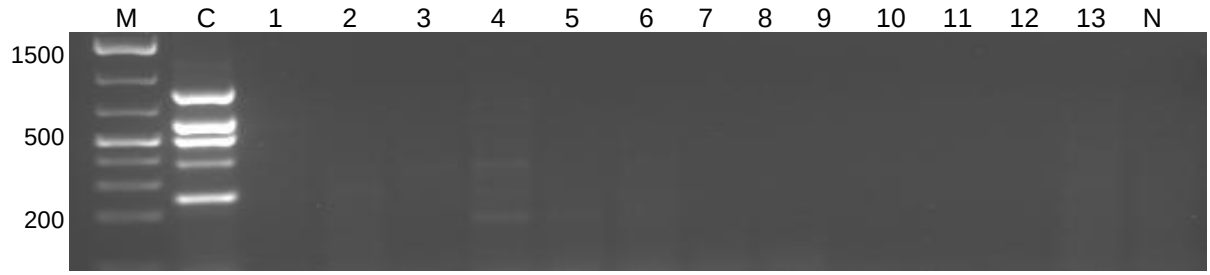

Figure 3.44 Agarose gel showing the multiplex assays performed on control *E. coli* strains and water samples of the Berg River targeting the four strains of *E. coli*. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex representing four strains of *E. coli;* lanes 1-13: represents water samples collected fortnightly from the Berg River throughout the sampling period and Lane N: Negative control.

3.4.6.2. Plankenburg River

The multiplex PCR was employed for the identification of the four different categories of pathogenic *E. coli* (EAEC, EHEC, EIEC and EPEC) in water samples collected from the Plankenburg River. The *eae* (EPEC), *IpaH* (EIEC) and *stx* (EHEC) genes were not detected by multiplex PCR for the entire sampling period. Faint DNA bands (254 bp) for the EAEC (*aggR* gene) strain were observed in weeks 3 (lane 2), 7 (lane 4) and 21 (lane 11) as shown in **Figure 3.45**, but they could not be sequenced due to the low concentration of PCR product obtained. Non-specific DNA bands (190 bp) were produced in lanes 2 (week 3), 4 (week 7), 12 (week 26) and 13 (week 28) as shown in **Figure 3.45**, but they fell outside the expected sizes of the four strains under investigation. Non-specific DNA bands in lanes 2, 3 and 13 were sequenced and confirmed negative for all the four enterotoxin genes (*eae*, *IpaH*, *stx* and *aggR*) representing each pathogenic *E. coli* strain under investigation but positive once again for *E. coli*.

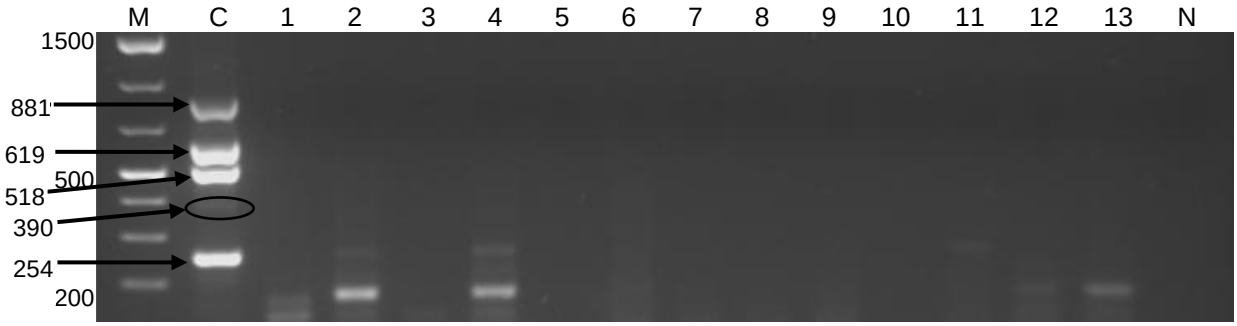

Figure 3.45 Agarose gel electrophoresis showing amplicons generated by *E. coli* strains specific primers from water samples collected along the Plankenburg River system. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex representing four strains of *E. coli;* lanes 1-13: represent water samples collected along the Plankenburg River system from week 1 (lane 1) to week 28 (lane 13) and lane N: Negative control.

The optimised PCR with reduced number of cycles (20 cycles) was then applied to Plankenburg River water samples as illustrated in **Figure 3.46**. The optimised multiplex PCR protocol was successful only for the control DNA templates and negative for all water samples collected from the Plankenburg River system.

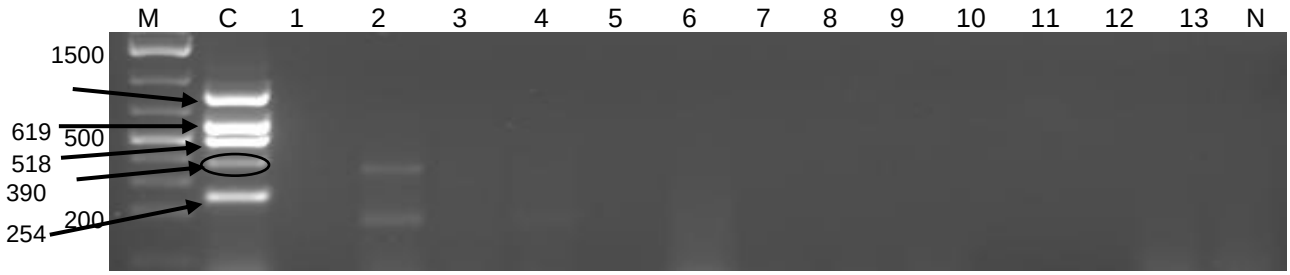

Figure 3.46. Agarose gel showing the multiplex assays performed on control *E. coli* strains and water samples of the Plankenburg River targeting the four strains of *E. coli*. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lane C: Multiplex representing four strains of *E. coli;* lanes 1-13: represents water samples collected fortnightly from the Plankenburg River (weeks 1-28) and Lane N: Negative control.

Faint DNA bands were observed in lane 2 in **Figure 3.46**, but the sizes did not correspond to the expected sizes displayed by the control strains in lane C.

Health risks associated with the exposure to pathogenic *E. coli* strains in river water samples should be assessed using rapid, cost-effective and sensitive methods. Multiplex PCR has been previously employed for the identification of various pathogenic *E. coli* from environmental and clinical samples and proved to be a versatile and efficient method to identify and differentiate pathogenic from non-pathogenic *E. coli* strains (Kong *et al.,* 1999; Eklund, 2005; Tamaki *et al.,* 2005; Omar and Barnard, 2010). It is time- and cost-effective for the simultaneous detection of numerous target genes in a single sample and also to differentiate between closely related pathogens such as the EIEC and *Shigella* species.

3.4.7. Optimisation for the Amplification of the Heat labile and the Heat stable Enterotoxins in ETEC

Amplification of the *elt* and the *est* genes in the ETEC strain (H10407) was performed in separate reaction tubes but under similar conditions as in the multiplex PCR. No DNA bands matching the expected sizes of 147 bp (*est*) and 322 bp (*elt*) were produced (results not shown) in both reaction tubes by decreasing and increasing the annealing temperature, *Go Taq* DNA polymerase, primer and dNTP mix concentrations. The *elt* was then amplified using different $MgCl_2$ concentrations (1.0 mM- 4.0 mM), and weak DNA bands were observed in lanes 3, 4, 5, 6 and 7 after analysis on 1.5% agarose gel as illustrated in **Figure 3.47**. Non-specific DNA bands of approximately 990 bp were also observed in lanes 4, 5, 6 and 7, but were outside the expected size range of 322 bp.

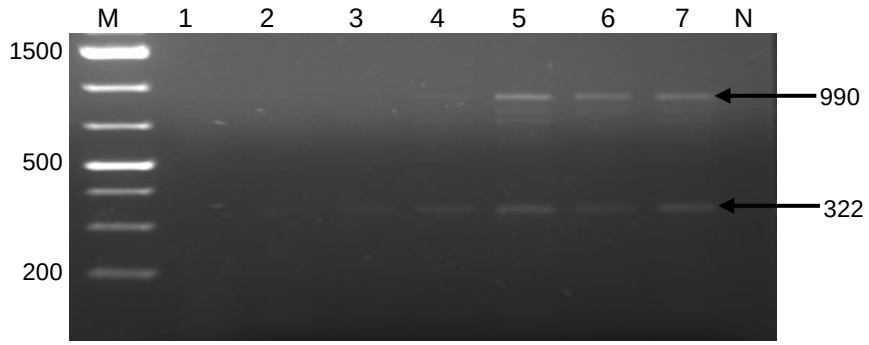

Figure 3.47 Agarose gel electrophoresis showing amplicons generated by *elt* specific primers from DNA extracted from ETEC (H10407) positive controls. Lane M: Molecular Weight Marker (Generuler^{TM} 1kb plus DNA ladder, Fermentas); lanes 1 - 7: represents amplicons generated using different concentrations of $MgCl_2$ with the primers targeting the *elt* gene (322 bp) in ETEC: lane 2: ETEC with 1.0 mM $MgCl_2$; lane 3: ETEC with 1.5 mM $MgCl_2$; lane 4: ETEC with 2.0 mM $MgCl_2$; lane 5: ETEC with 2.5 mM $MgCl_2$; lane 6: ETEC with 3.0 mM $MgCl_2$; lane 7: ETEC with 3.5 mM $MgCl_2$; lane 8: ETEC with 4.0 mM $MgCl_2$.

The amplicons of size 322 bp produced in lanes 5, 6 and 7 were sequenced and confirmed positive for the whole genome of *E. coli*, but negative for the *elt* gene using the online BLAST and the Ribosomal database programs (Altschul *et al.,* 1997; Cole *et al*., 2009). This led to the extraction of DNA using other methods (**Section 2.5.3.8.1**) previously used for the H10407 strain. Still no DNA bands were observed for the *elt* (322 bp) and *est* (147 bp) genes, after performing PCR using the multiplex conditions as listed in **Section 2.5.3.6**. Colony PCR was then performed on the H10407 strain to verify whether the strain obtained from National Institute of Communicable Diseases (NICD) was an *E. coli* strain. As illustrated in **Figure 3.48**, six colonies used as template DNA were positive for the *phoA* gene (956 bp) (lanes 1, 2, 3, 5, 6 and 7), while only one colony was *phoA* negative (lane 4).

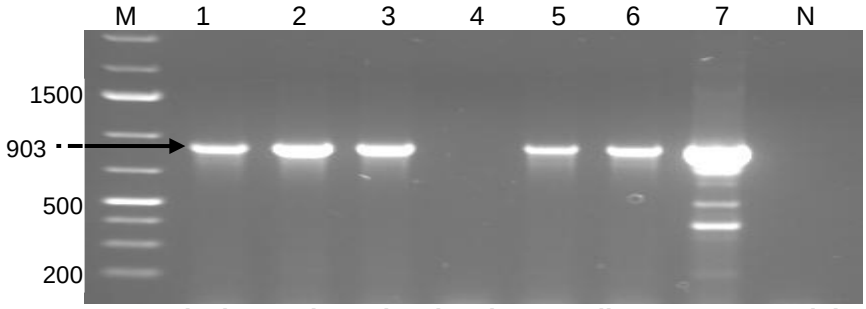

Figure 3.48 Agarose gel electrophoresis showing amplicons generated by *E. coli* specific primers from DNA extracted from the ETEC (H10407) strain. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane 1: *E. coli 1;* Lane 2*: E. coli 2;* Lane 3*: E. coli 3;* Lane 4*: E. coli 4;* Lane 5*: E. coli 5;* Lane 6*: E. coli 6;* Lane 7*: E. coli* 7 and lane N: *E. coli* negative control.

All amplicons were sequenced and confirmed positive for the *phoA* gene after analysis of the PCR products on agarose gel as illustrated in **Figure 3.48**. The NICD also performed tests to identify the two genes (*elt* and *est*) and found that the H10407 strain had lost the plasmid DNA carrying the *est* and *elt* genes.

3.4.7.1 Detection of the Enterotoxigenic *Escherichia coli* Strain in Water Samples

Even though the NICD confirmed that their positive control had lost the plasmid containing the *est* and *elt* genes, the *est* and *elt* genes specific for the ETEC strains were amplified in water samples collected from the two river systems in single reactions. They were amplified using the same cycling conditions used in the multiplex assay (**Section 2.5.3.7**). The negative control was run alongside the DNA extracted from water samples collected in both river systems. **Figure 3.49** shows the *elt* gene (322 bp) that was detected in the Berg River samples collected in weeks 7 (lane 4), 15 (lane 8), 26 (lane 12) and 28 (lane 13) were sequenced and confirmed positive using the online BLAST program (Altschul *et al.,* 1997). There were non-specific DNA bands produced in lanes 1, 2, 3, 4, 12 and 13, but they did not interfere with the DNA band (322 bp) of the *elt* gene under investigation.

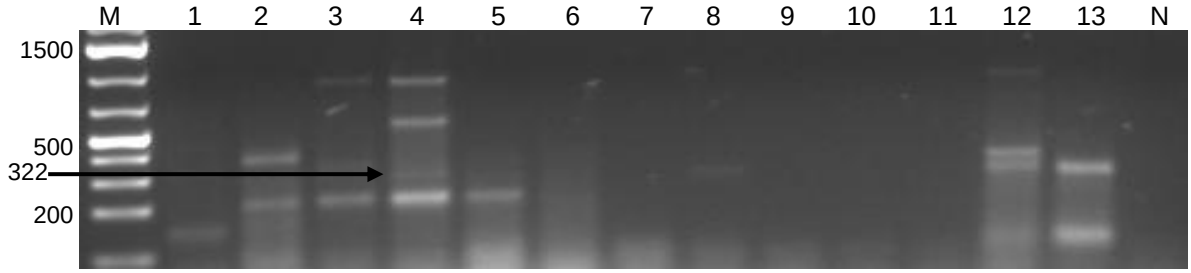

Figure 3.49 Agarose gel electrophoresis showing amplicons generated by ETEC (*elt* gene, 322 bp) strain specific primers from DNA extracted from water samples collected in the Berg River. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents samples from the Berg River from week 1 (lane 1) to week 11 (lane 6) and Lane N: ETEC negative control.

Figure 3.50 illustrates the DNA bands representing the *elt* gene (322 bp) (from water samples collected at the Plankenburg River) specific for the ETEC strain in lanes 1(week 1), 2 (week 3), 3 (week 5), 4 (week 7), 6 (week 11) and 12 (week 26). The *est* gene (147 bp) was detected (**Figure 3.51**) in water samples collected in weeks 3 (lane 2), 7 (lane 4) and 26 (lane 12) from the Plankenburg River.

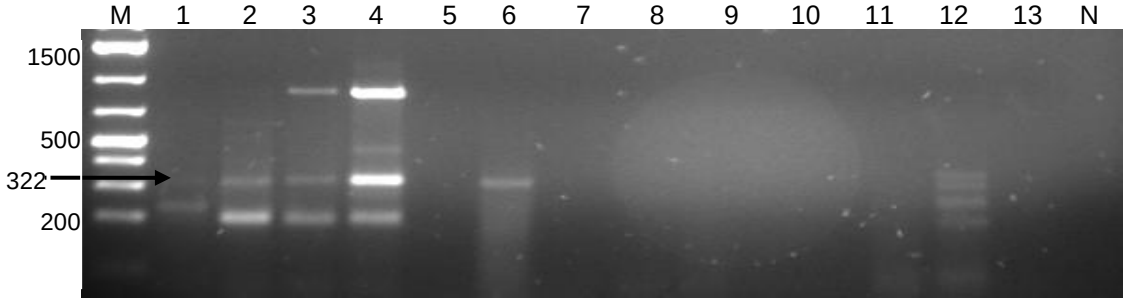

Figure 3.50 Agarose gel electrophoresis showing amplicons generated by ETEC (*elt* gene, 322 bp) strain specific primers from DNA extracted from water samples collected in the Plankenburg River. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents samples from the Plankenburg River from week 1 (lane 1) to week 28 (lane 13) and Lane N: ETEC negative control.

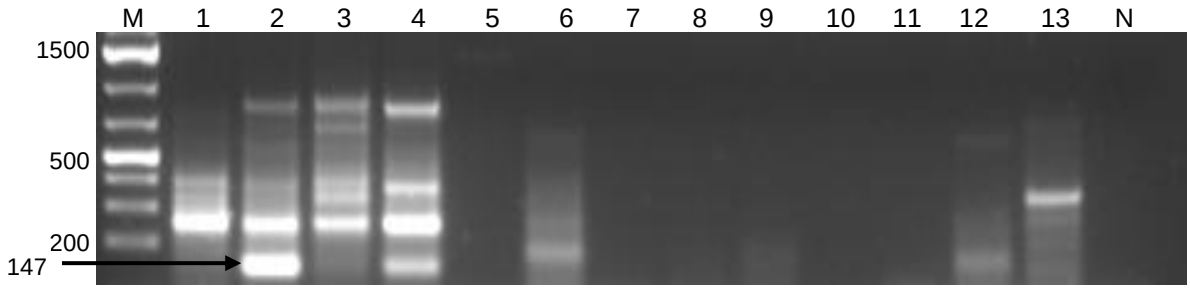

Figure 3.51 Agarose gel electrophoresis showing amplicons generated by ETEC (*est* gene, 147 bp) strain specific primers from DNA extracted from water samples collected in the Plankenburg River. Lane M: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); lanes 1-13: represents samples from the Plankenburg River from week 1 (lane 1) to week 28 (lane 13) and Lane N: ETEC negative control.

The 147 bp DNA bands were sent for sequencing and confirmed positive by using the online BLAST program (Altschul *et al.,* 1997). The *est* gene was not detected in the Berg River system during the study period.

Malfunctioning sewage systems, inadequately treated wastewater and general run off from the informal settlements carrying a variety of microorganisms could be discharged directly into the river systems (Winter and Mgese, 2011). Human waste, which generally contains high levels of possible pathogenic microorganisms, also commonly finds its way into rivers due to a lack of proper sanitation facilities (Barnes and Taylor, 2004; Gemmell and Schmidt, 2010). The results were in agreement with the previous study performed by Salem *et al.* (2011), where the *est* gene was detected in most of the wastewater effluents in different regions of Tunisia, which was to be discharged into the environment. Kong *et al.* (1999) also detected the *elt* and *est* genes in seawater samples, which were polluted by sewage using the multiplex PCR.

3.4.8 Optimisation of Real-time Polymerase Chain Reaction

Real-time PCR was optimised using different Sybr green master mixes and also different amplification conditions. The amplicons produced for multiplex and singleplex PCRs were analysed on a 2% agarose gel as illustrated in **Figure 3.52**. Lanes 1-10 and 11-15 represent the amplicons obtained by using the Sybr green master mix from Bio-Rad and Qiagen, respectively. Lanes 1-5 were produced with the cycling conditions similar to the optimised multiplex PCR as described in **Section 2.5.3.2.2**. Lanes 6-15 were amplified with optimised real-time PCR cycling conditions as described in **Section 2.5.3.3.1**, while lanes 11-15 using the Qiagen reagents produced stronger bands for the multiplex and single assays as illustrated in **Figure 3.52**. As can be observed in Lane 1 (Mulitiplex representing EAEC, EIEC, EHEC and EPEC), very weak bands were produced for the EPEC (*eae*, 881 bp), EAEC (*aggR*, 254 bp) and EHEC (*stx*, 518 bp), while the EIEC (*ipaH*, 619 bp) was the most pronounced.

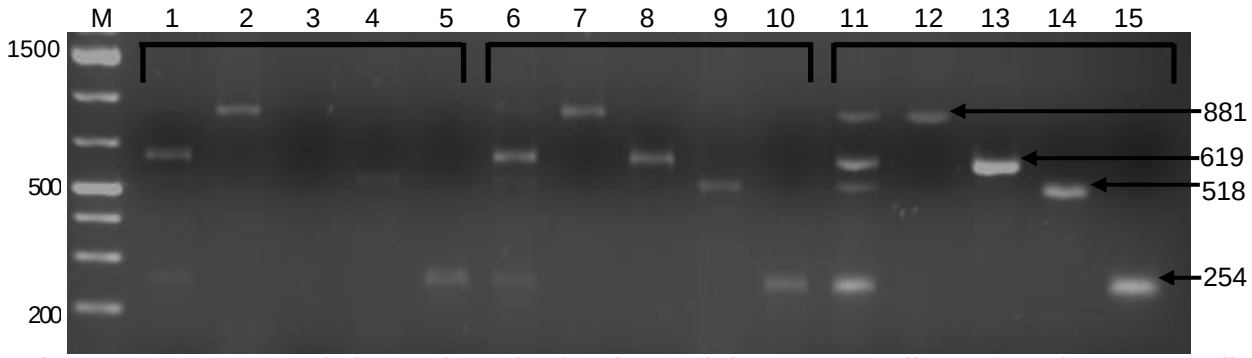

Figure 3.52 Agarose gel electrophoresis showing real-time PCR amplicons from known *E. coli* strains using strains specific primers. Lane M: DNA Ladder Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane 1: Multiplex with 4 sets of primers and DNA from 4 *E. coli* controls; Lane 2: EPEC; Lane 3: EIEC; Lane 4: EHEC; Lane 5: EAEC; Lane 6: Multiplex with 4 sets of primers and DNA from 4 *E. coli* strains; Lane 7: EPEC; Lane 8: EIEC; Lane 9: EHEC; Lane 10: EAEC; Lane 11: Multiplex with 4 sets of primers and DNA from 4 *E. coli* strains; Lane 12: EPEC; Lane 13: EIEC; Lane 14: EHEC; Lane 15: EAEC.

The multiplex assay in lane 6 produced weak DNA bands for the EPEC, EHEC and EAEC strains, but stronger DNA bands were observed in single assays [lane 7 (EPEC, 881 bp), lane 8 (EIEC, 619 bp), lane 9 (EHEC, 518 bp) and lane 10 (EAEC, 254 bp)] under same cycling conditions. Real-time PCR cycling conditions were further optimised by reducing the number of cycles using the Sybr green master mix (Qiagen) and the PCR products are shown in **Figure 3.53**. Lighter DNA bands were observed in lane 1 for the EPEC and EHEC strain while the non-specific bands were not obtained in the multiplex assay. More pronounced DNA bands were observed for the EIEC and EAEC in lane 1. Real-time PCR was used to amplify the genes found in the four strains of *E. coli* in a shorter time when compared to the conventional multiplex PCR. The Sybr green master mix obtained from Qiagen showed better bands of PCR products on 1.5% agarose gel (**Figure 3.52**), when compared to the Sybr green master mix from Bio-Rad.

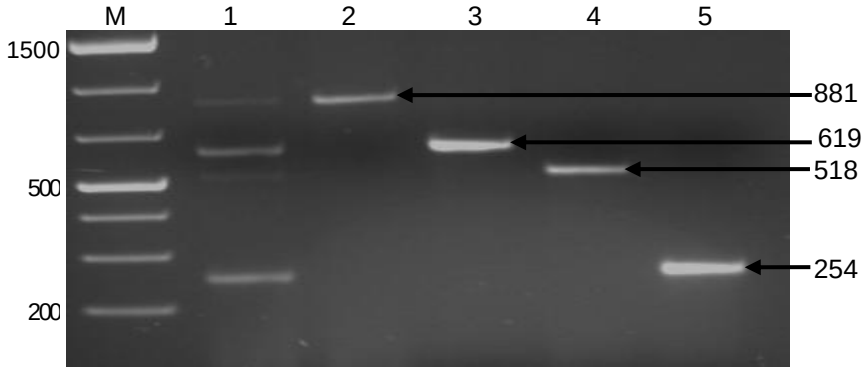

Figure 3.53 Agarose gel electrophoresis showing real-time PCR amplicons confirmation on a 2% agarose gel from control strains. Lane M: DNA Ladder Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane 1: Multiplex with 4 sets of primers; Lane 2: EPEC (881 bp); Lane 3: EIEC (619 bp); Lane 4: EHEC (518 bp); Lane 5: EAEC (254 bp).

3.4.8.1. Identification of *Escherichia coli* Strains in Water Samples Using Real-time PCR

The optimised real-time PCR assay (as described in **Section 2.5.3.10**) using the Sybr green master mix (Qiagen) enabled the detection of the pathogenic strains of *E. coli* from contaminated river water as shown in **Figure 3.54** which illustrates the expected amplicons. The expected sizes of 254 bp for the EAEC strain were detected in weeks 1 (lane 1), 3 (lane 2), 5 (lane 3), 7 (lane 4), 9 (lane 5), 11 (lane 6), 15 (lane 8), 26 (lane 12) and 28 (lane 13) in the Berg River while 619 bp for EIEC were detected in weeks 26 (lane 12) and 28 (lane 13) of the sampling period in the same river. There were light non-specific DNA bands (390 bp) produced in lanes 3 (week 5), 4 (week 7) and 12 (week 26), which corresponded in size to the positive control in lane C. As previously explained, these non-specific DNA bands contained sequences for *E. coli* but not any of the pathogenic strains of *E. coli* (EAEC, EHEC, EIEC and EPEC), which were tested. The *eae* gene representing the EPEC was detected once in week 28 (lane 13), while the *stx* gene representing the EHEC was detected in weeks 1 (lane 1) and 28 (lane 13). The DNA bands representing the latter two genes were light, as illustrated in **Figure 3.54**.

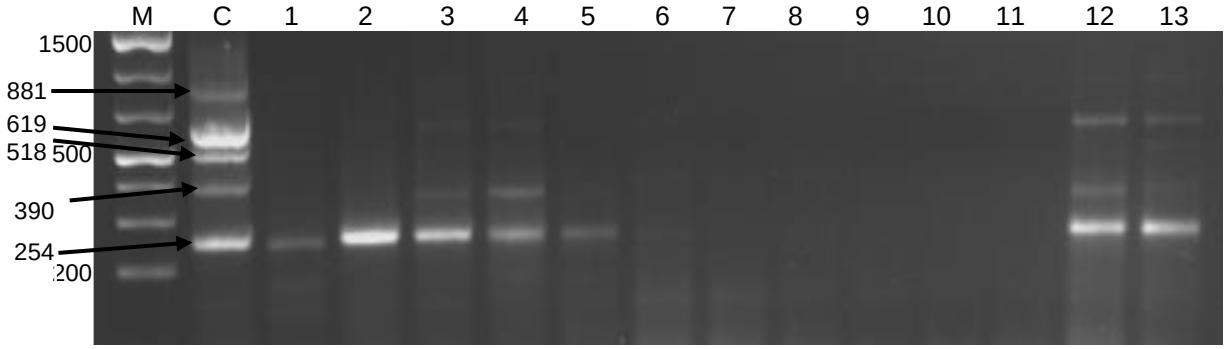

Figure 3.54 Agarose gel electrophoresis showing amplicons generated by real-time PCR using *E. coli* strains specific primers from DNA extracted from Berg River water samples. Lane M: DNA Ladder Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane C: Positive control for multiplexed *E. coli* strains; Lanes 1-13: Berg River water samples collected for the entire sampling period from week 1 (lane 1) to week 28 (lane 13).

In the Plankenburg River (**Figure 3.55**) the *aggR* gene for EAEC was detected in weeks 3 (lane 2), 5 (lane 3), 7 (lane 4) 26 (lane 12) and 28 (lane 13). No *stx* producing *E. coli* were detected, as the results of the real-time multiplex PCR (**Figure 3.55**) did not show any DNA band size of 518 bp (the size of *stx* gene in EHEC strain). The *eae* gene was detected in weeks 5 (lane 3) and 7 (lane 4), while the *ipaH* in weeks 7 (lane 4) and 28 (lane 13) for EPEC and EIEC, respectively. Real-time multiplex PCR seemed to be more sensitive when compared to the conventional multiplex PCR, as more *E. coli* strains were detected and also stronger DNA bands were produced for the same samples (**Table 3.7** and **Table 3.8**). The DNA band of 254 bp visible in lanes 2 (week 3), 4 (week 5) and 13 (week 28) were sequenced and confirmed positive for the *aggR* gene in EAEC. It can be concluded that

EAEC was found in 31% of water samples collected in the Plankenburg River. The results obtained in this study are in agreement with previous studies performed in Tunisia and in the Province of Gauteng, South Africa (Salem *et al.*, 2011; Omar and Barnard, 2010).

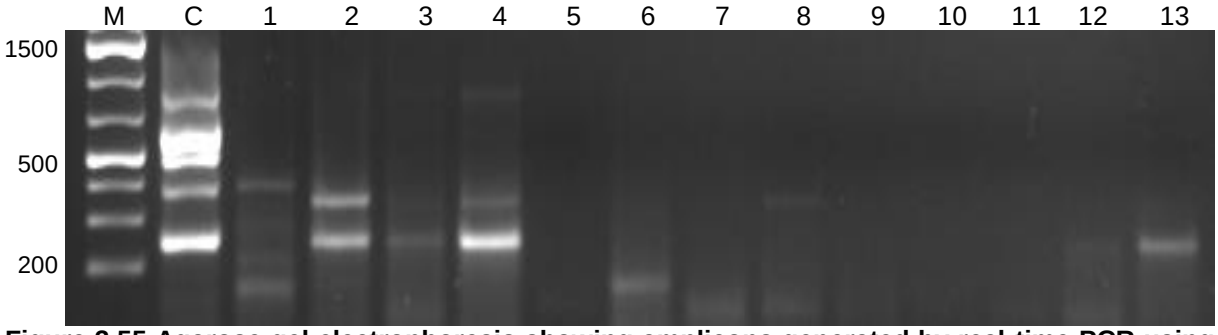

Figure 3.55 Agarose gel electrophoresis showing amplicons generated by real-time PCR using *E. coli* strains specific primers from DNA extracted from the Plankenburg River water samples. Lane M: DNA Ladder Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas); Lane C: Positive control for multiplexed *E. coli* strains; Lanes 1-13: Plankenburg River samples collected for the entire sampling period from week 1 (lane 1) to week 28 (lane 13).

Polymerase chain reaction has been found to be a reliable technique in the identification of microorganisms (Powledge, 2004). During the entire study period, real-time multiplex PCR was noted to be more sensitive compared to the conventional multiplex PCR in the identification of the *E. coli* strains in river water. **Tables 3.7** and **3.8** show the detected pathogenic strains of *E. coli* from both river systems using conventional, conventional-multiplex and real-time multiplex PCR.

Table 3.7 Pathogenic *E. coli* results obtained by the conventional, conventional-multiplex and real-time PCR in water samples collected from Berg River.

Samples in week	Conventional PCR for ETEC		Conventional multiplex PCR				Real-time PCR			
	elt gene	*est* gene	EPEC	EIEC	EHEC	EAEC	EPEC	EIEC	EHEC	EAEC
1	-	-	-	√	√	√	-	√	√	√
3	-	-	-	-	-	√	-	-	-	√
5	-	-	-	√	-	√	-	√	-	√
7	√	-	-	√	-	√	-	√	-	√
9	-	-	-	-	-	√	-	-	-	√
11	-	-	-	-	-	√	-	-	-	√
13	-	-	-	-	-	-	-	-	-	-
15	√	-	-	-	-	√	-	-	-	√
17	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
26	√	-	-	-	-	√	-	√	-	√
28	√	√	-	√	√	√	√	√	√	√

The real-time multiplex PCR was found to be time-effective for the identification of *E. coli* strains. Also more pronounced DNA bands were observed in real-time multiplex PCR

products (**Figures 3.54** and **3.55**) compared to conventional-multiplex PCR amplicons (**Figure 3.43** and **3.45**) in water samples collected in the Berg- and Plankenburg river systems, respectively. In addition, less non-specific DNA bands were observed in real-time PCR products (**Figure 3.54**) compared to conventional-multiplex PCR products (**Figure 3.43**) in samples obtained from the Berg River system.

These results are in agreement with a previous study by Mackay (2004) whereby real-time PCR was found to be more rapid, sensitive and reproducible when compared to conventional-multiplex PCR in the identification of microorganisms in clinical samples. The two techniques were similar in the identification of the *E. coli* strains, except that the real-time multiplex PCR showed more pronounced DNA bands than multiplex PCR. The number of cycles were reduced for the control organisms (EAEC, EHEC, EIEC and EPEC) using the real-time PCR to reduce the turnaround time for obtaining the results, but it did not produce any results with water samples collected from the Berg- and Plankenburg River systems. The reduced numbers of cycles were also used in the conventional-multiplex PCR, but were only producing positive results with control strains and negative with all river water samples.

Table 3.8 Pathogenic *E. coli* results obtained by the conventional, conventional-multiplex and real-time PCR in water samples collected from Plankenburg River.

Samples in week	Conventional PCR for ETEC		Conventional multiplex PCR				Real-time PCR			
	elt gene	*est* gene	EPEC	EIEC	EHEC	EAEC	EPEC	EIEC	EHEC	EAEC
1	√	-	-	-	-	-	-	-	-	-
3	√	√	-	-	-	√	-	-	-	√
5	√	-	-	-	-	-	√	-	-	√
7	√	√	-	-	-	√	√	√	-	√
9	-	-	-	-	-	-	-	-	-	-
11	√	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	√	-	-	-	-
26	√	√	-	-	-	-	-	-	-	√
28	-	-	-	-	-	-	-	√	-	√

4. GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusion

South Africa mainly relies on dams, rivers and streams as the most important sources of water for domestic, industrial, agricultural and recreational purposes, in the thermo-electric power industry to cool electricity-generated equipment as well as to naturally purify wastewater effluents. These important water sources are however threatened by mainly natural and anthropogenic pollution, both of which have a negative impact on the health of people relying on the water sources. In addition, the quality of water sources are being compromised by contamination from industries and waste from municipal sewage treatment plants as well as the excessive use of pesticides and fertilisers in the agricultural sector. Many of the urban rivers (including the Berg- and Plankenburg River systems) in the Cape Town area are also used as conduits for discharging treated or partially treated effluents into the ocean. Leaching of metals and other contaminants, present in sewage wastes and household products from the informal settlements, suburbs, hazardous waste dumpsites and effluent in treatment plants, also contributes to the contamination of both surface and groundwater sources. In addition, increased levels of microbial pollution are experienced during the rainy seasons as large numbers of microorganisms are washed from soil, faecal wastes (from humans and animals) and decaying vegetable matter into the river systems. The main aim of the study was thus to compare diagnostic tools and molecular based techniques for the rapid identification of *E. coli* and coliforms in contaminated water collected in the Berg- and Plankenburg River systems.

4.1.1 Enumeration of Coliforms and *Escherichia coli* in River Water

Numerous methodologies are currently available to monitor the quality of raw and treated water sources and the efficiency of treatment and disinfection processes. The three methods predominantly used for the routine detection and enumeration of microorganisms in the water industry, include the Multiple Tube Fermentation (MTF) and Membrane Filtration (MF) techniques as well as the Colilert 18® system. These techniques were employed in the current study to enumerate the total coliform, total faecal coliforms and *E. coli* counts. Sampling at the Berg- and Plankenburg River system sites (**Figures 2.1** and **2.2** in Chapter 2) started in July 2010 and continued until January 2011.

The faecal coliforms enumerated in the Berg River system using the MTF technique ranged from 1.1×10^3 microorganisms/100 ml in week 1, to 1.4×10^4 microorganisms/100 ml in week 28 as illustrated in **Figure 3.1** (Chapter 3). The corresponding total *E. coli* counts ranged from 5.4×10^3 microorganisms/100 ml in week 1 to 3.5×10^4 microorganisms/100 ml

in week 28. In comparison, the total coliform counts obtained using the MF technique ranged from 1.0×10^4 microorganisms/100 ml in week 1 to 7.8×10^3 microorganisms/100 ml in week 28 as illustrated in **Figure 3.2** (Chapter 3). The corresponding *E. coli* counts obtained by the MF technique ranged from 6.9×10^3 microorganisms/100 ml in week 1 to 5.6×10^3 microorganisms/100 ml in week 28. The Colilert 18® system was performed according to the Paarl municipality protocol (no dilution of river water samples before analysis), which resulted in the same total coliform counts of 2.42×10^4 microorganisms/100 ml being recorded. Results thus indicated that significantly high ($p < 0.05$) coliform and *E. coli* counts were obtained for the Berg River system throughout the sampling period, by means of the enumeration techniques (MTF, MF and Colilert 18® system) employed. According to the Department of Water Affairs and Forestry (DWAF, 1996), the maximum accepted limit for coliform bacteria present in recreational water should not exceed counts of 2000 microorganisms/100 ml. Where water is used for irrigational purposes the coliform count should not exceed a maximum of 10 000 microorganisms/100 ml. The total number of coliforms detected by the three enumeration techniques then exceeded the stipulated limit for recreational water throughout the study period except in week 1 where a count of 1.1×10^3 microorganisms/100 ml was obtained using the MTF technique. Total coliforms obtained by the MF (weeks 1, 9, 17, 19, 21 and 26) and MTF (weeks 3, 9, 11, 13, 17, 19, 21, 26 and 28) techniques also exceeded the stipulated guidelines of 10 000 microorganisms/100 ml (DWAF, 1996) where water is used for irrigational purposes.

The faecal coliforms in the Plankenburg River system enumerated by the MTF technique ranged from 1.1×10^3 microorganisms/100 ml in week 1 to 9.2×10^6 microorganisms/100 ml in week 28. The corresponding total *E. coli* counts ranged from 2.6×10^3 microorganisms/100 ml in week 1 to 1.6×10^7 microorganisms/100 ml in week 28. In comparison, the total coliform counts obtained using the MF technique, for the Plankenburg River system, ranged from 1.45×10^3 microorganisms/100 ml in week 1, to 1.82×10^4 microorganisms/100 ml in week 28 (**Figure 3.4** in Chapter 3). The total coliform counts obtained using the Colilert 18® system ranged from 2.42×10^3 microorganisms/100 ml in week 1 to 2.42×10^4 microorganisms/100 ml in week 28. As discussed in **Section 3.2.2** (Chapter 3), no variation in the *E. coli* and coliform counts (weeks 1 to 13) were obtained as the samples were analysed undiluted (Municipality protocol). In the Plankenburg River system, total coliform counts also exceeded the stipulated guidelines of 2000 microorganisms/100 ml (DWAF, 1996) for water used for recreational purposes, except in weeks 1 and 11, were counts of 1.5×10^3 microorganisms/100 ml and 1.8×10^3 microorganisms/100 ml (**Figure 3.4** in Chapter 3) were obtained respectively, using the MF technique. According to the Department of Water Affairs and Forestry (DWAF, 1996), the maximum accepted limit for *E. coli* present in recreational water should not exceed counts of 130 microorganisms/100 ml. Where water is used for irrigational purposes the *E. coli* count

should not exceed 1 microorganisms/100 ml. In the Plankenburg River system, all *E. coli* counts obtained by the Colilert 18® system, MF and MTF techniques exceeded the stipulated guidelines of 1 and 130 microorganisms/100 ml as stipulated by DWAF (1996) for water used for irrigational and recreational purposes, respectively.

The coliform and *E. coli* counts obtained by the enumeration techniques thus showed that the water in the Berg- and Plankenburg River systems is highly polluted and of great concern to fresh produce farmers, and consumers, as most farmers draw water from these rivers for irrigation without prior treatment. For the coliform and *E. coli* counts obtained using the three enumeration techniques, it was noted that the MTF method was more sensitive and obtained higher counts for most of the sampling weeks. The MTF technique was however, comparable to the MF technique in certain weeks of sampling in the Plankenburg (weeks 1 and 17) and Berg (weeks 1, 3, 7, 15 and 17) River systems. While *E. coli* and total coliforms were detected utilising the Colilert 18® system, accurate enumeration values for these two indicator groups were not obtained for the entire sampling period for both river systems. It has previously been shown that dilutions (up to 10^{-3}) of highly polluted waters increase the accuracy of the Colilert 18® system to enumerate colifoms and *E. coli* in marine waters (Pisciotta *et al.,* 2002). As the results obtained utilising the Colilert 18® system were also not comparable to the MF and MTF techniques it is recommended that highly polluted water samples be diluted to increase the accuracy of this system as a routine enumeration technique.

In should however, be noted that in the present study, the Colilert 18® system proved to be the most time-effective technique as final results were obtained after 19 hours. In comparison, the MF technique yielded results after 24 hours, while the MTF technique results were obtained after 75 hours. For the duration of the study, the MF technique and Colilert 18® system were found to be highly reproducible, implying that they could be used to test relatively large sample volumes and yielded results more rapidly than the MTF technique. The MTF technique was however, more cost-effective than the MF technique and Colilert 18® system materials as the media and reusable glassware utilised in the analysis allows for affordable routine analysis.

The selective media utilised in the Colilert 18® system and MF technique routinely utilised for the detection and enumeration of coliforms and *E. coli* has also been found to contribute to their efficiency. Fricker *et al*. (1994) discovered that MLGA showed lower recovery and produced smaller target colonies on prepared media stored for more than two weeks. However, the use of selective media such as CCA, on which coliforms and *E. coli* have been successfully cultured (from various water samples), could increase the sensitivity and selectivity of the MF technique. Previous studies have also shown that the media used in the Colilert method and MF technique influence the recovery of environmentally stressed microbial cells (maintain their viability and are still able to undergo metabolic activities), which

are unable to grow in general media used in the MTF technique (Fricker *et al.,* 1997; Eckner, 1998; Olstadt *et al.*, 2007; Nikaeen *et al.,* 2009; Fricker *et al*., 2010a). However, the media employed by the MTF technique is not specific for coliforms and *E. coli*, and elevated temperatures are used to cultivate these microorganisms.

Overall the MF technique is recommended as the best technique for the routine enumeration of *E. coli* and coliforms in terms of sensitivity, cost- and time-effectiveness (obtaining results in just over 24 hours from sampling time). The Colilert 18® system could be an alternative indicator technique to provide more rapid information for the assessment of coliforms and *E. coli* in river water. This technique has a low turn-around time to obtain final results (within 24 hours from sampling), requires less handling of samples and therefore less contamination, and is reproducible and sensitive in detecting coliforms and *E. coli* from diverse water sources. Dilution of highly polluted water samples is however, crucial when the Colilert 18® system is employed as an enumeration tool.

4.1.2 Identification of *Enterobacteriaceae* Species from River Water

4.1.2.1Selective Media Utilised for Water Samples

Sampling at the Berg- and Plankenburg River system sites (**Figures 2.1** and **2.2** in Chapter 2) started in July 2010 and continued until January 2011. Water samples were first diluted up to 10^{-2} and spread plated onto the different selective media. MacConkey and Violet Red Bile (VRB) agars were successfully employed for the isolation of lactose negative and positive *Enterobacteriaceae* from water samples for both river systems. However, it was not possible to distinguish between coliforms and *E. coli* using these two selective media. Based on phenotypical differences, the Chromocult Coliform Agar (CCA) media proved to be the most effective in distinguishing *E. coli* from other coliforms and non-coliform colonies. In addition, CCA proved to be more sensitive than MacConkey and VRB agar for the culturing of *E. coli* and coliforms as more colonies, representing the typical growth of these organisms, were observed.

4.1.2.2 Identification of *Enterobacteriaceae* in River Water Samples Using API 20 E and RapID ONE Identification Systems

The RapID ONE and API 20 E systems were employed for the identification of colonies cultured from river water samples. Microorganisms identified were obtained from the selective media (CCA, MacConkey and VRB agar), which were used for culturing water samples. Single isolated colonies, which morphologically resembled the *Enterobacteriaceae* group were then re-streaked onto nutrient agar (NA) plates. The Oxidase test was performed on each colony prior to identification. The API 20 E and RapID ONE systems

were then used according to manufacturer's instructions for the preliminary identification of *Enterobacteriaceae* species.

Tables 3.2 and **3.3** (Chapter 3) illustrate the *Enterobacteriaceae* species identified in the Berg- and Plankenburg River systems, respectively, by the API 20 E and RapID ONE identification systems. The API 20 E system successfully identified 87% and 85% of the colonies isolated from the Berg and Plankenburg River systems, respectively, while the RapID ONE system identified 82% and 75%, respectively. *Escherichia coli*, *E. cloacae*, *K. pneumonia*e and *K. oxytoca* were the most isolated organisms using the two identification techniques in both river systems for the duration of the study. The API 20 E system was more sensitive in the preliminary identification of the various isolates, as greater genus diversity was obtained in comparison to the RapID ONE system.

It was however demonstrated that the identification systems employed in the study involved the use of various materials, equipment and numerous steps were required before obtaining the final results. Approximately 48 hours was needed from the culturing of colonies and the incubation of test organisms in their respective biochemical wells [RapID ONE system (four hours) and the API 20 E system (18 hours)] for the identification up to species level. Apart from the identification systems being time consuming, the API 20 E and RapID ONE systems could not identify all the *Enterobacteriaceae* to species levels (approximately 10% of colonies from both river systems using both identification systems could not be identified) as additional tests were required, which increases the cost of analysis.

4.1.2.3 Identification of *Enterobacteriaceae* Species from River Water by Polymerase Chain Reaction

Faecal contamination from humans, livestock and wild animals are of great concern as they contain various pathogens. Increased levels of microbial pollution are also experienced during rainy seasons as large numbers of microorganisms are washed from various point- and non-point pollution sites into the river systems. Water then acts as an inert carrier of the pathogenic microorganisms, such as protozoa, helminths, viruses and bacteria, and humans can become infected with diseases such as diarrhoea, skin irritations, typhoid and respiratory disorders from the microbially contaminated water sources (Krantz and Kifferstein, 2003; Kong *et al.,* 2009).

An additional aim of this study was then to identify culturable and non-culturable *E. coli*, *K. pneumoniae*, *E. faecalis*, *S. marcescens*, *S. sonnei*, *S. typhimurium*, *M. luteus*, *B. cereus*, *P. aeruginosa*, *P. flourescens* and *V. cholerae* directly from river water using species-specific primers, targeting conserved gene regions within these microorganisms. The DNA extraction from the reference strains was optimised using two methods, namely the boiling method adapted from Watterworth *et al.* (2005) and the High Pure PCR Template

Preparation kit (used according to manufacturer's instructions). The extraction of DNA from the positive *Enterobacteriaceae* controls, utilising the High Pure PCR Template preparation kit, involved many steps and was more time consuming and costly compared to the boiling method. DNA concentrations were subsequently measured using the Qubit fluorometer and results showed that both methods successfully extracted DNA from all the *Enterobacteriaceae* species used as reference strains. While comparable genomic DNA concentrations were extracted with both methods, the boiling method was found to be cost- and time-effective, and less tedious when compared to the High Pure PCR Template Preparation kit and this method was then used for the extraction of the DNA from the river water samples. After extraction of DNA using both methods and the measurements of DNA concentration, optimisation of the conventional PCR was then performed as described in **Section 3.4.1.1** on all DNA samples extracted from reference strains used throughout the study. Previously published species specific primers were used to amplify specific genes in each *Enterobacteriaceae* species used in this study (**Table 2.2** in Chapter 2). The optimised conventional PCR cycling conditions were then applied to water samples collected from the Berg- and Plankenburg River sites (**Figures 2.1 and 2.2 in Chapter 2**), during July 2010 to January 2011.

After the DNA extraction and amplification using species specific primers (**Table 2.2** in Chapter 2) and the conventional PCR technique, sequencing and identification using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1997) was performed. **Table 3.6** (Chapter 3) illustrates the overall percentage of *Enterobacteriaceae* species detected in the Berg- and Plankenburg River systems by PCR. *Serratia marcescens* was detected throughout (100%) the sampling period in both river systems, while *V. cholerae* and *M. luteus* were not detected at any stage of the study. *Enterobacteriaceae* species such as the *E. faecalis, P. aeruginosa* and *S. sonnei* were more predominant in the Berg River system, while *S. typhimurium* was predominant in the Plankenburg River system. The detection of bacterial species belonging to the *Enterobacteriaceae* family in both river systems indicates that they were subjected to faecal pollutants.

Micrococcus luteus and *V. cholerae* were thus not detected at any point in the study by the three identification methods (PCR, API 20 E and RapID ONE identification systems) employed, while *E. coli* and *K. pneumoniae* were detected utilising all three techniques. Using the species specific primers, the PCR technique detected *E. faecalis*, *B. cereus* and *S. typhimurium* (detected once in the Plankenburg River), but these organisms were not detected using the API 20 E and RapID ONE system in both river systems. The API 20 E identification system and PCR technique also detected *P. aeruginosa* and *P. fluorescens* in certain sampling weeks in both river systems but these strains were not identified by the RapID ONE system, which however, detected *S. sonnei* in the Plankenburg River.

Conventional PCR proved to be sensitive in the identification of *Enterobacteriaceae* species in both river systems for the duration of the study. This technique also yielded results in a shorter time period when compared to the other identification systems (API 20 E and RapID ONE systems) utilised as results were obtained in just under 10 hours. Accurate laboratory analysis of river water is essential to enable informed decision-making and also to provide a proper indication of the potential risk of contracting waterborne diseases. The use of conventional PCR has also been shown to be rapid and sensitive for the successful identification of *Enterobacteriaceae* species in surface water sources (Østensvik *et al.,* 2004; Kumar *et al.,* 2010; Siri *et al.,* 2011). As *Enterobacteriaceae* species were found in the river water samples, it would suggest that the water is not suitable for use in irrigational and recreational purposes as these results indicates faecal pollution and possibly the presence of other pathogenic microorganisms (Leclerc *et al.,* 2001). These results correlate to the results obtained utilising the enumeration techniques, where significantly high ($p < 0.05$) coliform and *E. coli* counts were obtained. The presence of these organisms in river water also poses a threat to humans who ingest raw shellfish and fish, use the water for recreational purposes and consume raw fruits and vegetables irrigated with this water without any treatment (Ackermann, 2010).

The conventional multiplex PCR involved the extraction of DNA from the *E. coli* control strains [Enteroinvasive *E. coli* (EIEC) ATCC 43892, Enteropathogenic *E. coli* (EPEC) B170, Enterohaermorrhagic *E. coli* (EHEC) O157:H7, Enteroaggregative *E. coli* (EAEC) ATCC 3591-87 and the Enterotoxigenic *E. coli* (ETEC) H10407] using the boiling method (adapted from Watterworth *et al*., 2005). Multiplex PCR was initially performed using the template DNAs from all the five *E. coli* strains [EAEC, EHEC, EIEC, EPEC and ETEC], while single PCRs were also performed simultaneously for each primer set under the same conditions to evaluate the specificity of the primers used. The two genes (*elt* and *est*) in the ETEC control strain (H10407) could not be detected by both the multiplex and single PCR assay as discussed below, and this led to the exclusion of the ETEC strain in the optimisation of multiplex PCR. The multiplex PCR reaction was then optimised successfully for the simultaneous detection of the four remaining control strains of *E. coli* namely EAEC, EHEC, EIEC and EPEC strains and the optimised cycling conditions were then applied for the detection of these strains in the water samples collected from the two river systems. The EPEC strain was not detected in any of the river water samples collected from the two river systems, while the EIEC and EHEC were not detected by multiplex PCR for the entire sampling period in the Plankenburg River. As illustrated in **Figure 3.42** (Chapter 3), the EIEC, EHEC and EAEC strains were detected in certain weeks of sampling in the Berg River, while **Figure 3.44** (Chapter 3) indicates the detection of EAEC in a few water samples collected from the Plankenburg River.

The amplification of *elt* (322 bp) and *est* (147 bp) genes specific for the ETEC strain was performed separately as indicated in **Section 2.5.3.6.2**. Amplification of these genes, found in the H10407 strain, failed to yield the expected amplicons using different cycling conditions as described in **Section 2.5.3.6.2**, and led to the utilisation of DNA extraction procedures and primer sets adopted in previous studies. Colony PCR was then performed on the H10407 strain to verify whether the strain obtained from National Institute of Communicable Diseases (NICD) was an *E. coli* strain. The NICD also performed tests to identify the two genes (*elt* and *est*) and found that the H10407 strain had lost the plasmid DNA carrying the *est* and *elt* genes. The *est* and *elt* genes specific for the ETEC strains were then amplified in water samples collected from the two river systems in single reactions. They were amplified using the same cycling conditions used in the multiplex PCR assay (**Section 2.5.3.7**). The *elt* gene was detected in both river systems in certain weeks of sampling, while the *est* gene was only detected in the Plankenburg River system. The *elt* and *est* genes were both detected in weeks 3, 7 and 26 in the Plankenburg River, while in the Berg River there were both detected only in week 28.

The *E. coli* strains were amplified in a single reaction tube for each water sample collected, therefore fewer reagents were used, and hence it was cheaper and required less time to obtain results for different strains when compared to conventional PCR, the RapID ONE and API 20 E systems. The use of strains specific primers in the PCR reaction also increased the detection of these *E. coli* strains in the river water. However, the presence of pathogenic *E. coli* strains in the river systems confirms faecal contamination, and since sampling sites were situated close to the informal settlements, it raises concerns for humans who might be exposed to these strains.

The emergence of real-time PCR in the identification and quantification of DNA allows for the reliable detection and quantification of microorganisms in an original sample (Gizinger, 2003). In the present study, real-time PCR was firstly optimised using different Sybr green master mixes obtained from Qiagen and Bio-Rad on positive control *E. coli* strains. The Sybr green master mix obtained from Qiagen yielded better intensity bands of PCR products on a 1.5% agarose gel, when compared to the Sybr green master mix from Bio-Rad. The optimised real-time PCR assay (as described in **Section 2.5.3.10**) using the Sybr green master mix (Qiagen) enabled the detection of the pathogenic strains of *E. coli* from contaminated river water as shown in **Section 3.4.8.1**. The EAEC was not detected in weeks 13, 17, 19 and 21, while the EIEC (detected in weeks 1, 5, 7, 26, 28), EHEC (detected in weeks 1 and 28), EPEC (detected only in week 28) in the Berg River, while in the Plankenburg River system the EAEC (detected in weeks 3, 5, 7, 26 and 28), EPEC (detected in weeks 5 and 7) and EIEC (detected in weeks 5 and 7) strains were detected in some weeks using the real-time PCR.

Real-time PCR was found to be time-effective in the identification of *E. coli* strains, and more pronounced DNA bands were observed in real-time PCR products (**Figures 3.53** and **3.54** in Chapter 3) compared to conventional/multiplex PCR amplicons (**Figures 3.42** and **3.44** in Chapter 3) in water samples collected in the Berg- and Plankenburg river systems, respectively. In addition, less non-specific DNA bands were observed in the real-time PCR products (**Figure 3.53** in Chapter 3) compared to conventional-multiplex PCR products (**Figure 3.42** in Chapter 3) in samples obtained from the Berg River system.

4.2 Recommendations

To sustain the services provided by the Berg- and Plankenburg Rivers in the Western Cape (South Africa), these water sources should frequently be monitored, results assessed and reported according to the practices acknowledged by responsible bodies [Department of Water Affairs and Forestry (1996) and South African Bureau of Standards (1984)]. Based on the results obtained in this study:

- The MF technique is recommended as the best technique for the routine enumeration of *E. coli* and coliforms in terms of sensitivity, cost- and time-effectiveness. However dilutions should be performed if for the Colilert 18® system is to be employed in the analysis of highly polluted water samples.
- A growth medium, such as the Chromocult Coliform Agar (CCA), which is also more sensitive, selective and able to distinguish *E. coli* from other coliforms and non-coliforms should also be employed in the MF technique.
- The use of the enumeration techniques only is insufficient in the assessment of faecal contamination in river water samples. Enumeration techniques should thus be used in conjunction with the very sensitive PCR technique for the accurate detection of pathogens.
- For successful PCR amplification, there is a need for the extraction and extensive purification of DNA from all general environmental contaminants such as humic materials, clay, metals and organic material, which can interfere with the PCR.
- Since multiplex PCR is more cost effective compared to the conventional PCR, species-specific primers targeting a gene found in most *E. coli* strains (alkaline phosphatase gene) and the coliform group (β-galactosidase gene) should be used in a multiplex PCR to simultaneously detect these organisms in water samples.
- Real-time PCR proved to be more sensitive when compared to the multiplex PCR, however there is a need for further optimisation to routinely apply the real-time PCR technique in the detection and enumeration of coliforms and *E. coli* in river water.

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6. APPENDICES

APPENDIX A: Preparation of Reagents Used in the Extraction of DNA and Anaylsis of Polymerase Chain Reaction Products

Preparation of 0.5 M ethylenediaminetetraacetic acid (EDTA) with a pH of 8.0

- Add disodium EDTA to 800 ml of distilled water and stir vigorously on a magnetic stirrer
- Adjust the pH to 8.0 with sodium hydroxide (NaOH)
- NB: The disodium salt of EDTA go into solution when the pH is adjusted to 8.0 by the addition of NaOH

Preparation of 1 M of Tris-Hcl

- Dissolve 121.14 g of Tris-Hcl base in 800 ml of distilled water
- Adjust the pH to the desired value by adding concentrated Hcl
- Allow the solution to cool to room temperature before making final adjustments to the pH 8.0
- Adjust the total volume to 1 000 ml with distilled water

Preparation of ethidium bromide

- Add 1 g of ethidium bromide pellet to 100 ml of distilled water
- Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved
- Wrap the container in aluminium foil or transfer the solution to a dark bottle
- Store at room temperature

Preparation of 50 × Tris-acetate ethylenediaminetetraacetic acid (TAE) buffer

- Dissolve 242 g of Tris base in 600 ml of distilled water
- Add 57.1 ml of glacial acetic acid
- Add 100 ml of 0.5 M EDTA (at pH 8.0)
- Use distilled water to adjust to 1 000 ml
- Sterilise and store at room temperature

Preparation of Loading buffer

In 100 ml	65 g (w/v) sucrose
	1 000 µl of 1 M Tris-Hcl
	2 000 µl of 0.5 M EDTA
	300 mg of Bromophenol blue

Preparation of agarose gel for separating deoxyribonucleic acid (DNA)

- Add appropriate amount of agarose in appropriate volume of 1X TAE buffer
- Dissolve the agarose gel using a microwave
- Let it cool to about 55°C then add ethidium bromide of 0.5 µg/ml final concentration
- Pour the dissolved gel in casting tanks with the combs in place
- After it has solidified, remove combs gently to prevent the tearing of sample wells and it will be ready for sample loading

APPENDIX B: Enzymes Used During DNA Extraction

Preparation of 10 mg/ml lysozyme

- Dissolve solid lysozyme at a concentration of 10 mg/ml in 10 mM Tris-Hcl (pH 8.0) immediately before use
- Make sure that the pH for Tris-Hcl is at 8.0 for efficient functioning of lysozyme

Preparation of 20 mg/ml Proteinase K

- Dissolve an appropriate amount of proteinase K powder in sterile 50 mM Tris-Hcl (pH 8.0), 1.5 mM calcium acetate.
- Divide the stock solution into aliquots and store at –20 °C.
- Each aliquot can be thawed and refrozen several times for a month and then discarded.

Single colonies isolated from three selective media (MacConkey. Violet Red Bile agar and Chromocult Coliform agar) were restreaked onto nutrient agar and identified using the RapID ONE and API 20 E systems.

APPENDIX C: *Enterobacteriaceae* Identified from the Berg River Water Samples Using the RapID ONE System

Sampling week	Chromocult coliform agar	MacConkey	Violet Red Bile agar
1	*Shigella sonnei* *Citrobacter freundii* *Klebsiella oxytoca* *Salmonella* species *Enterobacter aerogenes*	*Klebsiella oxytoca* *Salmonella choleraesius* *Citrobacter freundii* *Salmonella* species *Citrobacter freundii*	*Enterobacter cancerogenus* *Enterobacter cloacae* *Citrobacter freundii*
3	*Enterobacter sakazakii* *Hafnia alvei* *Stenotrophomonas maltophila* *Escherichia coli*	*E. aerogenes* *E. asburiae* *C. freundii* *K. oxytoca*	*Kluyvera ascobarta* *Salmonella* species *E. cloacae* *E. cancerogenus*
5	*C. freundii* *E. cloacae* *Escherichia coli*	*E. cloacae* *E. aerogenes* *Escherichia coli*	*K. ornithinolytica* *Rahnella aquatilis* *E. gergoviae*
7	*Ent. asburiae* *Ent. cloacae* *E. coli* *E. coli*	*Ent. asburiae* *Cit. freundii*	*Ent.cloacae* *Ent.sakazakii* *K.oxytoca*
9	*Ent. aerogenes* *Hafnia alvei* *Cit. freundii* *Klu. cryocrescens*	*Cedecea neteri* *K. ornithinolytica* *Klu. ascobarta* *Proteus vulgaris*	*K. oxytoca* *Ent. cloacae* *E. coli* *K. ornithinolytica*
11	*Salmonella choleraesius* *Citrobacter freundii* *Salmonella* species *Escherichia coli*	*Ent. sakazakii* *K. oxytoca* *Escherichia coli*	*K. oxytoca* *Ent. cloacae* *E. coli*
13	*E. coli (O157:H7)* *K. pneumoniae* *Entrobacter* species	*Ent. cloacae* *K. oxytoca* *Cit. freundii*	*Ent. cloacae* *Cit. freundii*
15	*Cit. freundii* *Ent. Aerogenes*	*Ent. asburiae* *Cit. freundii* *K. oxytoca*	*Ent. asburiae* *Cit. freundii*
17	*Cit. freundii* *Entrobacter* species *Hafnia alvei* *Escherichia coli*	*Leminorella richardii* *K. pneumoniae* *Entrobacter* species *Hafnia alvei*	*K. pneumoniae* *E. coli* *Cit. freundii*
19	*S. sonnei* *Pr. mirabilis* *K. pneumoniae* *K. oxytoca*	*Ent.cloacae* *K. pneumoniae* *Acinetobacter calcoaceticus* *E. coli*	*K. pneumoniae* *E. coli* *Cit. freundii* *K. ornithinolytica*
21	*K. ornithinolytica* *E. coli* *Ent. aerogenes* *Cit. freundii*	*E. coli* *K. ornithinolytica* *Ent. sakazakii*	*Ent. cloacae* *Ent. cloacae* *Enterobacter* species
26	*Salmonella spp* *Acin. calcoaceticus* *E. coli*	*Cit. freundii* *Ent. cloacae* *K. pneumoniae*	*Ent. cloacae* *K. pneumoniae* *Cit. freundii* *K. pneumoniae*
28	*Ent. cloacae* *Salmonella 3* *Enterobacter* species	*Ent. cloacae* *K. oxytoca* *E. coli*	*K. pneumoniae* *Ent. cloacae* *Enterobacter* species

APPENDIX D: *Enterobacteriaceae* Isolated from the Berg River Water Samples Using the API 20 E System

Sampling week	Chromocult coliform agar	MacConkey	Violet Red Bile agar
1	*Ent. cloacae* *E. coli* *K. pneumoniae* species	*Vibrio fluvialis* *V. fluvialis*	*Cit. freundii* *Proteus vulgaris group* *K. pneumoniae*
3	*Ent. cloacae* *Shewanella Putrefacienss group* *Aeromonas hydrophila 1*	*V. fluvialis* *K. pneumoniae* *K. oxytoca* *Aeromonas hydrophila 1* *Pantoea* species 3	*E. coli* *Ent. asburiae* *E. coli* *Aeromonas hydrophila 1* *Enterobacter sakazakii*
5	*Aeromonas hydrophila* *Cit. freundii* *Proteus vulgaris group* *K. pneumoniae* *K. pneumoniae*	*Ent.cloacae* *Raoultella terrigena* *K. oxytoca* *Ent. gergoviae*	*E. coli* *P. flourescens/putida* *Salmonella* species *Shewanella putrefaciens* *K. oxytoca* *Aeromonas hydrophila 1*
7	*Kluyvera* species *Ent. cloacae* *E. coli*	*Ent. gergoviae* *Ent. cloacae* *Aeromonas hydrophila 1* *Hafnia alvei* 1	*Ent. cloacae* *Aeromonas hydrophila 1* *Kluyvera* species *K. oxytoca*
9	*Hafnia alvei 1* *E. coli* *E. coli* *K. oxytoca*	*K. pneumoniae* *Roultella terrigena* *Kluyvera* species *K. oxytoca* *Raoultella ornithinolytica*	*Serratia odorifera 1* *Aeromonas hydrophila 1* *Serratia liquefaciens* *E. coli*
11	*E. coli* *Ent.cloacae*	*K. oxytoca* *Roultella terrigena* *Kluyvera* species	*Cit.freundii* *Salmonella* species *Shewanella putrefaciens*
13	*K. pneumoniae* species *E. coli* *Aeromonas hydrophila 1*	*K. oxytoca* *Ent. cloacae* *Cit. freundii*	*K. oxytoca* *Acinetobacter baumannii 1* *Kluyvera* species
15	*K. oxytoca* *Cit. freundii*	*Shewanella putrefaciens* *K. oxytoca* *Aeromonas hydrophila 1*	*Aeromonas hydrophila* *Ent. cloacae*
17	*Kluyvera* species *K. pneumoniae* species *Providencia alcalifaciens* *E. coli* *Ent. Cloacae*	*K.pneumoniae spp pneumo* *Kluyvera* species *Ent. cloacae* *Ent. cloacae* *Kluyvera* species	*E. coli* *E. coli* *K. pneumoniae*
19	*K. pneumoniae spp pneumo* *Enterobacter intermedius* *K. oxytoca* *E. coli* 1	*Acinetobacter baumannii* *K. pneumoniae* species *E. coli*	*K. pneumoniae* *K. oxytoca* *K. oxytoca* *Acinetobacter baumannii 1*
21	*Cit. freundii* *K. oxytoca* *K. pneumoniae* species *E. coli 1* *Aeromonas hydrophila 1*	*Serratia liquefaciens* *K. oxytoca* *K. pneumoniae* species *K. oxytoca*	*K. pneumoniae* *Ent. asburiae* *Ent. cloacae*
26	*K. pneumoniae* species *E. coli 1* *Aeromonas hydrophila 1*	*Cit. cloacae* *Cit. koseri* *E. coli*	*K. pneumoniae* *Ent. cloacae* *Ent. cloacae*
28	*K. pneumoniae* species *E. coli 1* *K. pneumoniae* species	*K. oxytoca* *Salmonella* species	*K. pneumoniae* species *Shewanella putrefaciens* *K. oxytoca* *Aeromonas hydrophila 1*

APPENDIX E: *Enterobacteriaceae* Isolated from the Plankenburg River Water Samples Using the API 20 E System

Sampling week	Chromocult coliform agar	MacConkey	Violet Red Bile agar
1	*Citrobacter freundii* *Acinetobacter baumannii* *Providencia stuartii*	*K. pneumoniae spp ozaenae* *E. coli* *Vibrio fluvialis*	Possibility of *Erwina* species *Ent. cloacae* *Serratia fonticola* *Hafnia alvei*
3	*Aeromonas hydrophila 1* *V. fluvialis* *E. coli* *Ent. Cloacae*	*K. oxytoca* *E. coli* *Kluyvera* species *Aeromonas hydrophila 1*	*Citrobacter koseri* *Ent. sakazakii* *Pseudomonas aeruginosa* *K. oxytoca*
5	*K. oxytoca* *V. fluvialis*	*Salmonella* species *K. oxytoca* Cit. freundii *Serratia liquefaciens*	*Aeromonas hydrophila 1* *K. oxytoca* *Cit. koseri*
7	*K. oxytoca* *E. coli* *Kluyvera* species *Aeromonas hydrophila*	*E. coli* *Ent. sakazakii* *K. oxytoca*	*Cit. freundii* *Salmonella* species *Shewanella putrefaciens*
9	*K. oxytoca* *Aeromonas hydrophila 1* *E. coli* *Raoultella terrigena*	*K. oxytoca* *P. fluorescens* *Ent. sakazakii* *Aeromonas hydrophila*	*K. pneumoniae* *Pantoea* species *Raoultella ornithinolytica* *Ent. cloacae* *Aeromonas hydrophila*
11	*Aeromonas hydrophila* *E. coli*	*Providencia Alcalifaciens* *Cit. youngae* *Aeromonas hydrophila*	*E. coli* *Ent. sakazakii* *K. oxytoca*
13	*Cit. freundii* *K. pneumoniae* species *Enterobacter* species *E. coli*	*Salmonella* species *Serratia fonticola* *Hafnia alvei* *E. coli*	*Salmonella* species *E. coli* *Serratia fonticola* *Hafnia alvei*
15	*Salmonella* species *Serratia fonticola* *Hafnia alvei*	*K. oxytoca* *E. coli*	*K. pneumoniae* *K. oxytoca* *E. coli*
17	*Cit. freundii* *Ent. cloacae* *Kluyvera* species *K. oxytoca*	*K. oxytoca* *Aeromonas hydrophila* *Ent. cloacae*	*E. coli* *Ent. sakazakii* *Citrobacter koseri*
19	*Ent. cloacae* *Enterobacter* species *E. coli*	*Kluyvera* species *Enterobacter* species *E. coli*	*Lecleria adecarboxylata* *K. oxytoca* *Citrobacter koseri*
21	*K. pneumoniae* *Cit. freundii* *Ent. Cloacae*	*K. pneumoniae* *Ent. cloacae*	*Leclercia adecarboxylata* *Pseudomonas luteola* *K. oxytoca*
26	*Cit. freundii* *E. coli* *Ent. cloacae* *K. oxytoca*	*K. oxytoca* *E. coli* *Ent. cloacae* *Aeromonas hydrophila*	*Cit. freundii* *K. pneumoniae* *Ent. cloacae* *K. oxytoca*
28	*K. oxytoca* *K. pneumoniae* *Enterobacter* species	*Serratia marcescens* *K. oxytoca* *E. coli*	*K. oxytoca* *K. pneumoniae*

APPENDIX F: *Enterobacteriaceae* Isolated from the Plankenburg River Water Samples Using the RapID ONE System

Sampling week	Chromocult coliform agar	MacConkey	Violet Red Bile agar
1	*S. sonnei* *Cit. freundii* *K. oxytoca* *Salmonella* species *Ent. Aerogenes*	*K. oxytoca* *Salmonella choleraesuis* *E. cloacae* *C. freundii* *Salmonella* species	*E. cancerogenus* *K. oxytoca*
3	*E. coli* *Acinetobacter calcoaveticus* *Citrobacter freundii*	*K. oxytoca* *E. coli* *E. coli*	*K. oxytoca* *Shigella* species *E. sakazakii* *A. calcoaceticus* *E. aerogenes*
5	*Ent. aerogenes* *Ent. cloacae* *Cit. koseri*	*Cit. freundii* *K. ornithinolytica* *E. coli*	*S. macescens* *E. coli* *Cit. freundii*
7	*Cit. freundii* *K. oxytoca* *E. coli*	*S. marcescens*	*K. oxytoca* *Cit. freundii*
9	*K. oxytoca* *Shigella* species	*Kluyvera cryocrescens* *Serratia odorifera*	*Cit. freundii* *K. oxytoca*
11	*Ent. sakazakii* *Acin. calcoaceticus* *Ent. Aerogene*	*K. oxytoca* *Ent. cloacae* *Cit. freundii*	*K. oxytoca* *Ent. cloacae* *E. coli*
13	*Ent. cloacae* *K. ornithinolytica* *Cit. freundii*	*Lem. richardii* *K. oxytoca* *Ent. cloacae*	*Cit. freundii* *K. pneumoniae*
15	*K. oxytoca* *Ent. cloacae* *Cit. freundii*	*K. ornithinolytica* *Cit. freundii* *Ent. cloacae*	*K. oxytoca* *Prov. alcalifaciens*
17	*Ent. cloacae* *E. coli* *Cit. freundii*	*Lem. richardii* *K. oxytoca* *Ent. cloacae* *Cit. freundii* *Cedecea* species	*Salmonella* species *Ent. aerogenes* *K. oxytoca*
19	*Salmonella* species *Ent. cloacae* *Cit. freundii*	*K. oxytoca* *Cit. koseri* *Enterobacter* species	*Cit. freundii* *K. oxytoca*
21	*Cit. amalonaticus* *Ent. Cloacae*	*Ent. cloacae* *Ent. sakazakii*	*Kluyver cryocrscens* *K. oxytoca* *Prov. alcalifaciens*
26	*Amalonaticus* species *E. coli* *E. aerogenes*	*K. ornithinolytica* *Ent. aerogenes*	*K. oxytoca* *K. ornithinolytica* *Cit. freundii* *Ent. cloacae*
28	*Ent. cloacae* *Ent. aerogenes* *Enterobacter* species *Cit. freundii*	*Cit. koseri* *K. pneumoniae* *K. oxytoca*	*Ent. sakazakii* *Ent. cloacae* *K. pneumoniae*