# Toxicology and molecular epidemiology of microbes detected in surface water in the Western Cape: The Impact of Informal Settlement

ERNEST JM MABOZA

**A thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae, in the Department of Medical Bioscience, University of the Western Cape**



Supervisor: Prof. CWJ Africa

Co-Supervisor: Prof EJ Pool

Date: December 2012

**DECLARATION**

I, Ernest Maboza, of South Africa declare that the thesis entitled "**Toxicology and molecular epidemiology of microbes detected in surface water in the Western Cape: The impact of an informal settlement**" is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

Ernest JM Maboza

_____

December 2012

**ACKNOWLEDGEMENTS**

Many thanks to both my supervisors Prof CWJ Africa and Prof EJ Pool for their unbound patience, understanding and readiness to share their knowledge and expertise.

I would also like to thank Prof R Henkel for relentless work and encouragement, Prof D Fisher and Prof D Dietrich for their leadership, the whole Medical Bioscience Department for support through trying time.

I am indebted to NRF and WRC for funding this project.

Last but not least, thank you to all my friends and family who understood when I took their valuable time and resources to see this through.

JJ, mon frère, ngokubekezela nokucubulungisisa kwakho lelibhuku; Muneera, for patiently waiting for daddy for long hours on end after school before going home; Norman Coldrey, for your incisive and invaluable knowledge that you shared so unselfishly; Claude, mon ami, for pour votre encouragement; I am indebted to you guys!

Natalie Isaacs, your work is executed with distinction!

**TABLE OF CONTENTS**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| API | Analytical Profiling Index |
| CCA | Chromocult coliform agar |
| CDC | Center for Disease Control and Prevention |
| CFU | Colony forming unit |
| DWAF | Department of Water Affairs and Forestry |
| *E. coli* | *Escherichia coli* |
| FIB | Faecal indicator bacteria |
| GUD | β-D-glucuronide |
| NA | Nutrient agar |
| NMMP | National Microbial Monitoring Programme of South Africa |
| OD | Optical density |
| Post-K | Post-Khayamnandi |
| Pre-K | Pre-Khayamnandi |
| RS | Reference site |
| TC | Total coliforms |
| UNESCO | United Nations Educational, Scientific and Cultural Organization |
| UNICEF | United Nations International Children's Emergency Fund |
| USEPA | United States Environmental Protection Agency |
| WHO | World Health Organization |

**LIST OF FIGURES**

**LIST OF TABLES**

**ABSTRACT**

Informal settlements are often implicated in surface water pollution with faecal matter. In most instances faecal pollution in the associated surface waters persists despite improvements in sewage removal infrastructure. This study evaluates the importance of investigating the water quality of the Plankenbrug River before it reaches Khayamnandi settlement by comparing water quality in spring and in winter upstream (Pre-Khayamnandi) and downstream (Post-Khayamnandi) from the settlement.

In this study, faecal indicator bacteria (*Escherichia coli* and total coliforms) were enumerated using Chromocult agar. *E. coli* was further characterized with analytical profiling index (API) and haemolysis assays. Both Pre- and Post-Khayamnandi were not significantly different from each other for both total coliforms and *E. coli* in winter. Pre-Khayamnandi had between $10^5$ and $10^8$ cfu/100 ml for total coliforms while Post-Khayamnandi had total coliform colony count between $10^6$ and $10^7$ cfu/100 ml. *E. coli* also exhibited a similar pattern with slightly higher counts at Post-Khayamnandi with colony counts from $10^4$ to $10^7$ and $10^5$ to $10^7$ cfu/100 ml. Spring microbial count demonstrated a significant difference to winter counts within each test site ($p \leq 0.01$) and across the two sites ($p \leq 0.05$). Both total coliforms and *E. coli* were $10^2$ fold higher at Post-Khayamnandi than at Pre-Khayamnandi in spring.

The API assay demonstrated significant difference ($p \leq 0.05$) between the two test sites. Pre-Khayamnandi predominantly had two different profiles while Post-Khayamnandi had three. These profiles represented five distinct *E. coli* biotypes. Sorbitol and sucrose tests within the API assay demonstrated significant differences ($p \leq 0.05$) between the two test sites. The prevalence of sorbitol fermenters at Pre-Khayamnandi was 100% while at Post-Khayamnandi it was 73%. Pre-Khayamnandi also demonstrated a significantly higher prevalence of sucrose

fermenters than Post-Khayamnandi at 100% and 59% respectively. These differences indicated dissimilar sources of faecal contamination around these sites. Differences in the distributions of sorbitol and sucrose fermenting biotypes demonstrate different toxicity potentials across these two test sites.

The haemolysis assay demonstrated that 9% of isolates were haemolytic with reference to both known α- and β-haemolyitic streptococci at Post-Khayamnandi. At Pre-Khayamnandi there was a higher percentage of α- and β-haemolyitic species, 29% and 28%, respectively. Post-Khayamnandi and Pre-Khayamnandi were significantly different from each other with reference to both α- and β-haemolysis ($p \leq 0.05$). These haemolytic activities also demonstrate different toxicity potentials across the two sites.

In conclusion Khayamnandi contributes to an already heavy faecal load in the Plankenbrug River. Thus remedial measures to maintain high surface water quality of Plankenbrug River should be directed upstream from the Khayamnandi settlement as well as within the settlement equally. This study recommends integration of microbial loads with programs such as the National Microbial Monitoring Program of South Africa to drive prioritization process in directing reclaiming of water quality, *inter alia*.

**KEY WORDS:**

Analytical profiling index

β-D-glucuronide

Chromocult agar

Faecal contamination

Faecal indicator bacteria

Haemolysis assay

Khayamnandi

Plankenbrug River

Sorbitol fermentation

Sucrose fermentation

Surface water

Toxicity

## Chapter 1: Background

Fresh water is one of the most precious natural resources (Department of Water Affairs and Forestry, 2002; United Nations Educational, Scientific and Cultural Organization (UNESCO), 2003). Water is getting progressively scarce with the changing weather patterns. Water sources often get contaminated with hazardous chemicals and pathogenic microbes. This necessitates vigilance on the quality of surface water for microbial contamination. The need for microbial monitoring of water is in line with the National Microbial Monitoring Programme (NMMP) of South Africa (Department of Water Affairs and Forestry, 2002). UNESCO (2003) reports water contamination as one of the main causes of health problems in human beings with 2.3 billion people suffering from water-related diseases worldwide (UNESCO, 2003). The annual mortality rate due to water related diseases is more than 2.2 million people in developing countries. Furthermore, Ullah *et al* (2009) reported that 60% of infant mortality due to infectious and parasitic diseases worldwide is water related. With increasing pressure on surface water, there is an ever-increasing health risk to the end users. The NMMP seeks to provide information on the status of faecal pollution and its trends in surface water. This NMMP information can help assess the potential health risks posed by water sources (Department of Water Affairs and Forestry, 2002).



A problem inherent in microbial monitoring is the non-conservative behaviour of microbes in water. This means that bacterial concentration does not remain static in water. It may differ considerably from the point source of pollution. Bacterial concentration may increase or decrease according to ambient conditions, including nutrient availability (Adingra *et al*, 2012; Liu *et al*, 2010). This behaviour renders the national grid of monitoring sites impractical. Water systems tend to modulate either die-off or regrowth of microbes. This demands continual monitoring

especially in high-risk areas. Also when considering that South Africa has 278 tertiary catchment areas together with increasing problematic land use, it is clear that Department of Water Affairs and Forestry (DWAF) is faced with a daunting task (DWAF, 2002). The above two problems necessitate prioritisation or use of cost effective methods. Most water monitoring authorities in general still rely on pragmatic and subjective judgment such as focusing only on areas of known sensitivity (Park *et al*, 2006).

In the absence of data indicating previous contamination, prioritisation may leave out smaller rural communities that cannot afford regular testing. Studies in such communities are often initiated by epidemics, for example *Vibrio cholerae* at Eshowe, KwaZulu-Natal (www.kznhealth.gov.za/cholerareport1.pdf, 2001). Thus, it is ideal to resort to cost-effective methods for regular monitoring of water bodies to prevent epidemics. One of the most costly factors in this process is the human resource. Qualified personnel are mostly centralized in the more affluent municipalities. Consequently, methods that are readily interpretable to less skilled personnel are desirable. Such methods should be sensitive and informative too.

## Chapter 2: Literature review

### 2.1 Water quality

Water quality is very important in its functionality as a support to life. Water quality is defined in terms of its chemical, physical and biological characteristics (Anderson and Davidson, 1997; Queensland Water Recycling Strategy, 2000; United States Environmental Protection Agency, 2000; World Health Organization, 2002). Potable water must meet specific water qualities. The WHO proposed guidelines regarding potable water quality. These guidelines allow flexibility depending on each country's means. South African guidelines for domestic water use are outlined in the South African Water Quality Guidelines, Volume 1 (DWAF, 1996a). One of the primary means for securing high quality potable water is source protection which is preferred to treatment of water after pollution (DWAF, 2002), and involves preventing contamination of catchment areas with chemical and biological pollutants.



Water quality is frequently compromised by rapid urbanization, particularly where informal settlements are involved (Adingra *et al*, 2012; UNESCO, 2003). In most cases, chemical pollutants render water unpalatable and also alter its appearance, for example, colour and turbidity (USEPA, 2000). Because of these visible water changes, high chemical levels are not commonly associated with acute adverse health effects in humans (WHO, 2002; Yoshida *et al*, 2004). Chronic low-doses of chemical pollutants in water are associated with adverse health outcomes (Kapaj *et al*, 2006; Kazi *et al*, 2009; Yoshida *et al*, 2004). Low dosage has to be endured for a long time before effects are observed (Yoshida *et al*, 2004). With biological agents, particularly bacteria related to human excreta, there are almost always acute adverse health effects, making the control of bacterial contamination of paramount importance (WHO, 2002;

WHO/UNICEF, 2000). The fact that bacteria can multiply in water makes it even more essential to control bacterial contamination (Bengraïne and Marhaba, 2003; Brion and Lingireddy, 2003; Ketchum, 1955).

### 2.2 Biological water quality

Increased concentrations of bacteria of human origin increase the risk for disease in exposed populations. Thus screening for these microbes is one of the most important aspects of water quality monitoring (Field and Samadpour, 2007; Rodrigues *et al*, 2010; Wang and Fiessel, 2007; Wilkes *et al*, 2009; WHO/UNICEF, 2000). The mode of water contamination with such microbiota is normally via introduction of untreated faecal matter into water. In faecal matter, pathogenic bacteria may be excreted together with non-pathogenic gastro-intestinal tract (GIT) normal microflora (Becker *et al*, 2009; Jeng *et al*, 2005). Pathogenic organisms shed with faeces include bacteria, viruses, helminthes and protozoa (Azizullah *et al*, 2011; Baudart *et al*, 2011; Jeng *et al*, 2005; Oberholzer and Ashton, 2008). Some of these organisms can survive longer in water than others. Persistence of each organism in water together with its resistance to chlorine becomes a crucial factor in determining its pathogenicity (WHO, 2002).

Water-borne disease associated with faecal-oral transmission may manifest as mild to fatal diarrhoea, dysentery, hepatitis, cholera and typhoid, amongst others (Ashbolt, 2004; WHO, 2002). Inhalation of infected water aerosols transmits other diseases including meningoencephalitis caused by *Ancanthamoeba* (Dorch *et al*, 1983; Tyagi *et al*, 2006; WHO, 2002). Meningoencephalitis from contaminated water with pathogenic *Ancanthamoeba* and *Naegleria fowleri* is rare but it has high mortality (Puzon *et al*, 2009). Dermal and mucosal exposures are other possible routes of transmission (Fong and Lipp, 2005).

The presence of human pathogens in the environment is of concern because of increasing numbers of people with hypoactive immune systems, for example, HIV infected individuals and people suffering from malnutrition (Mayer and Wanke, 1995; Steiner *et al*, 2000). Non-tuberculous mycobacteria (NTM) such as *Mycobacterium avium* are examples of innocuous microorganisms that could be found in drinking water (Whiley *et al*, 2012). *Mycobacterium avium* can cause pulmonary infections, gastrointestinal tract infections and cutaneous or soft tissue infections in immunocompromised patients (Karakousis *et al*, 2004). Karakousis *et al* (2004) reported an annual frequency of 10-20% disseminated *Mycobacterium avium* infection in adults infected with HIV-1 in the developed world e.g. Australia, Hong Kong, South Korea, Japan, Thailand, Singapore, North America and the United Kingdom (Whiley *et al*, 2012).

In the developing world, faecal matter contaminates the majority of rural water supplies (WHO, 2002) with its population, demonstrating an infectious disease burden estimated at 4 billion cases of diarrhoea with 2.2 million fatalities annually, most of which are children younger than 5 years of age (Moyo *et al*, 2004; WHO/UNICEF, 2002). In the light of the report cited above and UNESCO (2003), these figures indicate an increase in water related morbidity and mortality.



The water pollution problem from human excrement is not limited to fresh water. Marine waters are also severely affected. Clark *et al* (2003) pointed out the potential global problem of faecal contamination of oceans due to sewer overflows and runoffs from farms. This became apparent when correlation between beach use and incidences of skin and enteric diseases, *inter alia*, were established. They suggest that faecal matter heavily contaminates beaches in Visakhapatnam, India. In one sampling site along the Visakhapatnam coast, 4140 viable counts/ml in water and 303 800 viable counts/ml in the sediments were found. At the same site, faecal coliforms recovered were 140 and 910 viable counts/ml, while faecal streptococci were 1455 and 3060

viable counts/ml, respectively (Clark *et al*, 2003). The trend in pollution was linked to human activity around the beach (Craig *et al*, 2002; 2004; Lipp *et al*, 2001). Queensland Water Recycling Strategy (2000) also reported that swimmers occasionally contract viral, as well as bacterial infections that may be linked to faecal pollution of neighbouring streams. This report further indicated that effluents with human excreta are more likely to pose a threat than that from intensive farming because most viruses are host-specific.

While water quality in all countries faces challenges from wastewater discharges, the low income countries have an added challenge in that it is faced with absent/insufficient sanitation infrastructure (Azizullah *et al*, 2011). As a result cities in the developing world put great stress on fresh water. Management of fresh water resources is becoming one of humanity's most important challenges (Fitzhugh and Richter, 2004). This necessitates proper infrastructural planning that takes population dynamics into consideration (Owusu-Asante and Ndiritu, 2009). The main causes of concern are peri-urban areas, particularly the informal settlements, as in this study.



In the developing world, rapid urbanization often results in population explosion in peri-urban areas (Ehrenberg and Ault, 2005). These areas are often characterized by poor infrastructure (Nomquphu, 2005). The infrastructure for sewage removal is often overwhelmed because it is not designed to cope with the population size (Fatoki *et al*, 2001; Owusu-Asante and Ndiritu, 2009; Ullah *et al*, 2009). The resultant raw sewage spillage often ends up in nearby rivers and underground water (Zingoni *et al*, 2005). This generally puts stress on surface water (Abu Amr and Yassin, 2008; Babah *et al*, 2012). Ehrenberg and Ault (2005) mention a scenario in Jaboatão, Brazil, where a peri-urban settlement with no sanitary infrastructure exists beside a lake receiving sewage from the city. This community uses lake water as food sources while its

practice of open defaecation contributes to pollution. As a result, lymphatic filariasis is endemic in the area.

Pollution of surface water can be a source of contamination of groundwater, particularly where the water table is low and where there are fissures in surface rocks. A study done in Epworth, an informal settlement outside Harare, Zimbabwe, showed the effects of absence of human excrement in a dense settlement (Zingoni *et al*, 2005). Water samples in this study were collected from well and boreholes within the settlement. Contamination of groundwater was correlated with the presence of pit latrines in the vicinity. However, Jeng *et al* (2005) further pointed out that storm-water microbes may travel some distance before die-off and sedimentation following a storm-water event. This alerts to the possibility that the pollution source may not be in close proximity to the polluted water body. They further reported correlation between the amount of rainfall and faecal indicator bacterial (FIB) concentration. Thus rainfall could be a vehicle for distribution of the pollutant. Jeng *et al* (2005) showed that FIB concentration dropped after heavy rains. This reduction of FIB was correlated to sedimentation that in turn depended on particle sizes in the environment. Sedimentation thus provided a mechanism for indicator organisms to return to baseline levels, whilst prolonging their survival time in an estuarine environment (Donovan *et al*, 2008; Haller *et al*, 2009; Lee *et al*, 2006; Potè *et al*, 2008). Craig *et al* (2004) reported consistent higher concentrations of FIBs in epilithic biofilms and sediments than in the overlying water. Corals and reefs are no exceptions to faecal contamination. Lipp *et al* (2001) detected bacterial indicators on coral surface microlayer samples. These sediments are likely to present with adverse human effects upon re-suspension and release of pathogens therein. Re-suspension of river and pond surface waters could be induced by flooding during

heavy rains and other disturbance of the water body such as wading in the water (Borst and Selvakumar, 2003; Craig *et al*, 2004; Donovan *et al*, 2008).

Increased faecal pollution increases the risk of contracting water-borne diseases (Abu Amr and Yassin, 2008). There is a positive correlation between the risk of contracting water-borne diseases and population density. The adverse health risk may be increased by lack of sanitation infrastructure. While in developed regions there is an increasing risk for water borne enteric viruses, in the developing regions, major concerns are enteric bacteria together with protozoa and worms (Ashbolt, 2004). The common factor for both is that they are enteric, thus they may have common indicator organisms. Difficulties arise in that indicator organisms may not have the same persistence in water as the endemic pathogens, for example, viruses can persist longer than faecal indicator bacteria. In such cases, research may miss out on detection of an enduring pathogen. For pathogens that persist only for a short period outside the human host, indicator organisms are ideal in that they can indicate recent faecal contamination (WHO, 2002).



Due to hazards and expenses inherent in handling pathogenic organisms, certain innocuous organisms are used instead of pathogens as indicators of faecal contamination (Ashbolt, 2004; WHO, 2002). The organisms often used are coliform bacteria, which include *Escherichia coli, Citrobacter, Enterobacter* and *Klebsiella* (Gemmell and Schmidt, 2011; Ishii and Sadowsky, 2008; Leclerc *et al*, 2001; USEPA, 2000). These organisms are found in the gut of all warm-blooded animals (Sotomayor-Ramírez *et al*, 2006; Stumpf *et al*, 2012; USEPA, 2000). Their association with pathogenic organisms stems from the fact that they are eliminated with faeces (Centers for Disease Control and Prevention, 2011).

## 2.3 Faecal indicator organisms

Currently there are three 3 groups of organisms that are used as indicators (USEPA, 2000a). Each group is best suited for different applications. Detection of coliforms is commonly used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Faecal coliforms are the preferred indicator for shellfish and shellfish harvest waters, while *Escherichia coli (E. coli)* is generally used to show recent faecal contamination (American Public Health Association (APHA), 1992). *E. coli* as a faecal indicator bacterium suits the current study best because the interest is on events immediately preceding the sample collection. *E. coli* in this case will be taken to be representing coliforms.

## 2.4 Coliforms



Coliforms have been used for indicating faecal contamination for a long time. In clean, natural surface water, the count can be between 1 and 10 per 100 ml. The total coliform count in raw sewage as well as in polluted waters is more than several millions per 100 ml (APHA, 1992; DWAF, 1996b; USEPA, 1991). Albeit the improvement that the setting of limits for the presence of coliforms has brought to water sanitation and public health, there are still some drawbacks, one of which is that coliforms are not exclusively of human origin, they are also found in all warm-blooded mammals (Sotomayor-Ramírez *et al*, 2006; Stumpf *et al*, 2010; USEPA, 2000a). This compromises the sensitivity of these indicators. It also makes bacterial source tracking difficult. Several methods have been developed to make this task easier, including antibiotic resistance profiling, polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (Fong *et al*, 2005).

## 2.5 Faecal indicator bacteria

The distribution of faecal indicator bacteria (FIB) of human origin in surface water is also influenced by many factors including urban and agricultural runoffs, waste water discharges, sewage leaks and spills, die-off, predation, faecal deposits from wild life, sedimentation and re-suspension and regrowth on sediments (Adingra *et al*, 2012; Craig *et al*, 2004; Donovan *et al*, 2008). This complicates identification of recent anthropogenic faecal contamination. Anthropogenic contamination could be direct discharge of domestic waste, leaching from poorly maintained septic tanks, overflow from beleaguered infrastructure and improperly managed farm waste (Adingra *et al*, 2012). Wetlands also often support wildlife in abundance and as a result can also be the source of contamination. This makes it essential that indicators used should be properly identified if they are to be linked to humans as a source (Fong *et al*, 2005; Sanders *et al*, 2005).



Another important short-coming of FIB is their survival rate in water where the conditions are not as favourable as in the gut of mammals. They may survive for shorter periods than other pathogens (Tyagi *et al*, 2006). Thus, they may only be indicative of recent faecal contamination. Some pathogenic organisms range from moderate to long persistence in water, e.g. Adenoviruses can survive up to 300 days at 4˚C and 80 days at 25˚C (Hurst *et al*, 2002; Kimberley *et al*, 2005). Yet another point to consider is survival of polluting species after water purification. While FIB measures the quality of purification system, some organisms survive primary and secondary water treatment, e.g. Rotaviruses can survive up to 30 days at 15˚C (Fong *et al*, 2005; QWRS, 2000; WHO, 2002). This indicates that FIB may fail in predicting outbreaks associated with faecal contamination. All the same, they are useful in monitoring water quality. Changes in FIB

can thus be used to trace a source of recent contamination and are ideal for implementing remedial measures.

One of the major problems in determining faecal contamination of water is bacterial source tracking. The University of Georgia developed target sampling as a method to solve this problem. In its study, four steps were set. First, sampling was divided into two temporal conditions, namely, base flow and storm flow conditions. Rigorous sampling of the contaminated water constituted the second step. In order to reduce temporal variability numerous samples were collected per day. The third step was to combine bacterial numbers with global positioning system data. Finally, it conducted bacterial source tracking in areas that had high faecal bacteria (Hartel *et al*, 2005).

Studies have shown that anthropogenic disturbances in estuarine surface waters are associated with elevated faecal coliform bacteria counts (Kirby-Smith and White, 2006). A positive correlation between bacterial counts and water level is often noted. In the Kirby-Smith and White study (2006), it was supposed that this correlation was due to flooding of marshes hence re-suspending and transporting faecal matter to open waters. An *et al* (2002) also found bacteria density increased with human activity. However, they assumed that it was because of re-suspension of bacteria from depths due to boating activity. They further speculated that bacterial density fluctuations could be linked to viability of *E. coli* at elevated temperatures and protozoan grazing. Targeted sampling during base-flow conditions has been conducted on the Sapelo River in Georgia, USA, with good results (Kuntz *et al*, 2003). Another study done in Georgia, USA, at St Andrews Park, suggested that wind is another important factor involved in bacterial density fluctuation. Data collected during stormflow conditions indicated double the concentration during windy conditions as compared to calm conditions (Hartel *et al*, 2005).

The three studies cited above indicate that disturbance of sediments may act as a resupply mechanism of faecal indicators. Such disturbance may be due to anthropogenic activity such as wading in water and boating activity or natural factors such as wind and runoff conditions.

Chattahoochee river survey (USA) shows a pattern of faecal contamination. There are higher FIB concentrations in wet weather as compared to dry weather, higher in summer than in winter, even at base flow, and a higher concentration in the tributaries than in the river (Frick and Gregory, 2001; Rose, 2007). Wet weather is also often accompanied by heavy and prolonged cloud cover. This may significantly reduce insolation. This, together with water turbidity, may prolong survival of FIB as well as that of other associated pathogens. In that event, FIB enumeration may be higher than that representing recent contamination (Borst and Selvakumar, 2003; Mohamed *et al*, 2008).

Faecal indicators belong to the family *Enterobacteriaceae* (Donnenberg, 2005). All organisms in this family are facultative Gram-negative rods that ferment glucose to pyruvic acid. They also reduce nitrate to nitrite. Members of this family are antigenically complex. They are identified with K, H and O antigens (Schleifer and Stackebrandt, 1983).

After glucose is broken down to pyruvate, two major pathways, namely mixed acid pathway and the 2,3-butanediol pathways, further degrade it. In the former pathway, the end products of glucose may include formic acid, acetic acid, lactic acid and succinic acid. Methyl red test is the method used to detect if an organism uses this method. In the latter pathway, butanediol and ethanol are the main end products with small amounts of acid produced. The detection method in this case is called the Voges-Proskauer test. This test detects production of acetoin, an intermediate on the way to butandiol. TC and *E. coli* are good representatives of faecal indicator

bacteria as they often are innocuous normal flora of the gut shed with faeces (Kotlowsky *et al*, 2007; Powers and Latt, 1977).

### 2.6 Methods for assessing faecal contamination

Numerous methods are used in assessing faecal water contamination. Most of these techniques are culture-based methods using selective media (Baudart *et al*, 2011). However, false-positive counts due to the growth of non-enteric bacteria and false-negative results due to non-growth of injured targeted cells may occur (Owen *et al*, 2010). In addition, culture-based methods are time-consuming and do not allow for a rapid contamination detection. These techniques include heterotrophic plate count and multiple tube fermentation for the enumeration of faecal indicator bacteria including total coliforms (TC), *E. coli* and enterococci (Sanders *et al,* 2005; WHO, 2002).



Alternative methods such as using fluorescent in situ hybridization-based assays (FISH) have been suggested for a more rapid detection of specific cells. Baudart *et al* (2011) recommended the FISH-based technique to identify and enumerate specific bacteria in environmental water samples. In their recommendation, they proposed combination of FISH technique with direct viable count (DVC) assay by using solid phase cytometry. They argued that this technique may detect even low viable quantities of cells within a few hours. The sensitivity of this technique is well below the lower limit of FIB for surface water monitoring (WHO, 2000; USEPA, 2000b). The DVC-FISH can further be enhanced by using appropriate probes to further characterize faecal indicator bacteria which can assist in bacterial source tracking (Baudart *et al*, 2011). The disadvantages with DVC-FISH technique is the practicality in small municipalities without appropriately trained personnel and the costs involved (Baudart *et al*, 2011).

## 2.7 Motivation for use of TC and *E. coli* as FIBs

Total coliform bacteria counts are less precise as faecal contamination indicators because several can live and reproduce in soil and water, without having a human host. However, if high numbers of faecal coliform bacteria are found in a sample of stream water, one may conclude that there has been recent faecal contamination, although not necessarily of human origin (Hartel *et al*, 2005). Other intestinal bacteria such as streptococci or enterococci (i.e. *Streptococcus faecalis*) may have a comparatively stronger correlation to human sewage, yet no indicator has been identified that is exclusive to human faecal matter (Kenner *et al*, 1961). The ratio of streptococci to faecal coliform was once considered to distinguish human from animal faecal contamination. This is no longer thought to be reliable because streptococci do not persist long in an open water environment, thus making it difficult to assess true concentrations. On the other hand, enterococcal bacteria seem to be consistently associated with human sewage and subsequent diseases, but testing for these organisms involves a lengthy and complicated procedure (Hartel *et al*, 2005).



Despite the fact that faecal coliforms cannot be linked directly to contamination by human faecal matter, faecal coliform bacteria counts are often used to regulate surface waters for recreational use, fishing and drinking purposes. Federal regulations in the USA stipulate maximum allowable numbers of these bacteria for various uses (USEPA, 2000). If faecal coliform counts are high (over 200 colonies per 100 ml of water sample) in the river or stream, there is a greater chance that pathogenic organisms are also present. An individual swimming in such contaminated water has a greater chance of illness from swallowing disease-causing organisms, or from pathogens entering the body through cuts in skin, the nose, mouth, or the ears. Diseases and illnesses such

as typhoid fever, hepatitis, gastroenteritis, dysentery and ear infections can be contracted in waters with high faecal coliform counts (USEPA, 2000).

Municipal and rural water supplies can transmit human diseases such as cholera (*Vibrio cholerae*), typhoid fever *(Salmonella typhi*), shigellosis (*Shigella spp.*), salmonellosis (*Salmonella spp.*), gastroenteritis (*Campylobacter jejuni, Escherichia coli, Giardia lamblia*) (Tyagi *et al*, 2006). The threat of such disease transmission becomes more serious as the population density increases and more sewage pollutes public water supplies, carrying with it human intestinal pathogens (Adingra *et al*, 2012; Ashbolt, 2004).

Various culture media have been used in microbial water assays (Finney *et al*, 2003; Grabow and Du Preez, 1979). In this study, chromogenic media that exploit certain characteristics of TC and *E. coli* together or separately, namely, Microbiology Chromocult agar (Merck. Cat. No. 10426.0500) and Dev Lactose Peptone 4-methylumbelliferyl-β-D-glucuronide (MUG) broth (Fluka. Cat. No. 31401) were chosen because one could identify both classes of faecal indicators on the same plate thus minimizing cost involved in identification. Both media reduced on human hours in preparation and in reading results. Furthermore they make the requirement of biochemical sugars redundant in confirming species of interest, e.g. with Lactose Peptone MUG broth, one only needs Kovac's reagent to confirm the presence of *E. coli* instead of the whole battery of biochemical sugars (Alonso *et al*, 1996; Manafi, 2000; Perez *et al*, 2001; Yanez *et al*, 2006).

## 2.8 Conditions influencing the indicator distribution

Environmental variables that need to be taken into consideration include pH, temperature, base flow, storm flow, presence of phosphate and nitrogen species, prevailing winds that could be linked to prevailing endemic bacterial species in a specific area (Burkhardt *et al*, 2007; Daskin *et al*, 2008; DWAF, 2006). The reduction of solar irradiation due to cloud cover or decrease length of daylight reduces inactivation of the transient bacteria (Burkhardt *et al*, 2007). The amount of solar irradiation lowers microbial loads in exposed surface water bodies as compared to those sheltered by steep south-east facing slopes in the Southern Hemisphere (Boyer, 2008).

## 2.9 Ecological impact of polluted surface water

Toxicology in this text is defined in terms of substances that can cause adverse effects on living organisms. This stance is assumed due to lack of consensus in accepted and comprehensive classification schemes for modes of toxicity and response endpoints for either toxicology or ecology (McCarty, 2002). Broadly speaking, the adverse effects range from those that cause immediate death to those that can cause subtle changes that are not perceived for months or years. Subtle changes include suppression of growth and reproduction in certain species (Hurter *et al*, 2002). Faecal contaminated water may be considered as eco-toxic on the whole as it can reduce or wipe-off fish populations downstream (Daskin *et al*, 2008; Nagvenkar and Ramatah, 2009). Raw faecal contaminated water may be considered as toxic as it induces micronuclei formation in pollen of *Tradescantia* (Monarca *et al*, 2000).

## 2.10 Microbial toxicity assays

Biotoxicity assays are considered relatively simple, rapid and cost effective compared to the more time-consuming microbial culturing (Farré *et al*, 2005). Microbial toxicity assays can also be used to evaluate potential toxicity of water (Guven et *al*, 2003). The luminescent bacterium *Vibrio fischeri* is normally used to test for toxicity reduction in water and evaluating sewage treatment effectiveness (Farré *et al*, 2005; Jung-Kon *et al*, 2007). Farré *et al* (2005) argued that use of bioluminescent bacteria is limited for fresh water because the assay has to be performed in 2% saline solution.

In the absence of toxic chemicals the microbiota could be pathogenic on their own, e.g. *E. coli*. Due to frequent outbreaks of food and water-borne epidemics, it is important to assay for the presence of *E. coli* pathotypes in the water test after determining the presence of faecal contamination thus assaying for toxicity potential of the water. This is especially important in recreational waters where the EPA's upper limits are 500 and 800 cfu (colony forming units)/100 ml for primary contact sport (PCS) and secondary contact sport (SCS), respectively. Primary and secondary contact recreational waters are defined according to whether a person is likely to be completely immersed or not. EPA further requires that faecal coliforms should not exceed 200 and 400 cfu/100 ml limit in 10% of samples over 30 days for PCS and SCS, respectively (Anderson and Davidson, 1997). With such high limits there is a chance of coming into contact with endemic pathogenic strains of *E. coli*. Surface waters should therefore be assessed because they feed into recreational waters. Besides, in some areas surface water is used for agricultural purposes downstream.

Zhu *et al* (2005) suggested the use of integrating waveguide biosensor in assaying for water-borne pathogens due to its ability to determine bacterial serotype, genotype, and viability. Cost and sophistication of the assay is the most important limiting factor, but there are numerous other cost-effective assays for determining the presence of *E.* coli including culture, PCR and serotyping. Moses *et al* (2006) cautioned that the presence of pathogenic *E. coli* strains should not be overlooked, particularly in the light of management of immuno-compromised people. Virulence factors in *E. coli* include adhesion-colonizing factors, toxins, secretions and plasmids (Clarke *et al*, 2003; Donnenberg, 2005; Guerrant and Steiner, 2005; Prats *et al*, 2003).

### 2.11 Characterization of *E. coli* with API

The profiling index (API) 20E system is a test kit for identification and characterization of enteric bacteria together with some non-fastidious Gram negative bacteria (Aldridge and Hodges, 1981; API Manual, BioMérieux, France; Holmes *et al*, 1978; MacDonell *et al*, 1982; Nucera *et al*, 2006; Peele *et al*, 1997). The API 20E system demonstrates (BioMérieux, Cat. No. B-20160) that different genera metabolize substrates differently. It further shows that variants occur even within a species. The API 20E is based on 21 conventional biochemical tests for identification of *Enterobacteriaceae* (Aldridge *et al*, 1978; Mugg and Hill, 1981; O'Hara *et al*, 1992) Microvolumes of bacterial suspension are grown in the desiccated medium of these biochemical tests and produce characteristics that can be used to discriminate bacterium from others after incubation.

It is helpful that organisms of the same group often utilize limited combinations of metabolic and enzymatic activities for identification of an unknown isolate. The numbers of tests that are performed on each test strip enable one to determine that even within species, there are variations

in these activities according to phenotypic expressions of genes. The index generated from the test has wide enough limits to allow identification of biotypes within a species (API 20E Instruction Manual version #2012). When this diversity is pronounced across two sites and particularly when samples show spatial diversity, it can be used to discriminate between two sources. The API system enables this diversity and can thus be used as a potential bacterial source-tracking tool (Soule *et al*, 2006; Wheeler *et al*, 2002) afforded by the distribution of positive and negative results for these tests in different animal species. Sorbitol and sucrose fermentation are examples of such tests (Soule *et al*, 2006; Wheeler *et a*l, 2002).

Sorbitol fermentation is traditionally used to discriminate asaccharolytic serotypes of *E. coli* O157:H7 (Bouvet *et al*, 1999; Griffin and Tauxe, 1991). It has been reported that these traditionally non-sorbitol fermenting serotypes can mutate to sorbitol positive variants (Fratamico *et al*, 1993). A *stx* strain of *E. coli* O157 (Shiga toxin producing *E. coli*) that ferments sorbitol and exhibits β-glucuronidase activity was identified for the first time in 1988 during an outbreak of haemolytic uremic syndrome (HUS) in Southern Germany (Ammon *et al*, 1999; Bielaszewska *et al*, 1998; Mellmann *et al*, 2008). The pathogen source implicated for these outbreaks was traced to sausage containing raw beef (Ammon *et al*, 1999).



After the initial outbreak of this strain, sorbitol fermenting *E. coli*-O157, was sporadically isolated from patients in paediatric hospitals throughout Germany (Karch *et al*, 1993). Karch and Bielaszewska (2001) reported that the relative frequency of the sorbitol fermenting O157 isolates among the *E. coli* O157 strains isolated in their studies ranged from 13.3% to 40.5% in HUS patients and from 7.4% to 25% in patients suffering from diarrhoea. Pollock *et al* (2010) suggest that while sorbitol fermenting-*E. coli* O157 is more associated with severe HUS than non-sorbitol fermenting strains, the latter results in more severe colitis.

Epidemiological studies have thus far failed to isolate sorbitol fermenting Shiga toxin producing *E. coli* (STEC) O157 from the cattle implicated in the outbreaks throughout Germany (Karch *et al*, 1993). It was Bielaszewka *et al* (1998) in the Czech Republic who first managed to elucidate cattle as the reservoir for the sorbitol fermenting STEC O157:H2 clone in central Europe. Although farm animals were implicated, previous studies failed to isolate sorbitol fermenting STEC O157 from cattle due to less sensitive methods than the immunomagnetic separation procedure used in their study (Bielaszewka *et al*, 1998). Lee and Choi (2006) however doubt that the human isolate originated from cattle. In their study, they demonstrated different virulence patterns from sorbitol fermenting STEC O157 isolates from cattle and from humans in Korea. Their study found that human and cattle strains are genetically closely related.

Interconversion between various *E. coli* pathotypes involving *stx2* gene has been demonstrated (Mellmann *et al*, 2008). These bidirectional interchanges result in ephemeral changes in the pathotypes during an infection, for an example, sorbitol fermenting *E. coli* O157:NM (non motile) strains could be converted to biologically potent enterohaemorrhagic *E. coli*. (Friedrich *et al*, 2007). These changes are mediated by a *stx2* encoding phage.



It has been observed that sorbitol fermenting enterohaemorrhagic *E. coli* (EHEC) O157:H- strains and non-sorbitol fermenting EHEC O157:H have distinct plasmids (Friedrich *et al*, 2004). This suggests that the ability to ferment sorbitol is related to the plasmid (Brunder *et al*, 2001). Sorbitol fermenting *E. coli* biotypes have been isolated in water (Prager *et al*, 2011). Ateba and Bezuidenhout (2008) have alluded to the presence of sorbitol fermenting *E. coli* O157 in livestock in South Africa.

Sucrose is also used to discriminate different biotypes of *E. coli*. This sugar is amongst the most highly water soluble components in plant tissue (Moniruzzaman *et al*, 1997). Hence it may be readily available for microbial uptake. However, some *E. coli* biotypes are known for their inability to ferment sucrose (Vaz *et al*, 2004). In some *E. coli* biotypes this ability is masked by competing genes and it can be realized by knocking off these genes. This was demonstrated by Zhu *et al* (2005) in designing SZ132 an ethanologenic derivative of *E. coli* K011. This derivative fermented sucrose completely at a rate twice that previously reported for SZ63 harbouring the sucrose plasmid (Shukla *et al*, 2004).

Alternatively, plasmids confer sucrose fermentation to *E. coli* via the expression of the *scr* regulon (Bogs and Geider, 2000; Wohlhieter *et al*, 1975). Sucrose fermentation is common in herbivores and avian populations (Carlos *et al*, 2010; Zinnah *et al*, 2007). In this study, the distribution of sucrose and non-sucrose fermenters is examined for use as a predictive tool for indicating source of river toxicity. This may also be used as a bacterial source tracking mechanism (Soule *et al*, 2006; Wheeler *et al*, 2002). Several studies have shown that the distribution of phylogenetic subgroups of bacteria is not random (Carlos *et al*, 2010; Baldy-Chudzik and Stosik, 2007; Orsi *et al*, 2008; 2007). Different groups of animals harbour diverse subgroups of the same organism. With respect to *E. coli*, omnivores (including humans) demonstrate more diverse populations compared with herbivores (Carlos *et al*, 2010).



**2.12 Haemolysis assay as a toxicity assay**

Water toxicity can also be demonstrated by showing the haemolytic ability of individual organisms. If the haemolytic patterns show spatial variation, this could be potentially indicative of different pollution sources. Some strains of *E. coli* elaborate an extracellular α-haemolysin

that is coded for by a plasmid (De La Cruz *et al*, 1980). These organisms are often pathogenic to both humans and animals. Ralph *et al* (1998) pointed out that these plasmids enhance *hlyE-lacZ* expression and confer a haemolytic phenotype onto an otherwise non-haemolytic one. They pointed out that when the *fnr* homologue of a pig pathogen is expressed on a non-haemolytic *E. coli*, it activates a dormant gene (*hlyE*) encoding for a haemolysin (Ralph *et al*, 1998). This raises concern that environmental *E. coli* under aerobic conditions may have AR1 or AR3 regions altered or acquire regulators of *hlyX* type from other environmental microbes. If this happens, pathogenicity could be enhanced. This makes it essential to monitor the haemolytic activity of environmental *E. coli*.

Haemolysis can be demonstrated on blood agar plates. However, when working with large numbers of samples it is advantageous to work on a microtitre plate. Assays have been developed to this end. Neely and Campbell (2005) used microplate assays to demonstrate haemolytic activity of crude algal extracts on *Sciaenops ocellatus* (fish) erythrocyes. Previously, Eschbach *et al* (2001) improved sensitivity of erythrocyte lysis assays from reading cuvettes at 540 nm with 3 ml sample to reading at 414 nm in microtitre plates. This drastically reduced the requirement for large volumes of blood.



*E. coli* haemolysin activity is rapid. Studies have shown that *in vitro* erythrolysis occurs within the first 5 hours after exposure. Eberspacher *et al* (1998) suggested that any lysis occurring after the first hour could be due to colloid-osmotic lysis.

### 2.13 Spatial distribution of pathogenic *E. coli* in surface water

Enterohaemorrhagic *E. coli* (EHEC) are a worldwide cause of outbreaks and sporadic cases of a variety of clinical syndromes such as bloody and non-bloody diarrhoea, haemorrhagic colitis and

haemorrhagic uraemic syndrome. Prevalence of *E. coli* O157 is a cause of concern with increased incidence of HIV/AIDS. Moses *et al* (2006) reported 3.8% recovery of *E. coli* O157 in 53 HIV sero-positive patients that had diarrhoea, while there was none among the 59 sero-negative patients in Nigeria.

EHEC has been linked with rivers, heavy rains and livestock in South Africa. The probability of getting infected by treated drinking water is negligible (Müller *et al*, 2003; Raji *et al*, 2006). Cases of haemolytic colitis caused by *E. coli* O157 have been reported in KwaZulu-Natal, Mpumalanga and Eastern Cape provinces of South Africa (Müller *et al*, 2001).

Out of 91 sewage and 40 river-water samples tested by Raji *et al* (2006) only 1.1% sewage samples tested positive for genes coding for *eae*, *Stx2* and e*hly*. None of the river samples turned positive for *E. coli* O157:H7 in this study. Müller *et al* (2001) obtained similar results on testing 204 samples from 15 sites on the Vaal River, South Africa. In their study they found strains elaborating *Stx1, Stx2* and enterohaemolysin genes. Both studies indicate low probability of infection with *E. coli* O157:H7 from river samples. These studies suggest that other EHEC strains could pose a health risk from these sources.

Hara-Kudo *et al* (2000) reported a possible loss of expression of verotoxin by *E. coli* O157:H7 isolated from water. They supposed this happens when the organism has been under starvation conditions for a prolonged period. These organisms can still produce verotoxin under suitable conditions. Similar results were reported on a dairy farm in Ontario, Canada (Jackson *et al*, 1998). This loss of verotoxin expression necessitates looking at these organisms at genetic level. McCleery and Rowe (2002) demonstrated genetic instability in *E. coli* O157:H7 serotypes. They showed that some strains apparently loose the *VT2* gene while some do not. Genotypic character

of these organisms is conserved despite the phenotypic changes. Galane and Le Roux (2001) demonstrated the useful nature of the PCR technique in epidemiological studies of *E. coli* in cases of diarrhoea.

Spatial information of distribution of particular strains could be useful in timely disease outbreak management. Geographic Information System (GIS) could be helpful at different stages of disease management. In the detection stage, aggregation of complaints may be located. Depending on availability and quality of information, point source of the outbreak may be located. This could help curtail further spread of the disease. During an outbreak assessment stage, positioning of short-term health care precautionary measures can be efficiently distributed. By providing detailed land use and aspects in and around catchment areas, GIS can support identification of cause together with insight into how the causative organism reached water. After an epidemic has been contained, GIS can be also used as one of the tools for long-term measures and for microbial risk assessment (MRA) (Kisteman *et al*, 2001). A GIS-based computer model was used in Nigeria to assess the risk of diarrhoeal disease outbreak in children using different water sources (Njemanze *et al*, 1999). A microbial risk assessment geographic information system (MRA-GIS) database can be built on spatial variation of bacterial contamination of surface water.



Management of health related water resource quality necessitates combination of several water screening methods. A holistic approach including culture, biochemical and toxicological methods combined with GIS could help trace and arrest potential epidemics. This approach may prevent persistent faecal pollution of surface water, thus eventually saving cost of water resource management and improving general water quality.

## 2.14 Background and motivation for the study

Khayamnandi was founded in the 1950s. It is predominantly an informal settlement with hostels built in 1966 for male migrant labourers (Darkwa, 2006). Erhard (2000) reported that public latrines built for the hostels had to cater for the township residents at large at a ratio of 1:136 (latrine: individual) in 1994. He deems the situation had improved by 2001 to 1 latrine per 75 residents. In 2001 the population of the township was estimated to be more than 22 000 people living in an area just over 1 $km^2$ with 208 public toilets (Erhard, 2000). Republic of South Africa population census (2012) has not been released to date, thus there is no record of the latest population of the township. The households in shacks were served with water by a single tap by the roadside. Erhard (2000) estimated that this tap served at least 600 households. The hostels which were better serviced in this regard had no baths or shower facilities. Refuse for the settlement is collected once a week (Erhard, 2000).



Plankenbrug River used to be an ephemeral river that runs past Khayamnandi, but is now perennial, most likely due to human activity. The river flows from the Bottelary mountains and is surrounded by farms. After crossing the road, R304, west of the Stellenbosch town, it skirts the informal settlement, Khayamnandi. The effluent from this settlement contributes to the water volume in the river. The contribution of the settlement is diffused, thus may not be singled out at a particular point. There might also be an industrial discharge contribution to the river, which could indirectly influence faecal pollution by facilitating sedimentation and affecting opacity of water (Adingra *et al*, 2012; Donovan *et al*, 2008).

Plankenbrug River skirting an informal settlement of Khayamnandi (Stellenbosch, Western Cape, South Africa) is one of the well-studied cases of the interplay between dense informal

settlements and faecal pollution of surface water (DWAF, 1993; Hendricks, 2003; RHP, 2005). Currently, it is believed that Khayamnandi severely compromises Plankenbrug River water quality (River Health Programme (RHP), 2005).

The current study aims to assess the impact of Khayamnandi on the water quality of the Plankenbrug River. The importance of the Plankenbrug River lies in the fact that it is one of the tributaries constituting the Eerste-Kuils River drainage basin (Department of Water Affairs and Forestry (DWAF) / National Microbial Water Quality Monitoring Programme (NMMP), 2004; Nleya, 2005). The Eerste-Kuils River system drains Jonkershoek Mountains east of Stellenbosch, the Bottelary Mountains west of the Stellenbosch and the eastern part of the Cape Flats. It then proceeds to run into False Bay at Macassar beach (http://www.saexplorer.co.za/south-africa/map/stellenbosch_map.asp). The Eerste-Kuils River drainage basin supplies fresh water to Stellenbosch and farms for irrigation (DWAF/NMMP, 2004). Therefore, its integrity is of high importance. Moreover, it impacts on the quality of marine waters too as it eventually drains into the Atlantic Ocean. The Atlantic Ocean at Macassar beach water occasionally has above 1000 colony forming units (cfu) faecal coliforms/100 ml (Ngwenya, 2006).



Plankenbrug River is one of the tributaries contributing to the Eerste-Kuils river catchment system. This is the only river that passes Khayamnandi before discharging into this catchment area (Jackson *et al*, 2009). Khayamnandi is a settlement consisting of both formal and inadequately serviced housing units. Informal settlements are notorious for affecting the integrity of surface waters (Adingra *et al*, 2012; Fatoki *et a*l, 2001; Gemmell and Schmidt, 2011). Studies have shown that water from Plankenbrug River consistently have high levels of faecal organisms (DWAF, 2001; DWAF/NMMP, 2004; Nleya, 2005). The Eerste River, upstream before joined by Plankenbrug River, is used to supplement the drinking water of Stellenbosch (RHP, 2005) and

thus needs protection as much as possible. In order to protect this water, the understanding of conditions in its tributaries is essential.

In recent years there have been infrastructural developments in the Khayamnandi settlement, but these have not translated to river water quality improvement (Darkwa, 2006; Department of Housing, 2004a; 2004b). The river itself has been transformed. It now flows throughout the year whereas earlier it used to flow only in winter (DWAF, 2001). These two latter points seem to indicate that there could be forces outside Khayamnandi that could be adding to the problem at hand. Only when such extra forces have been elucidated and dealt with together with the Khayamnandi problem, can water downstream be improved, that is, only if they exist. Thus, there is a need to find out whether such sources do exist or not. Informal settlements are generally underserviced with respect to sanitary infrastructure. This could be due to unpredictable demographics and it leads to excessive demand on the meagre existing amenities (Babah *et al*, 2012). Erection of structures generally tends to flood the existing drainage systems rather than allowing natural percolation of rain water into the soil, thus lending to stressing drainage systems (Owusu-Asante and Ndiritu, 2009). Intermittent water supply is also closely implicated in water contamination due to flooding of sewage drainage facilities (Abu Amr and Yassin, 2008).



Ngwenya (2006) pointed out that downstream from Khayamnandi, irrigation water samples have more than 1000 cfu of faecal coliforms/100 ml. A similar situation was reported by Gemmell and Schmidt (2006) downstream from Baynespruit River in Natal (South Africa) where analysis for faecal indicator bacteria was done on fresh produce irrigated with its water. Baynespruit River traverses a peri-urban settlement just as the Plankenbrug River passes an informal settlement. In the Limpopo province (South Africa), the Nyls River in the Waterberg catchment area is not

associated with any informal settlement albeit heavily contaminated. The major part of faecal contamination in this case is attributed to neighbouring intensive chicken and cattle farming (Greenfield *et al*, 2010).

However in the case of Plankenbrug River the question remains as to whether Khayamnandi is the only source or whether it merely exacerbates an already existing problem. Answering this question may go a long way in remedying the situation.

It is unquestionable that the presence of unplanned or improperly planned settlements on riverbanks poses serious negative impacts on both surface and underground water quality. The lack of sanitation and infrastructure tend to lead to faecal contamination and other pollutants of nearby rivers. Population density compounds the circumstances (Ehrenberg and Ault, 2005; Zingoni *et al*, 2005). Informal settlements are examples of unplanned settlements that result in sanitation infrastructure which often gets overwhelmed. As such these settlements are commonly blamed for nearby surface water contamination (Adingra *et al*, 2012; DWAF, 1993; Gemmell and Schmidt, 2011). However, it is also important to look beyond these apparent sources of pollution, especially after several attempts to correct the situation constantly seem to fail. It could happen that the apparent source is but a contributory although significant factor in the situation. Upstream evaluation of water quality can shed light on ambient conditions before the settlement, thus enabling alternative measures to curb the pollution. In this study, the impact of informal settlement (Khayamnandi) on the water quality of the Plankenbrug River is assessed.



**2.15 Hypothesis**

$H_0$: Khayamnandi does not impact on Plankenbrug River water quality

$H_1$: Khayamnandi impacts Plankenbrug River water quality

## 2.16 Aims and objectives

The current study entertains the idea that Khayamnandi may only be contributing to an already existing problem. Settlements on riverbanks affect surface water via faecal matter deposition amongst other things (Adingra *et al*, 2012; Fatoki *et al*, 2001; Gemmell and Schmidt, 2011). For this reason faecal bacteria are used as impact indicator in this study. First, this study aims at assessing the extent of faecal pollution that may be accrued to Khayamnandi by comparing water quality before reaching and after leaving the settlement. Secondly, the effect of Khayamnandi on potential toxicity of the river will also be assessed. Thirdly, it attempts to determine the distribution of biotypes which could help determine the common or diffused source of contamination of the river system. This could aid in directing remedial measures. Lastly, this study will propose recommendations to policy makers on possible methodologies that can be applied to monitor microbial contamination by municipalities.

## Chapter 3: The impact of Khayamnandi on Plankenbrug River water quality

### 3.1 Abstract

Protection of water quality from human faecal contamination is currently one of the most pertinent issues worldwide. Informal settlements are often implicated in surface water pollution with faecal matter. In most instances faecal pollution in the associated surface waters persists despite improvements in sewage removal infrastructure. This study evaluates the importance of investigating the water quality of the Plankenbrug River before it reaches Khayamnandi settlement by comparing water quality in spring and in winter upstream (Pre-Khayamnandi) and downstream (Post-Khayamnandi) from the settlement.

Chromocult coliform agar (CCA), analytical profiling index (API) and haemolysis assays were used in this study to count and characterize faecal indicator bacteria. Both Pre- and Post-Khayamnandi were not significantly different from each other for both total coliforms and *E. coli* in winter. Pre-Khayamnandi had between $10^5$ and $10^8$ cfu/100 ml for total coliforms while Post-Khayamnandi had total coliform colony count between $10^6$ and $10^7$ cfu/100 ml. *E. coli* also exhibited a similar pattern with slightly higher counts at Post-Khayamnandi with colony counts from $10^4$ to$10^7$ and $10^5$ to $10^7$ cfu/100 ml. Spring microbial count demonstrated significant difference ($p \leq 0.01$) and across the two sites ($p \leq 0.05$). Both total coliforms and *E. coli* were $10^2$ fold higher at Post-Khayamnandi than at Pre-Khayamnandi in spring.

The API assay demonstrated significant difference ($p \leq 0.05$) between the two test sites. Pre-Khayamnandi predominantly had two different profiles while Post-Khayamnandi had three. These profiles represented five distinct *E. coli* biotypes. Sorbitol and sucrose tests within the

API assay demonstrated significant differences ($p \leq 0.05$) between the two test sites. The prevalence of sorbitol fermenters at Pre-Khayamnandi was 100% while at Post-Khayamnandi it was 73%. Pre-Khayamnandi also demonstrated a significantly higher prevalence of sucrose fermenters than Post-Khayamnandi at 100% and 59% respectively. These differences indicated dissimilar sources of faecal contamination around these sites. Differences in the distributions of sorbitol and sucrose fermenting biotypes demonstrate different toxicity potentials across these two test sites.

The haemolysis assay demonstrated that 9% of isolates were haemolytic with reference to both known α- and β-haemolytic streptococci at Post-Khayamnandi. At Pre-Khayamnandi there was a higher percentage of α- and β-haemolytic species, 29% and 28%, respectively. Post-Khayamnandi and Pre-Khayamnandi were significantly different from each other with reference to both α- and β-haemolysis ($p \leq 0.05$). These haemolytic activities also demonstrate different toxicity potentials across the two sites.



In conclusion, Khayamnandi contributes to an already heavy faecal load in the Plankenbrug River. Thus remedial measures to maintain high surface water quality of Plankenbrug River should be directed upstream from the Khayamnandi settlement as well as within the settlement equally.

### 3.2 Introduction

Water is implicated in morbidity of a quarter of the world population (UNESCO, 2003). The developing world carries most of the water-related morbidity burden. The water-related morbidity manifests mostly as bacterial and parasitic infections. The people most affected are infants, the elderly and the immunocompromised, with highest mortality amongst infants (Ullah

*et al*, 2009; UNESCO, 2003). The most common mode of infection is the faecal-oral route. Skin and mucosal membranes are the other routes of infection (Ashbolt, 2004; Dorch *et al*, 1983; Tyagi *et al*, 2006; WHO, 2002).

Due to the presence of human pathogens in water, monitoring for the presence of faecal indicators in water is essential (Department of Water Affairs and Forestry, 2002). Human pathogens infecting the gut are often shed with high quantities of faecal indicators (WHO, 2002). The most useful faecal indicators in water are *E. coli* and total coliforms (USEPA, 2000). Total coliform (TC) includes *E. coli* and other gut flora. In South Africa, there is a National Microbial Monitoring Programme (NMMP) that uses *E. coli* and TCs to monitor the extent of faecal contamination of surface water (DWAF, 2002). The NMMP extrapolates the inherent potential risk in the assessed water by drawing from the extent of microbial contamination.

Under-resourced communities, particularly those with insufficient/poor sewage removal infrastructure, on the banks of surface waters are commonly associated with heavy faecal contamination of these water bodies (Adingra *et al*, 2012; Fatoki *et al*, 2001; Owusu-Asante and Ndiritu, 2009; Ullah *et al*, 2009). The informal settlements are examples of under-resourced communities. In most cases, prevention of faecal contamination is focused on correcting or improving the infrastructure and sewage removal resources in the implicated settlement (Erhard, 2000; Nomquphu, 2005). Localised measures tend to be ineffective in cases where faecal contamination begins before the water reaches the settlement. This failure necessitates the assessment of the discrepancy in water quality before and after the settlement. The resultant information could help direct efforts to preserve the integrity of the concerned surface water. The persistence of microbes in water is affected by several aspects including the physicochemical factors. The persistence and non-conservative nature of microbes in water may give a false

impression of the incident microbial load which calls for frequent monitoring during base flow and storm flow (Adingra *et al*, 2012; Liu *et al*, 2010; Kistermann *et al*, 2002). Microbial incidence into the water body due to the informal settlement may be assessed by evaluating the difference between the water before and after the informal settlement. Increased microbial incidence difference between these two points associated with wash-off due to heavy rainfalls may strongly implicate the informal settlement (USEPA, 2000). In South Africa, DWAF provides standard methods for quantifying microbial load. These methods are based on EPA and WHO standard methods *inter alia* (DWAF, 2002).

The monitoring of faecal microbes introduced in water may further enhance awareness of the potential environmental toxicity and health risks posed to water users, as articulated by NMMP (DWAF, 2002). Quality and diversity of the microbes may be assessed by looking at prevalence of different biotypes and haemolytic activity of the indicator organisms amongst other characteristics (De La Cruz *et al*, 1980). The resultant microbial profiles may show similar patterns (homogeneity) or distant relatedness (heterogeneity), for example, the profile may have a high percentage of α-haemolytic species or an equal distribution of α-, β- and γ-haemolytic species, respectively. Homogeneity may help lead to the source of faecal contamination thus helping in arresting future occurrence. Another example of such a case is that a high prevalence of sorbitol-fermenting *E. coli* in environmental water tends to indicate zoonotic rather than human point sources for faecal contamination (Bielaszewka *et al*, 1998; Karch *et al*, 1993).



The aim of this study was to assess the impact of the Khayamnandi informal settlement, Stellenbosch, South Africa on the surface water of the Plankenbrug River by evaluating faecal microbial contamination before and after the informal settlement.

Khayamnandi is often implicated as a sole source of faecal contamination of the Plankenbrug River. The current study attempts to demonstrate that Khayamnandi augments the *E. coli* population already existing in the river with biotypes that are normally associated with humans. Demonstration of different biotypes before and after the informal settlement may indicate that the new biotypes are due to the informal settlement.

## 3.3 Materials and methods

### 3.3.1 Site description

Water was sampled at two sites on the Plankenbrug River, west of Stellenbosch (Fig.3.1). One site is situated upstream from the Khayamnandi settlement while the other is downstream from Khayamnandi settlement at 18˚50′30″E, 33˚54′15″S and 18˚51′15″E, 33˚55′45″S, respectively (http://www.saexplorer.co.za/south-africa/map/stellenbosch_map.asp). The sites were chosen due to their accessibility. These sites were named Pre-Khayamnandi and Post-Khayamnandi, respectively. Pre-Khayamnandi is the last easily accessible point on the river before the settlement, while Post-Khayamnandi is the first easily accessible point after the settlement. Both sites are situated below bridges. For comparison, a nearby reference site (RS) was also chosen. This site is on Eerste River at 18˚53′45″E, 33˚53′45″S (http://www.saexplorer.co.za/south-africa/map/stellenbosch_map.asp) as it runs east of Stellenbosch before entering the town. This site is at Swaaibrug, Brummer Park. The aspects of the land around this river are such that it faces the sun more than the Plankenbrug River and is also steeper. This river runs the whole year round with an appreciable higher water volume than the former. These two rivers combine southwest of Stellenbosch and later join Kuils River which eventually drains into the Atlantic Ocean at Macassar (State of the river report, 2005).

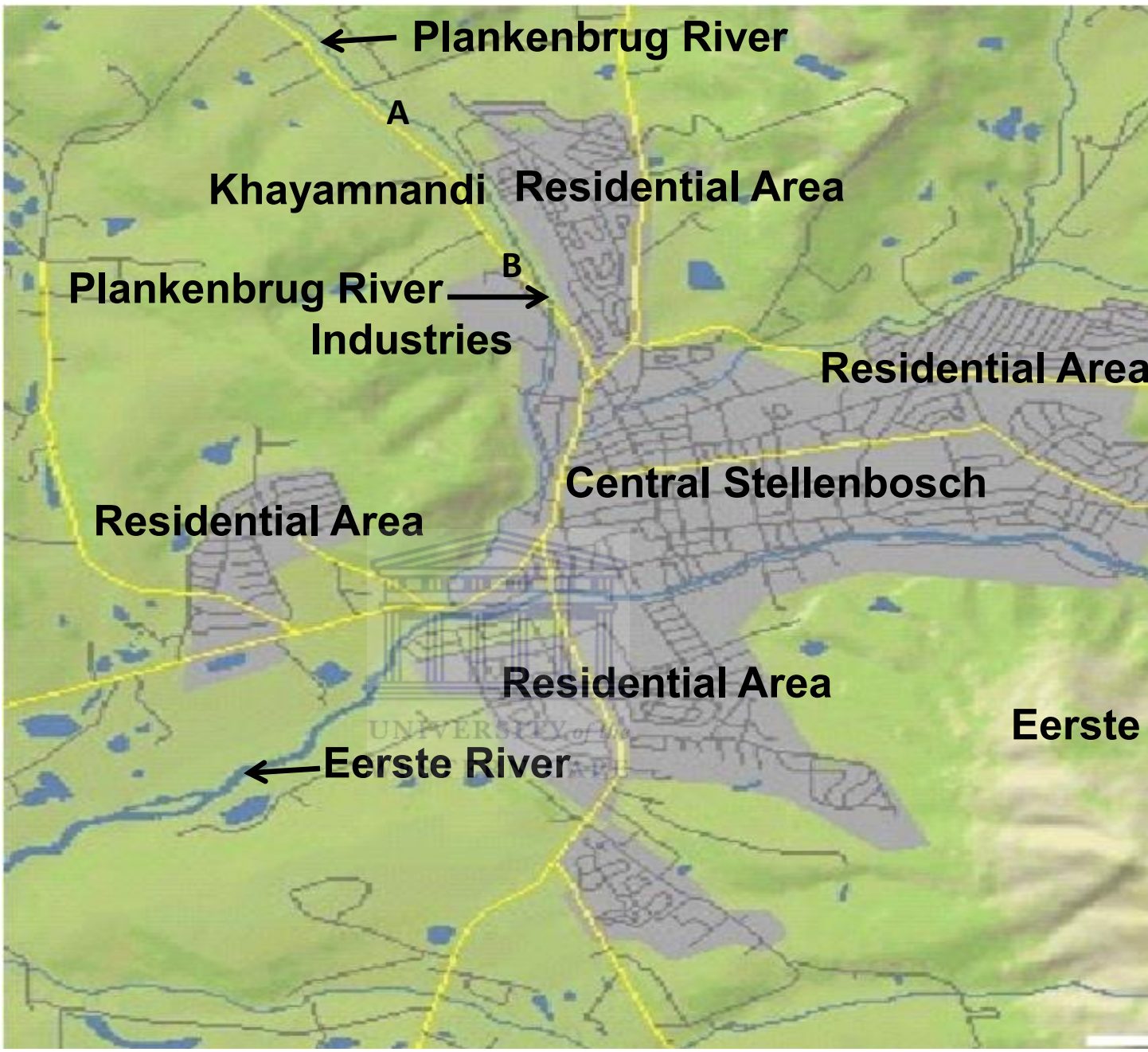


**Figure 3.1: Map of Stellenbosch, South Africa, between 18˚54′30″E; 18˚48′30″S and 18˚49′00″E; 33˚54′00″S, showing the location of the informal settlement of concern (Khayamnandi), the sampling sites: A (Pre-Khayamnandi), B (Post-Khayamnandi) and C (Reference site at Swaaibrug).**

**Source: http://www.saexplorer.co.za/south-africa/map/stellenbosch_map.asp**

### 3.3.2 Sample collection

**Table 3.1: Summary of samples used in the study**

| Sample Collection | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Winter Series | | | | | | | Spring Series | | | | |
| Sample No. | Date | Sample before rain | Sample during rain | Sample after rain | Total samples analysed | | Sample No. | Date | Sample 1 | Sample 2 | Total samples analysed |
| 1 | 11, 12 & 14 Jul | X | X | X | 3 | | 7 | 6 & 8 Sep | X | X | 2 |
| 2 | 18, 19 & 20 Jul | X | X | X | 3 | | 8 | 11 & 12 Sep | X | X | 2 |
| 3 | 25,2 6 & 29 Jul | X | X | X | 3 | | 9 | 16 & 20 Sep | X | X | 2 |
| 4 | 5, 6 & 9 Aug | X | X | X | 3 | | 10 | 29 & 30 Sep | X | X | 2 |
| 5 | 14, 15 & 18 Aug | X | X | X | 3 | | 11 | 4 & 5 Oct | X | X | 2 |
| 6 | 23, 24 & 24 Aug | X | X | X | 3 | | 12 | 14 & 15 Oct | X | X | 2 |



The samples were collected in tandem with heavy rain for the winter series. Each week, 3 samples were collected, one before rains start, one during heavy rain and the last one after heavy rain. Data from the three samples were pooled together and the average taken for that particular week. This comprised the winter series. For the spring series, water was collected twice a week for another six weeks, irrespective of rain because there were no heavy rains in this season. Data from the two samples were pooled together and the average taken as readings for that week. In all, 12 weeks' samples were analysed to generate data for this work.

Three 500 ml Schott bottles with screw caps were used for each sampling. The bottles were cleaned with detergent and rinsed repeatedly with hot water to remove any detergent residues. Thereafter they were rinsed with distilled water at least three times. These were then appropriately labelled according to site and date of collection, wrapped in aluminium foil and autoclaved at 121°C for 15 minutes.

A suitable and accessible sampling spot was chosen for each site. Sampling areas which may not be representative of the main channel, such as areas of stagnation and those located near the inside bank of a curve in the stream, were avoided.

Water temperature was recorded on the field sheet. The pH, total alkalinity, total hardness, and the concentration of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) ion were measured using test strips (Aquarium Multi-Test Kit, Jungle Laboratories Corporation) and posted on the field sheet. The water was classified as hard or soft in accordance with the guidelines in Table 3.2.

**Table 3.2: General guidelines for classification of waters (adapted from USEPA, 1985)**

| Water hardness (ppm) | Rating |
|---|---|
| <17.1 | Soft |
| 17.1-60 | Slightly hard |
| 60-120 | Moderately hard |
| 120-180 | Hard |
| >180 | Very hard |



For sample collection an appropriately labelled bottle was selected, unwrapped and held with one hand at its base. The cap was then unscrewed with the free hand and the bottle quickly dipped into the water mouth facing down. Once under the surface of water the bottle was allowed to fill by turning it upward with mouth angled against stream flow. After removal of the bottle from under stream, a small amount of water was poured off to allow air space. The cap was tightened and the sample volume was recorded in the field logbook. The sample was then transported to the laboratory and processed in the laboratory within six hours of collection. When this was not possible, they were stored at 4°C and processed within the next 24 hours.

### 3.3.3 Microbial assays

#### 3.3.3.1 Qualitative analysis for the presence of *E. coli*

The presence of *E. coli* in the sample was tested on Dev lactose peptone MUG Broth (Fluka, 31401) prepared by dissolving 26 g/l in distilled water. Ten ml of the medium was dispensed into Durham tubes and sterilized by autoclaving at 115°C for 20 minutes. The preparation was cooled, protected from direct light and stored below 8°C until use. One ml of sample was inoculated into the test tube and incubated overnight at 37°C. Turbidity of the medium accompanied by formation of gas overnight was used as a positive presumptive test for the presence of *E. coli* and/or other coliform organisms. In addition, the medium was viewed at 360-370 nm for a slightly blue fluorescence indicating the presence of *E. coli*. This is due to production of β-D-glucuronidase by *E. coli*, which cleaves 4-methylumbelliferyl-β-D-glucuronide to 4-methylumbelliferone and glucuronide.



Kovac's reagent was added to the tubes to confirm the presence of *E. coli* and read within 2 minutes. A cherry red layer on top of the medium was considered positive for the presence of *E. coli*. In case there was no fluorescence after 24 hours, Kovac's reagent was not added and the tubes were incubated for a further 24 hours. This was done because Kovac's reagent may destroy the growth conditions for cultures, as it is an alcoholic reagent.

#### 3.3.3.2 Quantitative analysis of colony forming unit and identification

Quantitative analysis of samples was done on Chromocult® coliform agar (CCA) (Merck; Cat.No.1.10426.0500). CCA was dissolved to a final concentration of 26,5 g/l in sterile water.

The medium was heated to assist dissolution then cooled down to about 45 – 55°C before pouring the plates. The plates were then marked with sample identification and dilution.

The sample was vigorously shaken for about 25 times to disperse bacteria evenly before measuring desired volumes for filtration. Filtration was done by placing a sterile membrane filter (Satorius cellulose nitrate filter, pore size 0.45 μm) grid-side up on a sterile filter base with the filter cup (Nalgene, 500 ml) then firmly secured. Water samples were analysed neat or diluted in distilled water. The volume of neat or diluted samples analysed was kept at 100 ml. The neat or diluted water sample was transferred into the cup and filtration was assisted by suction with a vacuum pump (Gast, Model DOA-P730-BN). The cup was then rinsed with 30 ml of sterile buffered water. The first sample collected was diluted serially and filtered to establish the dilutions that produce 20 – 80 colonies on the membrane filter. This was done to establish the potential pollution levels. After filtration was completed, the vacuum was turned off and the membrane aseptically removed with sterile forceps. It was then rolled onto sterile Chromocult agar with care taken to avoid the formation of bubbles. A minimum of three dilutions (in triplicate) of each sample were filtered. The plates were incubated at 37°C for 18-24 hours after which they were investigated for growth of red and dark-blue pigmented colonies on the agar (qualitative analysis).



Colonies on filter membranes were counted using a colony counter (Gallenkamp, UK). Colonies identified as *E. coli* were confirmed by their ability to ferment biochemical sugars, namely production of gas from glucose, fermentation of lactose, sucrose and mannitol together with reaction on the IMViC tests (Kotlowsky *et al*, 2007; Powers and Latt, 1977). Membrane filters with 30 to 250 colonies were selected for computation of the concentration of both *E. coli* and total coliform (TC) per 100 ml. This concentration was calculated as follows:

TC = (Number of red colonies + Number of dark-blue colonies)/Volume of sample filtered X 100 ml.

*E. coli* = (Number of dark-blue colonies)/Volume of sample filtered X 100 ml.

Results were then reported as cfu/100 ml and log transformed. Bacterial concentration below detection was recorded as zero.

### 3.3.4 Analytical profiling index (API)

Each test kit is composed of a plastic sterile strip with 20 mini-tubes. Each tube contains desiccated medium for a specific biochemical test. A saline suspension of pure culture is inoculated into each of the tubes. Thereafter, some of the tubes are overlaid with sterile mineral oil allowing for anaerobic reactions by preventing oxygen from further entering the medium (BioMérieux, France). The 20 reactions on the API 20E test strip are presented in Table 3.3. ADH, LDC, ODC, $H_2S$, and URE in Table 3.3 are anaerobic reactions that are overlaid with mineral oil. Reactions from 1 to 20 in Table 3.3 are performed on the API test strip.



Reaction 21, i.e. the oxidase reaction, is done separately on a piece of sterile filter paper impregnated with tetramethyl-p-phenylene-diamine dihydrochloride (Halpern *et al*, 2009; Rodriguez *et al*, 2011). In this reaction an overnight colony is applied to the filter paper with a wooden applicator stick. Oxidative positive reactions turn purple, while negative samples remain white or turn light pink. All *Enterobacteriaceae* are negative for oxidase test. After overnight incubation, reactions 8, 9 and 10 in Table 3.3 need appropriate supplementary reagents to be added before they can be interpreted (BioMérieux, France). The results are then reported as negative or positive.

**Table 3.3: Reading frame for the API 20E**

| READING TABLE | | | | | |
|---|---|---|---|---|---|
| Test | Active ingredients | Quantity (mg/cup) | Reactions/ Enzymes | Results Negative | Positive |
| ONPG | 2-Nitrophenyl-BD-galactopyranoside | 0.22 | B-galactosidase | Colourless | Yellow |
| ADH | L-arginine | 1.9 | Arginine DiHydrolase | Yellow | Red/orange |
| LDC | L-lysine | 1.9 | Lysine DeCarboxylase | Yellow | Red/orange |
| ODC | L-ornithine | 1.9 | Ornithine DeCarboxylase | Yellow | Red/orange |
| CIT | Trisodium citrate | 0.76 | Citrate utilization | Pale green/yellow | Blue green/blue |
| $H_2S$ | Sodium thiosulfate | 0.08 | $H_2S$ production | Colourless/greyish | Black deposit |
| URE | Urea | 0.75 | Urease | Yellow | Red/orange |
| TDA | L-tryptophane | 0.38 | Tryptophane deaminase | Yellow | Reddish brown |
| IND | L-tryptophane | 0.19 | Indole production | Colourless/pale green/yellow | Pink |
| VP | Sodium pyruvate | 1.9 | Acetoin production | Colourless/ pale pink | Pink/red |
| GEL | Gelatin | 0.6 | Galatinase | No diffusion | Black pigment |
| GLU | D-glucose | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| MAN | D-mannitol | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| INO | Inositol | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| SOR | D-sorbitol | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| RHA | L-rhamnose | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| SAC | D-sucrose | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| MEL | D-melbiose | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |
| AMY | Amygdalin | 0.57 | Fermentation/oxidation | Blue/blue green | Yellow |
| ARA | L-arabinose | 1.9 | Fermentation/oxidation | Blue/blue green | Yellow |

Table 3.3 gives the key for reading the API tests showing the 20 tests used for generating the seven-digit code. Source: (BioMérieux, France).

In order to generate the analytical profiling index, the API 20 E tests are grouped into triads. The first triad is composed of the first 3 tests in Table 3.3; the second triad is the next 3, etc according to the manufacturer's instructions.

The first test in the API 20E strip is for action of β-galactosidase on o-nitrophenyl-8-D-galactopyranosidel (ONPG). β-galactosidase is one of the enzymes that are required in bacteria for lactose utilization (Lapage *et al*, 1973). In this assay ONPG, an analogue of lactose, is used

as a decoy molecule. ONPG can break down to produce a yellow coloured end-product, O-nitrophenol, in the presence of β-galactosidase. Most enteric bacteria produce this enzyme. Positive tests will turn yellow, while negative tests will remain colourless (API 20E Instruction Manual version #2012). The results will be scored as 1 or 0 respectively. *E. coli* is commonly positive for this test.

In the test, the presence of arginine dihydrolase is assayed. Numerous microorganisms use L-Arginine to generate ATP by the arginine dihydrolase 1 pathway. Arginine dihydrolase produce ornithine, ammonia and carbon dioxide from L-arginine (Galkin *et al*, 2004; Morada *et al*, 2010; Venugopal and Nadkarni, 1977). These products cause a rise in pH and subsequently a change in the indicator from yellow to red. Consequently, a positive arginine dihydrolase test will be red while a negative one will be yellow. As this result is the second in the triad, it will be scored 2 or 0, respectively. There are *E. coli* isolates that are positive for this test, although they are rare.



The next two reactions are decarboxylation reactions where the production of lysine and ornithine decarboxylases by bacteria are tested. Phenotypic expression of these enzymes is demonstrated by the media turning red or pink due to the production of cadaverine and putracine, respectively. The medium will remain yellow in the absence of these enzymes. The LDC has a high prevalence of positive *E. coli* while there are equal chances of encountering both positive and negative ODC biotypes of *E. coli*.

In the fifth tube, bacteria are tested for their ability to use citrate as a sole source of carbon (Kanungo, 2009). Traditionally citrate utilization is evaluated by observing turbidity in a tube that had been lightly inoculated after overnight incubation (Smith *et al*, 1978). This is, however, confounded by heavy initial inocula (Vaughn *et al*, 1950). Simmons formulated a preparation

that incorporated bromothymol blue as an indicator to identify growth on the medium. When citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are ultimately produced. This causes an increase in pH of the solution to above 7.6 which causes the indicator to change from pale green (negative) to blue (positive) (API 20E Instruction Manual version #2012; Kanungo, 2009). Although *E. coli* is commonly citrate negative, positive isolates have been reported (Blount *et al*, 2008; Hall, 1982; Ishiguro *et al*, 1979; Pos *et al*, 1998).

The sixth tube contains Sodium thiosulfate as a reactive ingredient. Some microorganisms can use sulphates as a sulphur source. These organisms reduce sulphur to hydrogen sulphide (Huang and Barrett, 1991). Organisms that produce hydrogen sulphide ($H_2S$) cause precipitation of a black deposit (Treleaven *et al*, 1980) due to a reaction between $H_2S$ and iron salt in the medium. $H_2S$-positive *E. coli* is rare (API 20E Instruction Manual version #2012; Magalheas and Vance, 1982; Marker and Washington II, 1974; Treleaven *et al*, 1980).



In the seventh tube the reactive agent is urea with phenol red as an indicator (API 20E Instruction Manual version #2012). Some organisms produce an enzyme called urease (Collins and Falkow, 1990). These organisms produce ammonia causing a rise in pH. The medium turns red indicating a positive reaction. Negative reactions remain yellow. *E. coli* are commonly negative for this test (Steyert *et al*, 2011). The API data sheet reports no prevalence of urease and L-tryptophan positive *E. coli* from the API system (API 20E Instruction Manual version #2012).
The eighth tube tests for the production of tryptophan deaminase. This reaction is tested on L-tryptophan. This reaction is visualized by addition of 10% ferric chloride whereupon it turns red (API 20E Instruction Manual version #2012).

In the 9th tube, the production of indole from tryptophan is tested. Some organisms produce the enzyme tryptophanase which breaks down tryptophan to produce indole (Wang *et al*, 2001). A positive test is indicated by the pink colour produced following the addition of Kovac's reagent to the medium. In negative tests the medium remains yellow (Sasaki-Imamura *et al*, 2010). There is a high prevalence of indole positive *E. coli* (LeClercq *et al*, 2001; Wang *et al*, 2001).

The tenth tube is the Vogues Proskauer (VP) test where the production of acetoin from sodium pyruvate is determined (Iverson *et al*, 2007). In order to visualize acetoin, 40 % KOH and 6 % α-napthol are added. Positive tests will turn red while negative tests may turn pale pink. *E. coli* is known to be VP-negative (API 20E Instruction Manual version #2012; Vinué *et al*, 2008).

The 11th test is to determine whether test organisms produce gelatinase. Gelatinase is a proteolytic enzyme that causes gelatin liquefaction and release of a black pigment which diffuses throughout the medium (Kohn, 1953). There are no reports of *E. coli* testing positive for gelatin liquefaction (API 20E Instruction Manual version #2012).



Reactions 12 to 20 are fermentation /oxidation tests. In these reactions the substrates are glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose, respectively. The oxidative-fermentative tests determine whether Gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are asaccharolytic (API 20E Instruction Manual version #2012). Asaccharolytic bacteria have no ability to use the carbohydrate in the media and therefore will not alter the colour of the media. Fermentation is an anaerobic process, where pyruvate is converted to a variety of mixed acids (Hallenbeck and Ghosh, 2009). Because of the high concentration of acid produced during this process, the bromothymol blue indicator in oxidation-fermentation media will turn from blue to yellow in the presence or absence of oxygen, starting from the bottom of the tube (De Mora *et al*, 2011). On

the other hand, oxidative reactions are aerobic and produce small amounts of weak acids. Colour change of the medium tends to be towards the top of the tube after the first 24 hour incubation (Zhu *et al*, 2003). It may subsequently diffuse throughout the tube. Reading the tubes carefully after 24-hour incubation is important in determining whether the tested bacterium metabolized carbohydrates oxidatively or by fermentation (API 20E Instruction Manual version #2012). Positive results will be yellow while negative results will be blue. Each positive result will be scored 1, 2 or 4 according to its position in the triad, irrespective of whether the reaction was oxidative or fermentative. Asaccharolytic reactions are scored zero. Fermentation of carbohydrates is positive for *Enterobacteriaceae* while oxidative processes are negative (Bouvet *et al*, 1989). All *E. coli* metabolize glucose by fermentation producing acid and gas (Trinh *et al*, 2008). Next to glucose comes mannitol, followed by sorbitol, arabinose, rhamnose and melibiose respectively (API 20E Instruction Manual version #2012). Saccharose (sucrose) fermentation is not uncommon with *E. coli* (Ratnam *et al*, 1998; Spyropoulou *et al*, 2001). Inositol and amygdalin fermentation is rare (Ratnam *et al*, 1998; Yoon and Horde, 2008; Souza *et al*, 1999).



#### 3.3.4.1 Preparation and inoculation of API strips

Two hundred (100 from Pre-Khayamnandi and 100 from Post-Khayamnandi) single well-isolated purple colonies (previously identified as *E. coli*) from the membrane were picked with a sterile loop and streaked onto nutrient agar (NA) plates. Each plate was given a reference number (1-100) that included the site and colony number. The NA plates were incubated for 18-24 hours at 37ºC. Young cultures were now ready for API and haemolysis assays. Isolates for API test were picked with a sterile pipette and carefully emulsified into 4 ml saline to achieve a homogeneous suspension in a test tube with the same reference number as the plate. The suspension was then used immediately for inoculating the API strips.

Five ml of distilled water was distributed into the honeycomb wells of the incubation box to create a humid environment. The reference number was written on the elongated flap of the tray to facilitate identification of isolates. The API strip was then removed from its packaging and placed into the incubation box.

The pipette previously used to transfer the isolate from the NA plate into saline was used to distribute the bacterial suspension into the tubes of the strip. The strip was tilted forward and the pipette tip was placed against the side of the capule to avoid bubbles in the ampule of the strip. For citrate, Voges Proskauer and gelatin hydrolysis, both tubes and capules were filled. For the remaining tests, only the tubes were filled. The tubes for ADH, LDC, ODC, $H_2S$ and URE were overlaid with mineral oil to favour anaerobic reactions.

The incubation box was then closed and incubated for 18-24 hours at 37°C. After incubation, the colour reactions were read. For TDA, one drop of TDA reagent was added and a reddish brown colour was considered as positive. For IND test, one drop of JAMES reagent was added and a pink colour interpreted as positive. For VP test, one drop of VP1 and VP2 were added and results read after 10 minutes. A pink or red colour indicated a positive test.



**3.3.4.2 Reading and interpretation of API results**

After incubation, the reading API frame (Table 3.3), supplied with the kit, was used to determine the nature of the results. All 20 reactions were recorded on the result sheets that were provided with the API kit. The tests are grouped into threes on the result sheet, with each test given a score of, 1, 2 or 4 (Table 3.4). These scores were then added together per group to give a seven-digit numerical value for each isolate. This seven-digit value is the API for the isolate tested (Table 3.4). This index was then fed into a computer and read with the api*web*$^{TM}$ identification software.

**Table 3.4: API 20E triads**

| Triad | I | | | II | | | III | | | IV | | | V | | | VI | | | VII | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | oxidase |
| Reaction | + | + | + | - | - | - | + | + | - | + | - | - | - | + | + | + | + | + | - | + | - |
| Points | 1 | 2 | 4 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 0 | 0 | 2 | 4 | 1 | 2 | 4 | 0 | 2 | 0 |
| Profile Index | 7 | | | 0 | | | 3 | | | 1 | | | 6 | | | 7 | | | 2 | | |

Table 3.4 represents the layout of the triads, where tubes 1, 2 and 3 are the first 3 reactions in Table 3.3 constituting the first triad. The positives in a triad are given a score of 1, 2 and 3 respectively. All negatives are scored as zero. The values in each triad are added together. The resulting sum would thereafter represent the digit of that particular triad, as shown in Table 3.4. This seven digit figure is the analytical profiling index coding for a specific bacterium.

### 3.3.5 Haemolysis assay



Inocula were prepared as described for API (3.2.4). Three percent (3%) blood solution was prepared by mixing 15 ml fresh horse blood in 485 ml of Dulbecco purified salts. One and a half millilitres of this solution was dispensed into each of 212 Eppendorf tubes. One hundred tubes were for the Pre-Khayamnandi isolates and the second hundred was for Post-Khayamnandi. The remaining twelve tubes were 3 control groups, namely, blood blanks and two sets of positives (i.e. β and α-haemolytic streptococci).

Each isolate was picked from the nutrient agar plate with a sterile pipette tip and emulsified in an Eppendorf tube with 2 ml Dulbecco salts. The isolates were then incubated at 37ºC for 4 hours to reach the log phase. Aliquots of 200 µl were then dispensed into 96-well Nunc maxisorb microtitre plates and the plates read for opacity at 600 nm with a spectrophotometer. The aliquots

to be assayed for haemolysis were then calculated from the lowest spectrophotometer reading to eliminate variation due to bacterial concentration. The formula used was, $V = C_0/C_n*20$ µl, Where V= volume to use

$C_0$= Lowest microbial density

$C_n$= Microbial density of the aliquot to be assayed.

The isolate sample at V was gently mixed with blood in an Eppendorf tube by inversion and incubated at 37ºC for 1 hour. The mix was tapped every 10 minutes during the incubation period. After incubation, the mix was centrifuged at 1000 rpm for 10 minutes. Fifty microlitres of the supernatant was transferred from their respective tubes into a new microtiter plate and read for haemolysis. The first column in the plate was reserved for controls with blood blank in the first two wells, followed by two wells of the negative control and the next two wells contained known positive controls. The last two wells were left blank in order to determine the background due to the plate itself. The plates were read with the spectrophotometer at 405 nm for haemolysis and the data captured on the computer. Deducting the plate's average background and the average blood blank OD readings from the samples' OD readings normalized the readings. Statistical analysis was done using SigmaPlot 11 version.



### 3.3.6 Statistical analysis of data

One-way ANOVA (SigmaPlot 11 version) was used to demonstrate significant differences in bacterial concentrations between sites. Different sampling stages were compared to see if any consistency could be deduced with regard to prevalence of certain subtypes.

## 3.4 Results and discussion

### 3.4.1 Physicochemical parameters

**Table 3.5: Physicochemical parameters across study sites**

| | | T (ºC) | pH | TA (ppm) | TH (ppm) | $NO_2$- (ppm) | $NO_3$- (ppm) |
|---|---|---|---|---|---|---|---|
| Winter | RS | a12 ±2.76 | 6.3 ±0.24 | 6.67 ±16.33 | 4.17 ±16.33 | 0 ±0.00 | 3.33 ±8.16 |
| | Pre-K | a*14.33 ±1.86 | **8.17 ±0.27 | **220 ±90.33 | **300 ±0.00 | *0.25 ±0.27 | **23.33 ±8.16 |
| | Post-K | a*13.83 ±1.72 | **8 ±0.44 | a**163.33 ±77.37 | **245 ±77.37 | *0.42 ±0.38 | **36.67 ±23.38 |
| Spring | RS | a15.4 ±1.52 | 6.57 ±0.43 | 0 ±0.00 | 0 ±0.00 | 0.17 ±0.26 | 3.33 ±8.16 |
| | Pre-K | a15.8 ±0.45 | **7.8 ±0.42 | **170 ±70.14 | **250 ±77.46 | 0.17 ±0.26 | *23.33 ±15.06 |
| | Post-K | a16 ±0.71 | **7.57 ±0.41 | αa**100 ±21.91 | α**145 ±83.07 | 0.33 ±0.26 | 16.67 ±19.66 |

**Key:**

**T – Temperature, TA - Total alkalinity , TH - Total hardness, $NO_2^-$ - Nitrites, $NO_3^-$ - Nitrates**

*** p < 0.05 (significantly different to RS for a specific season)**

**** p < 0.01 (significantly different to RS for a specific season)**

**[a] p < 0.05 (significantly different between winter and spring for the same site)**

**[b] p < 0.01(significantly different between winter and spring for the same site)**

**[α] p < 0.05 (Pre-K is significantly different to Post-K)**

**[β] p < 0.01 (Pre-K is significantly different to Post-K)**



Table 3.5 presents the averages and standard deviations of the physicochemical parameters studied across the three sites (n=6). RS, Pre-K and Post-K represent the reference site, Pre-Khayamnandi and Post-Khayamnandi, respectively. These averages represent measurements over winter and spring, that is, rainy and dry seasons respectively. Table 3.5 also depicts season and site specific p values comparing the physicochemical parameters across the sites.

Temperature is the single most important factor for longevity of bacteria in water. Hara-Kudo *et al* (2000) illustrated the importance of temperature on the survival of bacteria in water under starvation conditions. In their study, bacterial populations decreased faster at 4ºC than at 18ºC.

They also showed that the initial bacterial population correlated positively with its survival. Assuming that temperature was the only major factor, lower temperatures at the reference site could have negatively affected its microbial population. Low temperatures together with lower population density could have facilitated die-off.

There was no seasonal significant difference in temperature between Pre- and Post-K sites (Table 3.5). The slight difference in temperature across the settlement could be due to the aspects of land at the sampling positions. Temperature also affects a wide array of physical and chemical factors such as pH, nitrate and nitrite concentration, to name a few (Jaynes *et al*, 2004; LeChevallier *et al*, 1996). Due to the insignificant differences in temperature across the three sites and the different distribution of other physicochemical properties, a correlation of temperature with these variables has no merit. Hence the other parameters would be discussed individually or in small groups without considering the influence of temperature.



The pH is determined by the concentration of hydrogen ions and alkalinity by concentration of hydroxyl, bicarbonate and carbonate ions in water (Palmer *et al*, 2004). Rivers in the Western Cape are naturally acidic because of fynbos vegetation (Dallas and Day, 2004). A sustained low pH would indicate the integrity of these rivers.

The three sites used in the current study have two different origins (Fig. 3.1). Test sites are on Plankenbrug River while the reference site is on Eerste River. This could explain the difference in pH of the two rivers (Table 3.5). However, the variation in the two rivers may indicate pollution. The spring pH difference between RS and the two test sites was $\geq 1$. This indicates more than 10-fold increase in $H^+$ concentration. The winter series exhibited almost double this increase (Table 3.5). Post-K also had a slightly wider range than Pre-K in winter. Total alkalinity

could have maintained high pH in the two test sites in both seasons. RS was significantly different to test sites (Table 3.5). The two test sites were also significantly different from each other ($p < 0.05$). These differences in TA could have affected pH. All three sites are within the survival pH range ($4.4 \leq pH \geq 9.0$) for *E. coli* (Aronsson and Rönner, 2001). Therefore, pH could not have had any negative impact on the population density of the faecal indicators.

RS had little buffering capacity (TA = 6.67 ppm and 0.00 ppm) in winter and spring, respectively. This indicates that this water was susceptible to pH changes. Such susceptibility could positively influence microbial die-off, given that fynbos vegetation tends to make river water acidic (Dallas and Day, 2004).

Closely associated with buffering capacity in water, is the total hardness which is the molar sum of calcium and magnesium found in water. The overall mean for RS (2.08 ppm) was due to a single event over the whole sampling series as demonstrated by a high standard deviation. This is demonstrated in Table 3.5 with 4.17±16.33 and 0.00±0.00 for winter and spring, respectively. In general the total hardness (TH) detection levels were below threshold. This single event was after the first heavy rains, after which the TH readings levelled off at 0.00 ppm. These readings set RS apart from the other two test sites where there was constant presence of TH detection (Table 3.5). The two test sites were significantly different ($p < 0.05$) from each other in spring. Thus, the RS is different from Pre-K, and Pre-K is different from Post-K. This means that the two test sites are subjected to different influences.

Hard water has benefits; for example, it can protect fish embryos in the presence of silver toxicity (Morgan *et al*, 2005). It can also influence turbidity of water (Homendra, 2004). In the latter capacity, it may also offer shielding of microbes from the sun and offer a medium to form

surds, thus perpetuating their persistence. So in terms of toxicity with reference to total hardness, Post-K may have more remedying capacity than Pre-K with persistent higher TH.

The other parameters, namely, nitrites and nitrates also show that the two test sites were both significantly different to RS in winter ($p < 0.05$ and $p < 0.01$ for nitrites and nitrates, respectively) (Table 3.5). The [$NO_3^-$] at RS differed significantly from Pre-K (Table 3.5) in spring. Post-K exhibited a high [$NO_3^-$] mean in spring but was not significantly different to RS. This mean was due to one episode and is demonstrated by the high standard deviation (Table 3.5).

The above physicochemical tests indicate that RS is different from Pre-K. The question now is how different are the two sites from each other? The p values in Table 3.5 do not show any appreciable differences in physicochemical parameters across the two test sites except for TA and TH in spring.

### 3.4.2. Microbial assays

#### 3.4.2.1 Qualitative analysis

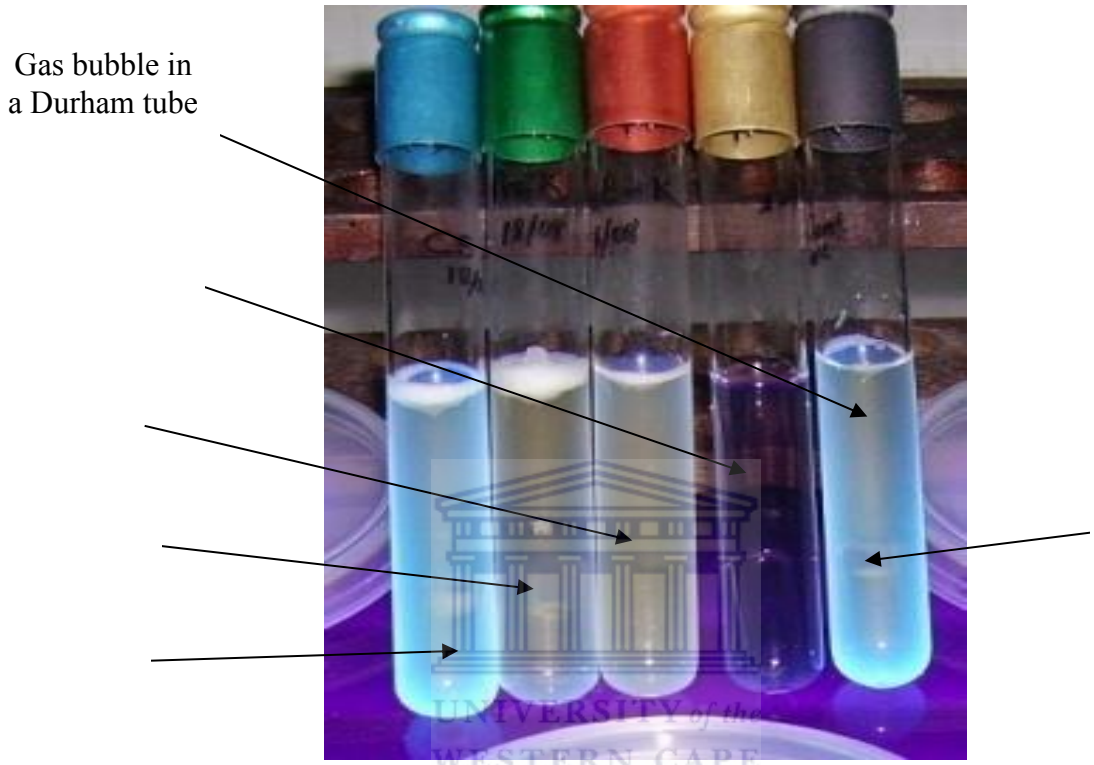




**Figure 3.2: Presumptive test for the presence of *E. coli* with DEV Lactose Peptone MUG broth after inoculation with sample and incubation over-night at 37˚C, viewed under UV light**.

From left to right are RS, Pre-K and Post-K samples, negative and positive control, respectively (Fig.3.2). Tubes 1, 2, 3 and 5 (from left to right) fluoresced blue when irradiated with long-wave ultra violet light (λ = 366 nm) while tube 4 remained negative. In these tubes the enzyme *β*-D-glucuronidase produced by *E. coli* had hydrolyzed 4-methylumbelleryl- *β*-D-glucuronide in the medium to produce a fluorescent end product, 4-methylumbelliferone. In tube 4 there was no reaction because there was no *E. coli*. Tubes 2 and 3 demonstrated weaker fluorescence, possibly due to the diffusion of the fluorogen into the particulate matter in these tubes. All samples were

treated in the same way to avoid any discrepancies. This was a presumptive test for faecal contamination. The first step for confirming the presence of *E. coli* was investigating the inverted Durham tube inside the test tube for gas production. In tubes 1, 2, 3 and 5, there was a gas bubble in each of the Durham tubes. In the negative control (test tube 4) no gas formed (Fig.3.2).



**Figure 3.3: Confirmatory test for presence of *E. coli* on DEV Lactose Peptone MUG Broth using Indole reaction.**

The front row shows the confirmatory test for the presence of *E. coli* in the samples from the three sites with Kovac's reagent after incubation of sample O\N at 37˚C (Fig. 3.3). A qualitative presumptive test for the presence of *E. coli* (Fig.3.3) was done for all samples collected. All samples tested positive. However, the fluorescence intensities and amount of gas formed varied, indicating inhibition of the enzyme in turbid water. The presence of *E. coli* was then confirmed with the Indole reaction (Fig. 3.3).

Due to financial implications of quantitative tests, it is ideal to initially do a presence or absence assay for faecal contamination. The results from such an assay would then inform the subsequent

steps. Some chromogenic and fluorogenic media serve this purpose well. Similarly in the current study, it first had to be established whether there was faecal contamination or not before proceeding to elucidate the extent of contamination through which the difference or similarity of the two sampling sites were assessed.

The three sampling events around one episode of heavy rain were aggregated into a single event in order to avoid serial correlation yet capturing both events around the episode (Table 3.1). Richards and Grabow (2003) warned about error due to independent observations. They warned that the first observation may carry influence over into the subsequent one, thus creating noise in the overall observation. Grabow *et al* (1999) suggested that the easiest way to correct serial correlation is to aggregate data into a larger step, for example, average data around a single event.



In the current study the presence or absence of faecal matter at the sampling sites was assessed using a fluorescence medium called DEV Lactose Peptone MUG Broth exploiting the presence or absence of *E. coli* as an indicator. Figure 3.2 shows the activity of *β*-D-glucuronide in all experiment test tubes. However, the RS in test tube 1 showed the brightest fluorescence. This correlates with the comparative clarity of water at this site (Fig. 3.2). The turbidity of water from Pre- and Post-K interfered with fluorescence in test tubes 2 and 3 respectively (Fig. 3.2). The high affinity of 4-methylumbelliferone for soil particles lowered fluorescence (Manafi, 2000; Marx *et al*, 2001). The intensity of fluorescence appeared not to be related to the amount of *E. coli* in the sample. Therefore *E. coli* was presumed present in all sites.

The presumptive presence of *E. coli* was then fully confirmed by indole reaction (Fig. 3.3). All samples tested positive for indole reaction verifying the presence of *E. coli* in all samples. The

need for verification arises from the fact that *Salmonella, Shigella* and *Yersinia spp.* are also GUD positive but are indole negative (Heizmann *et al*, 1998; Manafi, 2000). For assessing whether water is fit for drinking purposes, no further testing is required if these tests are negative.

### 3.4.2.2 Quantitative analysis

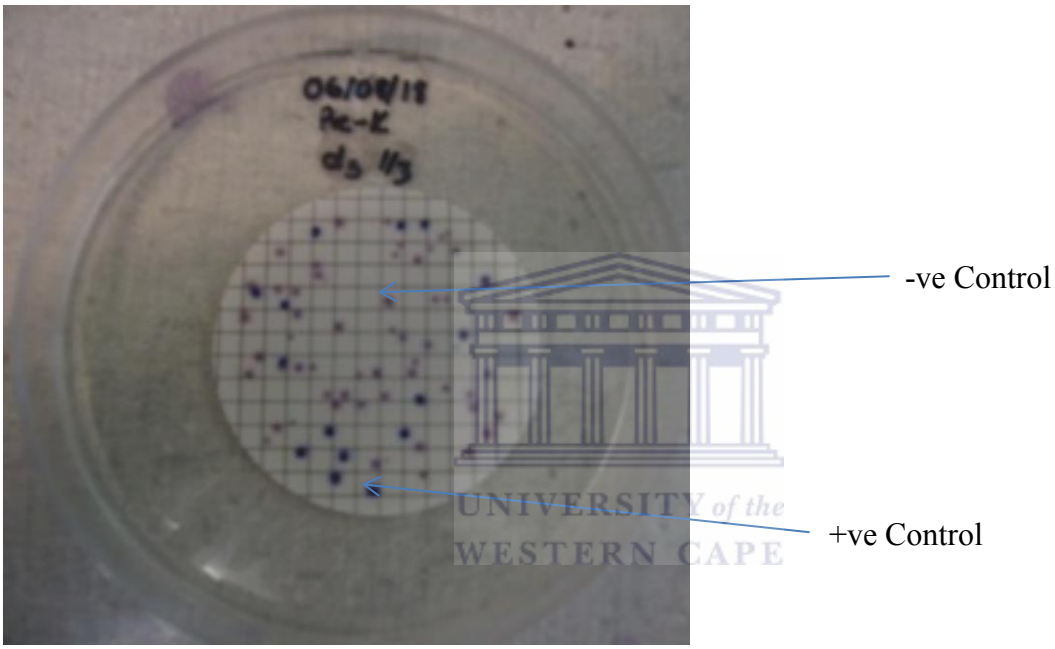


**Figure 3.4: Indicator organisms on Chromocult agar plate**

Figure 3.4 shows a membrane filter with filtered sample on a Chromocult agar plate after incubation overnight at 37˚C. The dark blue colonies are *E. coli* colonies and pink colonies indicate other coliforms.

A quantitative analysis on the membrane filter-Chromocult agar system (Fig. 3.4) showed diverse amounts of the indicator organisms across the different sites. The red pigmented colonies were identified as total coliform (TC) while blue to dark blue colonies were identified as *E. coli*. It was not possible to count from neat samples, particularly for the test sites. Countable colony

forming units started at 1 in 4 dilutions for most cases. Countable colonies ranged from 30 to 250 per membrane filter. The average colony count for Post-Khayamnandi was the most diverse for both total coliforms and *E. coli* on the whole (Table 3.6).

**Table 3.6: Average colony counts for winter and spring**

| Average microbial counts (cfu/100 ml) | | | | | | |
|---|---|---|---|---|---|---|
| | Total coliforms | | | *E. coli* | | |
| Sample series | RS | Pre-K | Post-K | RS | Pre-K | Post-K |
| 1 | 4.62E+01 | 9.61E+08 | 1.46E+08 | 1.03E+01 | 7.38E+05 | 3.10E+07 |
| 2 | 1.25E+01 | 9.25E+07 | 2.45E+06 | 3.33E+00 | 1.60E+07 | 6.83E+05 |
| 3 | 1.25E+01 | 7.69E+05 | 1.10E+06 | 3.33E+00 | 7.23E+04 | 3.91E+05 |
| 4 | 4.62E+01 | 2.15E+06 | 1.38E+06 | 1.03E+01 | 3.17E+05 | 6.70E+05 |
| 5 | 2.00E+01 | 2.82E+06 | 6.56E+06 | 5.00E+00 | 8.47E+05 | 2.01E+06 |
| 6 | 6.00E+01 | 3.31E+05 | 2.35E+06 | 2.00E+01 | 1.64E+04 | 2.35E+06 |
| 7 | 2.50E+01 | 1.46E+07 | 2.53E+06 | 5.00E+00 | 3.90E+06 | 1.73E+06 |
| 8 | 7.50E+01 | 5.47E+06 | 8.90E+08 | 2.25E+01 | 1.63E+06 | 2.80E+08 |
| 9 | 4.42E+01 | 2.50E+06 | 2.75E+07 | 1.20E+01 | 2.50E+05 | 8.95E+07 |
| 10 | 4.95E+01 | 2.85E+06 | 3.45E+08 | 4.57E+01 | 1.30E+06 | 1.20E+08 |
| 11 | 1.50E+00 | 3.20E+06 | 4.30E+08 | 8.33E-01 | 1.80E+06 | 1.98E+08 |
| 12 | 6.50E+01 | 6.70E+06 | 6.55E+08 | 4.18E+01 | 3.40E+06 | 2.30E+08 |



Table 3.6 represents average microbial concentration per 100 ml over winter and spring series, i.e. 1-6 and 7-12, respectively. Each series (n=6) was comprised of 3 and 2 sampling episodes in winter and spring, respectively. Each episode is a mean of three samples. The microbial density in RS is in the region of $10^1$, while the other two sites range between $10^5$ and $10^8$ both seasons.

**Table 3.7: Summary of seasonal variation of TC and *E. coli* during sampling series**

| Comparison of Seasonal Microbial Averages | | | | | | |
|---|---|---|---|---|---|---|
| Series | Total coliforms | | | *E. coli* | | |
| Winter | RS | $^{**}$Pre-K | $^{**}$Post-K | RS | $^{**}$Pre-K | $^{**}$Post-K |
| Spring | RS | $^{\alpha **}$Pre-K | $^{a **}$Post-K | RS | $^{\alpha}$**Pre-K | $^{a}$**Post-K |

**Key:**

*** - p < 0.05 (significantly different to RS for a specific season)**

**** - p < 0.01 (significantly different to RS for a specific season)**

**$^{a}$ - p < 0.05 (significantly different between winter and spring for the same site)**

**$^{\alpha}$ - p < 0.05 (Pre-K is significantly different to Post-K)**

Table 3.7 presents the seasonal microbial averages of TCs and *E. coli* (n=6) compared across the three sites. Significant differences within and across the three sites are also presented as against RS seasonally, within each site across the two seasons and seasonally across the two test sites.

CCA contains two chromogenic enzyme substrates 6-chloro-3-indoxyl-3-ß-D- galactopyranoside (SALMON-GAL) and 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid, cyclohexylammonium salt (X-GLUC), for detection of activities of ß-D-galactosidase from coliforms and ß-glucuronidase from *E. coli*, respectively. After hydrolysis SALMON-GAL releases a chromogenic compound, chloroindigo forming salmon to red coloured colonies, while X-GLUC releases bromochloroindigo forming light blue to turquoise colonies (Finney *et al*, 2003; Merck, 2000).

CCA exploits the discrimination of both TC and *E. coli*. This enables the evaluation of the same scenario using these two parameters separately. Therefore the trend in faecal pollution was observed for both *E. coli* and TC simultaneously. Data for Table 3.6 was generated from counting the pink/red plus blue colonies for TC on the filtration membranes (Fig. 3.4) grown on CCA while blue colonies indicated the presence of *E. coli* only.

The two test sites were significantly different ($p < 0.01$) to RS for both winter and spring (Table 3.7). Pre-K and Post-K further differed ($p < 0.05$) from each other in spring. Table 3.7 further showed difference ($p < 0.05$) within Post-K across the two seasons. The Ln [TC] increased by a factor of 2 in spring at Post-K (Table 3.6).

The difference between RS and Pre-K *E. coli* population density was pronounced for both seasons ($p < 0.05$). There was also a significant difference between Pre-K and Post-K ($p < 0.05$, Table 3.7).

In general, linear transformations of average Ln (TC) population across the three sites show different responses to decrease in water flow. Contrary to expectation, the population at Post-K increased, while that at the other two sites responded according to norm. The decline at RS is pronounced ($p < 0.05$) while a flatter gradient at Pre-K was observed. This was also visible when comparing the winter to the spring population densities (Table 3.7). It is worth noting that in winter both Pre- and Post-K were in the same range on average count, i.e. $10^7$(Table 3.6). They were different in range, where Post-K had a broader range (Table 3.7). Turning to spring, the averages of the two sites were $10^2$ fold apart (Table 3.6). This indicates that microbial presence is indeed heavily increased by conditions surrounding Khayamnandi though it may not be merely due to wash-off from the settlement itself. The persistent microbial presence at Post-K may also be influenced by anthropogenic activities and vegetation immediately at the banks and above the sampling point of the river, respectively. *E. coli* population followed a similar trend.

Hara-Kudo *et al*, 2000, made an observation that bacterial decrease with time under starvation is less pronounced when the initial density was high. This could be one of the explanations of the patterns seen in the trend analysis. Linear transformations of the microbial population indicate an

increase in microbial density for both populations despite reduced water flow; hence water influx from the settlement. The increase in *E. coli* was however slightly higher ($p \leq 0.05$).

Taking into consideration that microbial density at RS and Pre-K declined after heavy rains, it seems that there were persistent contributing factors within the boundaries of Khayamnandi (Table 3.6). It appears that these factors were not directly linked to rains, although such conditions exacerbated the situation as indicated by sample series points 1 in winter and 8 in spring (Table 3.6). The points that are farthest above the linear transformation represent samples at storm flow or when water velocity had settled down from rapid flow (e.g. point 8 in Table 3.6) after wash-off had taken place. Overall, the significant increase ($p \leq 0.05$) in *E. coli* at Pre-K suggests that the surrounding environment plays a major role in polluting the Plankenbrug River (Table 3.7).



All data collected refutes the assertion that the degree of pollution at RS is equivalent to that at Pre-K. Instead, data points out that Pre-K faecal pollution is much higher than that at the reference site. Further the assertion that Pre-K pollution is different to that at Post-K is affirmed. It could be thought that these assertions are influenced by run-offs during heavy rains. Therefore, analyzing the two seasons separately may lend perspective to that event.

However, in the case of *E. coli*, the difference between Pre- and Post-K was more prominent compared to that of TC (Tables 3.6, 3.7). The reference site showed perceptive seasonal variation, from winter to spring in both total coliform and *E. coli* prevalence. There was however more average decrease in total coliforms while a slight but significant increase in *E. coli* was observed. In the Pre-K site there was an increase of both classes, however these changes were not statistically significant. In contrast to both Pre-K and RS, Post-K reflected appreciable

average increase in both classes ($p \leq 0.01$). *E. coli* increase was more marked of the two classes with 1000x increase as compared to a 100x increase in total coliforms. Spring *E. coli* count increased from an average $10^6$ to the $10^8$. Despite this increase, there was least variation, Ln (cfu/100 ml = 1.14); within the spring samples on this site (Table 3.7). The highest microbial variation within a season was observed at Pre-K in *E. coli*, that is Ln (cfu/100 ml) = 6.88 (Table 3.7). Spring samples had insignificant variation although *E. coli* concentrations were higher than in winter. This could be due to increased temperature in spring that encourages microbial growth in water meaning that this site continually responded to rain even in spring, the drier season. The earlier conclusion stands firm that the informal settlement contributes to an already dire situation. All these results support that the river is already polluted as it reaches Pre-K.

### 3.4.3 Analytical profiling index


**Key:**

| | | | | | |
|---|---|---|---|---|---|
| **1-ONPG** | **2-ADH** | **3-LDC** | **4-ODC** | **5-CIT** | **6-$H_2S$** |
| **7-URE** | **8-TDA** | **9-IND** | **10-VP** | **11-GEL** | **12-GLU** |
| **13-MAN** | **14-INO** | **15-SOR** | **16-RHA** | **17-SAC** | **18-MEL** |
| **19-AMY** | **20-ARA** | | | | |

**Figure 3.5: API reactions from isolates of Pre-K**




**Key:**

| | | | | | |
|---|---|---|---|---|---|
| **1-ONPG** | **2-ADH** | **3-LDC** | **4-ODC** | **5-CIT** | **6-$H_2S$** |
| **7-URE** | **8-TDA** | **9-IND** | **10-VP** | **11-GEL** | **12-GLU** |
| **13-MAN** | **14-INO** | **15-SOR** | **16-RHA** | **17-SAC** | **18-MEL** |
| **19-AMY** | **20-ARA** | | | | |

**Figure 3.6: API reactions from isolates of Post-K**

Isolates from Figure 3.5 had slight differences in their respective reactions. As a result, the isolates shown above were identified as variants of *E. coli* 1. In contrast to Figure 3.5, isolates

from Post-K (Fig. 3.6) exhibited a number of differences in their biochemical reactions. The *apiweb*$^{TM}$ identification software again identified them as variants of *E. coli*.

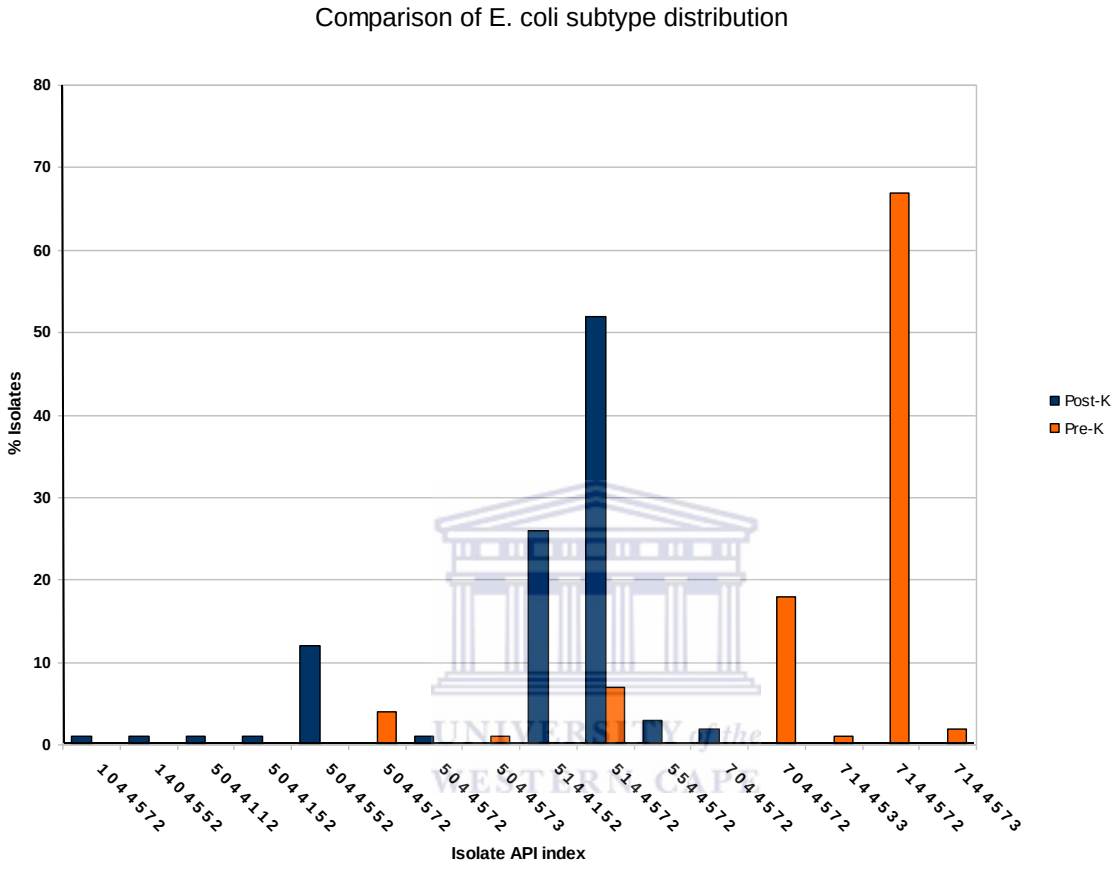


**Figure 3.7: Graph representing % distribution of *E. coli* subtypes within and across Pre- and Post-K**

Pre-K isolates were dispersed, while those of Post-K seemed clustered in two prominent groups (Fig. 3.7). After entering the 7 digit codes, which resulted from the respective API reactions, all isolates were identified as *E. coli* 1 with confidence ranging from 86.2% to 99.9% (Table 3.8), except for two isolates from Post-Khayamnandi. The first deviant isolate had a profile index of 1 404 552. It was identified as *Citrobacter freundi* with the second best identification as *E. coli* 1.

The second deviant isolate had a profile of 5 044 112 and was identified as *E. coli* 2, with second best identification as *E. coli* 1.

Having enumerated the extent of pollution across the two sites, the distribution of isolates was determined, via API 20E and haemolytic assay for similarities or dissimilarities. Isolates from Pre-K site gave 7 distinct analytical profiles, while Post-K yielded 10 (Fig. 3.5, 3.6). Pre-K API's ranged from 5 044 572 to 7 144 573 and Post-K 1044572 and 7 044 572. Pre-K profiles had two peaks, one at 7 044 572 (18%) and the other at 7 144 572 (67%) (Fig. 3.7). These API peaks stood out at the opposite end of the range to those that characterized Post-Khayamnandi isolates. The whole of Post-K isolates occupied the lower range with peaks at 5 144 152 (26%) and 5 144 572 (52%). The only common index across the two sites was at 5 144 572.

Figure 3.7 reflects stark contrast between the two sites. Post-K has strains that are not available at Pre-K. The three peaks at Post-K rivaled by the two at Pre-K invariable show distinction between the two sites. Post-K demonstrates diverse population while Pre-K almost points at a sole source. The sum total of results from both sites substantiates that the integrity of water across the two points is affected differently. These differences point out once more that Post- and Pre-K are impacted differently. These differences also suggest a probability of using the prominent strains for bacterial source tracking and/or as biomarkers for pollution.

Isolates within each site reacted differently to some of the API tests. These tests could then be used as markers for identifying different biotypes. The API isolates from Pre- and Post-K were investigated in order to establish if they could point at different sources of pollution. Sucrose and sorbitol fermentation in the API assay amongst other tests seemed to provide the answer to this question as biotyping assay.

Table 3.9 showed skew distribution of sucrose fermenters towards Pre-K. Both test sites exhibited high prevalence of sorbitol fermenters. The distributions of the biotypes across the two test sites indicate that their sources are different ($p \leq 0.05$). This together with the almost exclusive prevalence of one subtype of bacteria in at one site, carry a strong probability of point source impact on that site while diversity implies diffused source. This means that sorbitol fermentation can be used as a biotyping tool in tracing the source of faecal contamination in water.

**Table 3.8: Percentage positives for individual API tests**

| Tests | Percentage positives of Pre-k | Percentage positives of Post-k | Significant difference across sites |
|---|---|---|---|
| ONPG | 100% | 99% | None |
| ADH | 95% | 1% | Yes |
| LDC | 100% | 97% | None |
| ODC | 78% | 81% | None |
| CIT | 3% | 2% | None |
| $H_2S$ | 0% | 4% | Yes |
| MAN | 100% | 99% | None |
| INO | 2% | 0% | None |
| SOR | 100% | 73% | Yes |
| RHA | 100% | 99% | None |
| SAC | 100% | 59% | Yes |
| MEL | 99% | 97% | None |
| AMY | 4% | 0% | None |

Table 3.8 shows the distribution of positive results for each API test for Pre-K and Post-K. Differences across the two tests sites are also reflected.

**Table 3.9: Prevalence of sorbitol and sucrose fermenters across the two sites**

| Site | | Pre-Khayamnandi (n=100) | Post-Khayamnandi (n=100) |
|---|---|---|---|
| Prevalence (%) | Sorbitol fermentation | 100 | 73 |
| | Sucrose fermentation | 100 | 59 |

Table 3.9 was adapted from the API test (Table 3.8) to be interpreted as possible biotyping and toxicologic assays. There were significant differences across the two sites for both sorbitol and sucrose fermentation tests ($p \leq 0.05$ for both assays).

The prevalence of such high percentages of sorbitol fermenters (100% and 73% for Pre- and Post-K, respectively) is alarming because sorbitol fermenting *E. coli* is closely associated with haemolytic uremic syndrome (HUS). Its association with fatality talks to the toxicity of the river across the two sites and beyond (Karch *et al*, 1990; Karch and Bielaszewska, 2001). The lower prevalence downstream seems to indicate dilution of the river population by influx of non-sorbitol fermenting *E. coli*. This is true under the assumption that the river population should be evenly distributed all things being equal. Alternatively, assuming the river population is discrete one would draw a conclusion that there is a skew distribution of biotypes across the two sites. This population difference negates the hypothesis that the informal settlement is the sole contributor to the river's pollution. As is, it reinforces that it might heavily add to the river's pollution.

Since there is no specific treatment for HUS and there is no consensus on the reservoir of sorbitol fermenting *E. coli*, prevention is of essence (Werber *et al*, 2011). It is therefore important to further study in detail the prevalence of an organism with such great probability of

virulence in water. Such information would enhance the epidemiological knowledge of this pathotypes.

Table 3.9 also demonstrates a skew percentage distribution of sucrose fermenters towards Pre-K with 100% of isolates positive ($p \leq 0.05$). This significant difference in distribution indicates homogenous distribution of sucrose fermenters in this site while downstream it shows heterogeneity. Firstly, it can be deduced from this that the two sources are polluted from different sources. Secondly, with the background that omnivores have heterogeneous *E. coli* flora (Carlos *et al*, 2010), Post-K site is most likely having direct anthropogenic contamination. This indicates that the settlement augments the river's pollution with new pollution markers. Concern, however, is raised when considering that sucrose fermenting ability is plasmid mediated (Bogs and Geider, 2000). This means that downstream (Post-K) there could be intermixing of different subgroups. This could result in new and more virulent pathotypes as these plasmids are readily transmissible (Orsi *et al*, 2007; Shukla *et al*, 2004; Bogs and Geider, 2000).



Homogeneity at a site may be a strong pointer towards herbivores and avian population as potential pollutants (Carlos *et* al, 2010; Zinnah *et al*, 2007). As such, the sucrose fermentation assay can be used as a biotyping assay for surface water pollution. Hence, the *E. coli* sucrose fermentation assay can help in bacterial source tracking where pollution has already occurred. This may help direct remedial measures appropriately and save resources.

Both sorbitol and sucrose fermentation assays lean toward considering of the river across the two points as toxic with Pre-K more toxic. The distribution of the pathotypes, if it is shown to be conserved over a long time at one point, may go a long way in tracing the epidemiology due to

these fermenters and the associated pathogens via endemicity. In a similar way, the persistent sustained skew distribution of biotype could be used for bacterial source tracking. The skew prevalence of *E. coli* with pathogenic potential suggests that remedial measures must also encompass beyond the informal settlement. Neglecting this could result in misdirected efforts in curtailing the problem at hand.

### 3.4.4 Haemolysis assay

**Table 3.10: Haemolytic activity of isolates from Post-K**

| Post-K OD | % Ref β | % Ref α | Post-K OD | % Ref β | % Ref α | Post-K OD | % Ref β | % Ref α | Post-K OD | % Ref β | % Ref α |
|---|---|---|---|---|---|---|---|---|---|---|---|
| -0.0305 | -1.88 | -3.10 | 0.025 | 1.54 | 2.54 | 0.047 | 2.90 | 4.78 | 0.061 | 3.76 | 6.20 |
| -0.0273 | -1.68 | -2.78 | 0.025 | 1.54 | 2.54 | 0.047 | 2.90 | 4.78 | 0.064 | 3.95 | 6.51 |
| 0.0085 | 0.52 | 0.86 | 0.025 | 1.54 | 2.54 | 0.047 | 2.90 | 4.78 | 0.064 | 3.95 | 6.51 |
| 0.0105 | 0.65 | 1.07 | 0.0255 | 1.57 | 2.59 | 0.047 | 2.90 | 4.78 | 0.064 | 3.95 | 6.51 |
| 0.0125 | 0.77 | 1.27 | 0.026 | 1.60 | 2.64 | 0.047 | 2.90 | 4.78 | 0.064 | 3.95 | 6.51 |
| 0.013 | 0.80 | 1.32 | 0.026 | 1.60 | 2.64 | 0.047 | 2.90 | 4.78 | 0.068 | 4.19 | 6.91 |
| 0.013 | 0.80 | 1.32 | 0.026 | 1.60 | 2.64 | 0.0495 | 3.05 | 5.03 | 0.0705 | 4.35 | 7.17 |
| 0.013 | 0.80 | 1.32 | 0.0305 | 1.88 | 3.10 | 0.0525 | 3.24 | 5.34 | 0.079 | 4.87 | 8.03 |
| 0.0145 | 0.89 | 1.47 | 0.0305 | 1.88 | 3.10 | 0.053 | 3.27 | 5.39 | 0.0835 | 5.15 | 8.49 |
| 0.0165 | 1.02 | 1.68 | 0.031 | 1.91 | 3.15 | 0.053 | 3.27 | 5.39 | 0.0895 | 5.52 | 9.10 |
| 0.0195 | 1.20 | 1.98 | 0.031 | 1.91 | 3.15 | 0.053 | 3.27 | 5.39 | 0.0945 | 5.83 | 9.61 |
| 0.02 | 1.23 | 2.03 | 0.031 | 1.91 | 3.15 | 0.0545 | 3.36 | 5.54 | 0.0945 | 5.83 | 9.61 |
| 0.02 | 1.23 | 2.03 | 0.0325 | 2.00 | 3.30 | 0.0545 | 3.36 | 5.54 | 0.1 | 6.17 | 10.17 |
| 0.02 | 1.23 | 2.03 | 0.0345 | 2.13 | 3.51 | 0.055 | 3.39 | 5.59 | 0.102 | 6.29 | 10.37 |
| 0.02 | 1.23 | 2.03 | 0.036 | 2.22 | 3.66 | 0.055 | 3.39 | 5.59 | 0.1025 | 6.32 | 10.42 |
| 0.024 | 1.48 | 2.44 | 0.0365 | 2.25 | 3.71 | 0.0565 | 3.49 | 5.74 | 0.3595 | 22.18 | 36.55 |
| 0.0245 | 1.51 | 2.49 | 0.037 | 2.28 | 3.76 | 0.058 | 3.58 | 5.90 | 0.963 | 59.41 | 97.92 |
| 0.0245 | 1.51 | 2.49 | 0.037 | 2.28 | 3.76 | 0.0585 | 3.61 | 5.95 | 0.9745 | 60.12 | 99.08 |
| 0.0245 | 1.51 | 2.49 | 0.037 | 2.28 | 3.76 | 0.0595 | 3.67 | 6.05 | 0.979 | 60.39 | 99.54 |
| 0.0245 | 1.51 | 2.49 | 0.037 | 2.28 | 3.76 | 0.0595 | 3.67 | 6.05 | 0.988 | 60.95 | 100.46 |
| 0.025 | 1.54 | 2.54 | 0.0405 | 2.50 | 4.12 | 0.06 | 3.70 | 6.10 | 0.9935 | 61.29 | 101.02 |
| 0.025 | 1.54 | 2.54 | 0.0425 | 2.62 | 4.32 | 0.06 | 3.70 | 6.10 | 1.003 | 61.88 | 101.98 |
| 0.025 | 1.54 | 2.54 | 0.0425 | 2.62 | 4.32 | 0.06 | 3.70 | 6.10 | 1.0515 | 64.87 | 106.91 |
| 0.025 | 1.54 | 2.54 | 0.0425 | 2.62 | 4.32 | 0.06 | 3.70 | 6.10 | 1.0595 | 65.36 | 107.73 |
| 0.025 | 1.54 | 2.54 | 0.0465 | 2.87 | 4.73 | 0.061 | 3.76 | 6.20 | 1.1135 | 68.69 | 113.22 |

Table 3.10 shows 100 isolates in sets of 25. Columns 1, 2 and 3 are corrected OD readings of isolates' haemolytic activities against the media blank, followed by correction against positive controls, i.e. β and α-haemolytic streptococci, respectively. This represents the first set of 25 isolates, followed by the next 3 sets of 25 isolates, thus making 100 in total. Percentage Ref β and α = isolate activity / average activity of β- or α-streptococci (respectively) X100.

Haemolytic activities were measured at 405 nm using an ELISA microtiter plate reader. All Table 3.10 and Table 3.11 values below were initially calculated from the actual OD readings by adjusting for the average media blank reading, thus explaining the negative results of the first two readings. The negative is due to dilution of the haemolytic preparation by low or unsuccessful growth of isolates in Dulbecco salts. These readings make up the first column of each of the four sets in both Tables 3.10 and 3.11. The subsequent two columns were calculated from the haemolytic activities of the respective positive controls by dividing each value in the first column by the average haemolytic activity reading of the controls. These were then multiplied by 100 to get percentage haemolytic activity with reference to each of the two controls.

**Table 3.11: Haemolytic activity of isolates from Pre-K**

| Pre-K OD | % Ref β | % Ref α | Pre-K OD | % Ref β | % Ref α | Pre-K OD | % Ref β | % Ref α | Pre-K OD | % Ref β | % Ref α |
|---|---|---|---|---|---|---|---|---|---|---|---|
| -0.003 | -0.22 | -0.30 | 0.047 | 3.47 | 4.75 | 0.064 | 4.72 | 6.47 | 0.969 | 71.47 | 97.98 |
| 0.01 | 0.74 | 1.01 | 0.047 | 3.47 | 4.75 | 0.067 | 4.94 | 6.77 | 0.969 | 71.47 | 97.98 |
| 0.013 | 0.96 | 1.31 | 0.047 | 3.47 | 4.75 | 0.068 | 5.02 | 6.88 | 0.97 | 71.55 | 98.08 |
| 0.013 | 0.96 | 1.31 | 0.047 | 3.47 | 4.75 | 0.068 | 5.02 | 6.88 | 0.971 | 71.62 | 98.18 |
| 0.013 | 0.96 | 1.31 | 0.047 | 3.47 | 4.75 | 0.07 | 5.16 | 7.08 | 0.974 | 71.84 | 98.48 |
| 0.014 | 1.03 | 1.42 | 0.053 | 3.91 | 5.36 | 0.07 | 5.16 | 7.08 | 0.975 | 71.92 | 98.58 |
| 0.015 | 1.11 | 1.52 | 0.053 | 3.91 | 5.36 | 0.07 | 5.16 | 7.08 | 0.975 | 71.92 | 98.58 |
| 0.018 | 1.33 | 1.82 | 0.053 | 3.91 | 5.36 | 0.08 | 5.90 | 8.09 | 0.975 | 71.92 | 98.58 |
| 0.02 | 1.48 | 2.02 | 0.053 | 3.91 | 5.36 | 0.083 | 6.12 | 8.39 | 0.976 | 71.99 | 98.69 |
| 0.023 | 1.70 | 2.33 | 0.054 | 3.98 | 5.46 | 0.086 | 6.34 | 8.70 | 0.977 | 72.06 | 98.79 |
| 0.025 | 1.84 | 2.53 | 0.054 | 3.98 | 5.46 | 0.087 | 6.42 | 8.80 | 0.98 | 72.28 | 99.09 |
| 0.025 | 1.84 | 2.53 | 0.055 | 4.06 | 5.56 | 0.087 | 6.42 | 8.80 | 0.981 | 72.36 | 99.19 |
| 0.025 | 1.84 | 2.53 | 0.055 | 4.06 | 5.56 | 0.096 | 7.08 | 9.71 | 0.981 | 72.36 | 99.19 |
| 0.025 | 1.84 | 2.53 | 0.058 | 4.28 | 5.86 | 0.097 | 7.15 | 9.81 | 0.982 | 72.43 | 99.29 |
| 0.026 | 1.92 | 2.63 | 0.058 | 4.28 | 5.86 | 0.098 | 7.23 | 9.91 | 0.983 | 72.51 | 99.39 |
| 0.026 | 1.92 | 2.63 | 0.058 | 4.28 | 5.86 | 0.101 | 7.45 | 10.21 | 0.986 | 72.73 | 99.70 |
| 0.026 | 1.92 | 2.63 | 0.058 | 4.28 | 5.86 | 0.108 | 7.97 | 10.92 | 0.988 | 72.87 | 99.90 |
| 0.031 | 2.29 | 3.13 | 0.058 | 4.28 | 5.86 | 0.124 | 9.15 | 12.54 | 0.988 | 72.87 | 99.90 |
| 0.035 | 2.58 | 3.54 | 0.058 | 4.28 | 5.86 | 0.13 | 9.59 | 13.14 | 0.989 | 72.95 | 100.00 |
| 0.037 | 2.73 | 3.74 | 0.059 | 4.35 | 5.97 | 0.13 | 9.59 | 13.14 | 0.989 | 72.95 | 100.00 |
| 0.037 | 2.73 | 3.74 | 0.06 | 4.43 | 6.07 | 0.137 | 10.11 | 13.85 | 0.992 | 73.17 | 100.30 |
| 0.037 | 2.73 | 3.74 | 0.06 | 4.43 | 6.07 | 0.496 | 36.58 | 50.15 | 0.994 | 73.32 | 100.51 |
| 0.043 | 3.17 | 4.35 | 0.06 | 4.43 | 6.07 | 0.965 | 71.18 | 97.57 | 0.996 | 73.46 | 100.71 |
| 0.046 | 3.39 | 4.65 | 0.062 | 4.57 | 6.27 | 0.968 | 71.40 | 97.88 | 0.998 | 73.61 | 100.91 |
| 0.046 | 3.39 | 4.65 | 0.064 | 4.72 | 6.47 | 0.968 | 71.40 | 97.88 | 1.012 | 74.65 | 102.33 |

Table 3.11 (n=100) is similar to Table 3.10, but represents isolates from Pre-K. The resultant percentage activities were arranged in ascending order so that the 50% cut-off mark could easily be seen. The values above and including 50% were considered positive for haemolysis and those below 50% were considered negative. The two haemolytic patterns represented by β- and α-

haemolysis are complete and partial haemolysis due to *Streptococcus pyogenes* and S*treptococcus pneumoniae*, respectively.

**Table 3.12: Actual number of haemolytic vs. non-haemolytic isolates**

| | Number of haemolytic species | | | |
|---|---|---|---|---|
| | Post-K (n=100) | | Pre-K (n=100) | |
| | Ref β | Ref α | Ref β | Ref α |
| Non-haemolytic isolates | 91 | 91 | 72 | 71 |
| Haemolytic isolates | 9 | 9 | 28 | 29 |

Ref β and Ref α indicate that the percentages given were calculated with reference to average activity on the haemolytic assay of β- and α-haemolytic streptococci, respectively. Activity below 50% in each category was taken as negative (Tables 3.10, 3.11). Nine (9) of 100 isolates from Post-K showed ≥ 50% activity with reference to both β- and α-streptococci (Table 3.12).

**Table 3.13: Average percentage haemolysis vs. non-haemolysis OD**

| | Mean % haemolysis | | | |
|---|---|---|---|---|
| | Post-K (n=100) | | Pre-K (n=100) | |
| | Ref β | Ref α | Ref β | Ref α |
| Non-haemolysis (Mean±STDEV) | 2.81±2.54 | 12.48±8.55 | 4.53±4.40 | 5.59±3.06 |
| Haemolysis (Mean±STDEV) | 62.55±2.91 | 100.00±4.79 | 72.37±0.80 | 97.51±9.02 |

Table 3.13 shows the average percentage OD readings of isolates with reference to β and α-haemolytic streptococci. These readings were grouped according to whether the activity was considered haemolytic or non-haemolytic as referred to Table 3.12. The average haemolytic activity was 62.55±2.91 % and 100.00±4.79 %, respectively (Table 3.13). Pre-K, on the other hand, had 28 and 29 isolates above and including 50% activity for β- and α-streptococci, respectively. These isolates had average percentage activities of 72.37±0.80 and 97.51±9.02, respectively (Table 3.13).

Average non-haemolytic activities for Post-K were 2.81±2.54% and 12.48±8.55% with reference to β- and α-streptococci, respectively. Those from Pre-K were 4.53±4.40 and 5.59±3.06.

The means of % haemolysis were 8.18 and 23.35 for Post- and Pre-K with reference to β-haemolysis ($p \leq 0.05$) and with reference to α-haemolysis, they were 13.49 and 32.25, Post- and Pre-K, respectively, with $p \leq 0.05$ (Table 3.13).

The haemolytic assay was then visited to investigate if there are any discernible patterns supporting our hypothesis. The haemolytic patterns of the river agreed with the other patterns in reinforcing the assertion that pollution already exists before the settlement. These patterns also suggest that the sources of pollution at these two sites are different. This is demonstrated by the relative means across the two test sites for both β- and α-haemolysis in Table 3.13 ($p \leq 0.05$).

There is a higher prevalence of "haemolytic *E. coli"* before the settlement (Table 3.12). However, after the settlement, the average haemolytic activity with reference to the α-streptococci is 103.10±4.79% compared to 97.51±9.02% before the settlement (Table 3.13). This difference in average haemolytic activity means that the extent of haemolysis due to subspecies found after the settlement is more than before it. This brings another point of concern regarding toxicity after Khayamnandi. It indicates that these waters could be more toxic. It should, however, not be missed that even before the river passed the settlement it was already showing high toxicity in accordance with *E. coli* haemolysis as shown by the fact that Pre-K had more than double the number of haemolytic isolates (Table 3.12). The prevalence of Pre-K haemolytic activity indicates that it is necessary to broaden remedial measures beyond Khayamnandi in order to improve the river's integrity.

The variation in haemolytic patterns across the two sites could serve as bacterial source tracking. These patterns seemed to point out a possibility of difference in the origin of the microbes. This difference can help one to rule out contamination beyond a certain area and therefore focus remedial measures to a limited area.

### 3.5 Conclusion

In order to interpret microbial test results, the standards for drinking water have to be taken into account. USEPA (2000) established two categories, namely, the Primary Standards and the Secondary Standards. The bases for Primary Standards are health considerations. These standards aim to protect the public from three classes of pollutants: pathogens, radioactive elements and toxic chemicals. Bacterial pollution falls into the category of pathogens. As such, there is EPA Maximum Contamination Levels (MCL).



Pathogens are discrete and not in solution. They often form flocs or adhere to suspended solids in water; as a result, the chance of acquiring an infective dose cannot be predicted from their average concentration in water (Boyer, 2008). The likelihood of a successful challenge by a pathogen, resulting in infection, depends upon the invasiveness and virulence of the pathogen, as well as upon the immunity of the individual (Clark *et al*, 2003). If infection is established, pathogens multiply in their host. Other pathogens can multiply in food or beverages, in this manner increasing their pathogenicity (CDC, 2011). Furthermore, unlike many environmental pollutants, the dose response of pathogens is not cumulative (WHO, 2003). Because of these properties, there is no acceptable upper limit for pathogens (WHO, 2002; USEPA, 2000). Thus, water intended for consumption or for personal hygiene should contain no human pathogens. Pathogen-free water is possible by selection of high-quality uncontaminated sources of water and

maintaining the integrity of such sources. This begins by being aware of the sources of pollution and the mechanisms thereof.

Plankenbrug River downstream Khayamnandi has had an ongoing problem of high microbial pollution to the magnitude greater than $10^5$ microorganisms per 100 ml of water (Rossouw, 2004). The informal settlement has had high incidences of drinking untreated water, limited water treatment and full contact recreation with polluted water (Rossouw, 2004). Progress has been reported with regard to pollution due to Khayamnandi after the local municipality had spent several thousands of rands on waste control (Department of Housing, 2004a). The progress alluded to above, included construction of diversion channels for storm water to direct storm water into the sewerage system and prevent it from reaching the river (Department of Housing, 2004a). Despite these attempts, faecal pollution still prevails in the river as demonstrated in the current study.



The microbial data suggests that in one way or another, faecal matter still finds its way into the river system. The relationship between storm water and high microbial incidence affirms this. Sample 1 in the winter sampling series (Table 3.6) was at storm flow after the first heavy winter rains whereas samples 3 and 4 in the same series were at base flow. Storm water readings show that *E. coli* concentration peaked at storm flow and then seemed to return to relatively constant levels as the river water volume abates. Thus it may be supposed that wash-off from the settlement filtered into the river.

It could be speculated that among factors influencing these trends are human behaviour and vegetation around the river together with water velocity and temperature (Craig *et al*, 2002). Water velocity may impact on resident microbial flora in a number of ways. Sudden increase in

water flow may cause deep flora to come to the surface, thus increasing microbial density in the flow (Craig *et al*, 2002). It can also dislodge microbial populations resident in the river vegetation to the same effect. Rapid flow on the other hand can cause a decrease in the flow population by reducing the time it spends at one point (Boyer, 2008). Water flow drops drastically at base flow. This enables microbes to multiply in one place and therefore increase the spring count. Rain drop on open faecal matter can serve as both a source and a vehicle for transportation into the water bodies farther away (Boyer, 2008).

Vegetation can provide a substrate for the attachment of microbiota. This could be a conditioning layer that could develop into a biofilm. Organic matter can act as a buffer that neutralizes excessive charge, thus enhancing cells to aggregate. Microbial cells attach to surface and begin to form a glycocalyx. This in turn encourages accumulation of nutrients and as a result, growth of microbial flora. Incidentally such a microcosm makes disinfection difficult as the polysaccharide network protects the organisms. Temperature is another factor that can determine microbial population stability. A number of strains tend to decrease more rapidly around 18˚C than at lower temperature (Hara-Kudo *et al*, 2000). These authors observed that this decay is dependent on the initial population. Over 48 days at 18˚C, they found that the population was only reduced by less than 1 order of magnitude when the initial population was at the level of $10^7$ cfu/100 ml. In the same study, it was observed that populations of $10^5$ cfu/100 ml were reduced to $10^2$ cfu/100 ml under the same conditions (Hara-Kudo *et al*, 2000). Therefore, the higher winter population at Post-K could be the source of sustained higher population in spring. Road construction and tarring facilitates the water movement during heavy rains from the settlements into river ways. Thus pollutants tend to reach rivers more readily. Beside such remote activities, the abuse of river banks is one of the major concerns at Post-K. Figures 3.8, 3.9 and 3.10 show

clearly the extent of pollution due to direct human activity. Throughout water collection there were fresh faecal deposits under the bridge. The number of individual deposits seemed to correlate with occurrence of rain. In contrast, no human faecal matter could be detected at the reference site at any given time.




**Figure 3.8: Photo of Post-Khayamnandi site at base flow after a heavy rain**


**Figure 3.9: Conditions under the bridge at Pre-Khayamnandi at base flow after a heavy rain showing faecal deposits and vegetation in the river way.**

**Figure 3.10: Photo of the Eerste River at Swaaibrug (Reference site) showing spring water flow. During heavy rain in winter the water covered the grass and stony island to the right.**

Individual tests within assays as well as complete assays were used to elucidate important information from this study. The choice of individual tests within assays to emphasize was in most cases prompted by pronounced differences across the test sites. Some of the tests were grouped together as they may be informing each other or simply because they best form a comprehensive picture together.

Ideal assessment of water integrity with regard to faecal contamination starts and ends with determining the presence or absence of faecal indicators (WHO, 2002). This is functional in assessing whether the water is potable or not (DWAF, 2000; WHO, 2002). USEPA's recommendations are 1 cfu/ml of water for drinking (USEPA, 2000). There are different limits for other water uses (DWAF, 2000). The point is that enumeration of the extent of contamination would be futile and/or expensive without having first ascertained whether the contaminant is present or not. In this study a sequence is followed. This study starts by ascertaining the presence of faecal contamination after having assessed the field parameters. The discussion of the presence of faecal contamination is then followed by quantitative assessment of the extent of the problem and discussion of characterization of a faecal indicator (*E. coli*). Characterization of *E. coli* will thereafter enable determination or suitability of use of the indicator organism as a biomarker and/or a bacterial source-tracking tool. At each step an effort is made to compare and contrast the two sampling sites, hence assessing the impact of the informal settlement on the nearby body of water.



In conclusion, Khayamnandi impacts adversely on Plankenbrug River. However, focusing remedial measures only in the settlement may not alleviate faecal pollution in the river system. The problem already exists before the river passes Khayamnandi. Different toxicological profiles and microbial distribution indicate that those sources before Khayamnandi are different from

those within the settlement. In conclusion assessment of waters associated with informal settlements should include points upstream of the settlements to produce a more comprehensive picture.

## Chapter 4: Recommendations and further studies

The aim of this study was to assess the impact of Khayamnandi settlement on Plankenbrug River by comparing faecal contamination and potential toxicity upstream and downstream from the settlement. Microbial culture on chromogenic media, analytical profiling index (API) and haemolysis assays demonstrated that the Plankenbrug River already has a high faecal load upstream from Khayamnandi. Khayamnandi added onto this heavy faecal load. These assays also demonstrated prevalence of different biotypes across the two test sites. The distribution of *E. coli* biotypes downstream from Khayamnandi was narrower than upstream. To improve the water quality of the Plankenbrug River holistically, measures should be taken to trace and remediate sources of contamination from Khayamnandi and also upstream from Khayamnandi along the river.



### 4.1 Integrating microbial data generated into National Microbial Monitoring Programme (NMMP framework) of South Africa

Microbial studies on surface water, in line with NMMP of South Africa, should be done before and after the suspected source of faecal contamination. The NMMP extrapolates the inherent potential risk in the assessed water by drawing from the extent of microbial contamination and linking it to intended water use. The NMMP links *E. coli* concentrations to the adverse health risk index (HRI) associated with different water uses. Differences in HRI upstream and downstream to the settlement could help direct prioritization of remediation resources for the relevant water use.

### 4.2 Informal settlement development

Changing high population density of the informal settlement into low density housing is desired for settlements on river banks. But this entails removal of some of the households, which is not easy. The alternative is converting informal settlements on environmentally sensitive areas onto high density housing with appropriate road and waste removal infrastructure in order to reduce impact on the environment.

### 4.3 Education to communities

First and foremost, water quality is not entirely the responsibility of the authorities. It is also important to involve the resource users. In order to maintain the integrity of potable water, end-users should be educated with respect to the fragility of water. This awareness is even more important to local communities around surface waters. Ignorance of this could exacerbate water pollution or could increase the health risks due to community exposure. Thus, the water end-users should be incorporated into maintaining high water quality and made aware of their importance. This could be achieved by incorporating water integrity into the school curriculum. Involving communities in preventing water pollution may be a powerful tool in water resource management particularly in developing areas with insufficient infrastructure (Steynberg, *et al*, 1995).



### 4.4 Recommendation to small municipalities

Although 3 – 5% of *E. coli* are ß-glucuronidase (GUD) negative and some *Salmonella, Shigella* and *Yersinia spp.* are GUD positive, GUD tests are used to monitor water and food. Chromocult® coliform agar is one of the GUD tests. Total coliform and *E. coli* recoveries on this

media are significantly higher than some other standard methods. Enzymatic activity is a powerful tool in applied research fields. It enables rapid detection and identification of microorganisms. This advantage could prove useful to local municipalities in monitoring faecal contamination.

### 4.5 Further research

River systems such as the Plankenbrug River that show persistently high faecal pollution should be explored thoroughly using geographical data models such as ArcGIS (Diefenbach, 2004). ArcGIS could help in mapping out drainage systems, hydro-network systems to microbial pollution. Monitoring hydro edges and junctions could help redefine apparent diffused pollution sources to multiple point sources. Comparative studies should thereafter be undertaken followed by prioritization process within that river system. Data acquired through this process could further aid in speedily arresting pollution especially when coupled with full characterization of indicator microbes per site. However, high faecal indicator bacteria may also be due to microbial regrowth. In our study, physicochemical parameters, turbidity and presence of nutrients appear to be liked with microbial regrowth especially in the absence of apparent run-offs. Therefore regrowth has to be taken into consideration when analyzing such water.

Furthermore, full characterization of *E. coli* isolates could be beneficial as it could give a distribution profile that can help predict and trace possible *E. coli* related disease outbreaks. The need for characterization is important because of the potential adverse effects due to environmental microbial exposure is not incremental like other pollutants. Development of disease in humans depends on the susceptibility of the host, for example, age or immune status as well as to the virulence of the pathogen. Thus, thorough knowledge of regular prevailing

pathotypes in local surface water could prove useful in tracing the sources of epidemics (Do *et al*, 2006).

## References


Abu Amr, S.S and Yassin, M.M. 2008. Microbial contamination of the drinking water distribution system and its impact on human health in Khan Yunis Governorate, Gaza Strip: Seven years of monitoring (2000–2006). *Public Health*, Vol. 122(11): 1275–1283.

Adingra, A.A., Kouadio, A.N., Ble, M.C and Kouassi, A.M. 2012. Bacteriological analysis of surface water collected from the Grand-Lahou lagoon, Côte d'ivoire. *African Journal of Microbiology Research*, Vol. 6(13): 3097-3105.

Aldridge, K.E and Hodges, H.L. 1981. Correlation studies of Entero-Set 20, API 20E, and conventional media systems for *Enterobacteriaceae*. *Identification Journal of Clinical Microbiology*, Vol. 13(1): 120-125.

Aldridge, K.E., Gardner, B.B., Clark, S.J and Matsen, J.M. 1978. Comparison of Micro-ID, API 20E, and Conventional Media Systems in Identification of *Enterobacteriaceae*. *Journal of Clinical Microbiology*, Vol. 7(6): 507-513.

Alonso, J.L., Amoros, I., Chong, S and Garelick, H. 1996. Quantitative determination of *Escherichia coli* in water using CHROMagar *E. coli*. *Journal of Microbiological Methods*, Vol. 25(3): 309-315.

American Public Health Association (APHA). 1992. Compendium of Methods for the Microbiological Examination of Foods, 3rd ed. Washington, DC.

Ammon, A., Petersen, L.R and Karch, H. 1999. A Large outbreak of hemolytic uremic syndrome caused by an unusual sorbitol-fermenting strain of *Escherichia coli* O157:H2. *The Journal of Infectious Diseases*, Vol. 179: 1274–1277

An, Y.J, Kambell, D.H and Breidenbach, G.P. 2002. *Escherichia coli* and total coliform in water sediments at Lake Marinas. *Environmental Pollution*, Vol. 120(3): 771-778.

Anderson, K.A and Davidson, P.M. 1997. Drinking Water and Recreational Water Quality: Microbiological Criteria, University of Idaho. *Available at* http://info.ag.uidaho.edu

API 20E Identification system for *Enterobacteriaceae* and other Gram-negative rods. Instruction Manual version E (#2012).

Aronsson, K and Rönner, U. 2001. Influence of pH, water activity and temperature on the inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* by pulsed electric fields. *Innovative Food Science and Emerging Technologies*, Vol. 2: 105-112.

Ashbolt, J.N. 2004. Microbial contamination of drinking water and disease outcomes in developing world. *Toxicology,* Vol. 198: 229-238.

Ateba, C.N and Bezuidenhout, C.C. 2008. Characterization of *Escherichia coli* O157 strains from humans, cattle and pigs in the North-West Province, South Africa. *International Journal of Food Microbiology*, Vol. 128: 181-188.

Azizullah, A., Khattak, M.N.K., Richter, P and Häder, D.P. 2011. Water pollution in Pakistan and its impact on public health: A review. *Environment International,* Vol. 37: 479- 497.

Babah, I.H., Deida, M.F., Blake, G and Froelich, D. 2012. Fresh water distribution problematic in Nouakchott. *Procedia Engineering*, Vol. 33: 321-329.



Baldy-Chudzik, K. and Stosik, M., 2007 Prevalence of antibiotic resistance profile in relation to phylogenetic background among commensal *Escherichia coli* derived from various mammals.*Polish Journal of Microbiology*, Vol. 56(3):175-83.

Baudart, J., Robyns, A., Peuchet, S., Drocourt, J.L and Lebaron, P. 2011.Sensitive counting of viable *Enterobacteriaceae* in seawaters and relationship with fecal indicators. *Journal of Microbiological Methods*, Vol. 84(3): 482–485.

Becker, B., Weiss, C and Holzapfel, H.W. 2009. An evaluation of the use of three phenotypic test-systems for biochemical identification of *Enterobacteriaceae* and *Pseudomonadaceae*, *Food Control*, Vol. 20: 815-821.

Bengraïne, K and Marhaba, T.F. 2003. Using principal component analysis to monitor spatial and temporal changes in water quality, *Journal of Hazardous Materials,* Vol. 100 (1): 179–195.

Bielaszewska, M., Schmidt, H., Karmali, M.A., Khakhria, R., Janda, J., Blahova, K and Karch, H. 1998. Isolation and characterization of sorbitol fermenting Shiga toxin-producing *Escherichia coli* O157H2 strains in the Czech Republic. *Journal of Clinical Microbiology*, Vol. 36: 2135-2137.

Blount, Z.D., Borland, C.Z and Lenski, R.E. 2008. Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. *Proceedings of the National Academy of Sciences*, Vol. 105(23): 7899-7906.

Bogs, J and Geider, K. 2000. Molecular analysis of sucrose metabolism of *Erwinia amylovor*a and influence on bacterial virulence. *Journal of Bacteriology*, Vol. 182(19): 5351-5358.

Borst, M and Selvakumar, A. 2003. Particle-associated microorganisms in stormwater runoff. *Water Research*, Vol. 37: 215-23.

Bouvet, M.M.O., Lenormand, P and Grimont, A.D.P. 1989. Taxonomic diversity of the D-glucose oxidation pathway in the *Enterobacteriaceae*. *International Journal of Systematic Bacteriology*, Vol. 39(1): 61-67.



Bouvet, O.M.M., Pernoud, S., and Grimont, P.A.D. 1999. Temperature-dependent fermentation of D-sorbitol in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, Vol. 65(9): 4245-4247.

Boyer, D.G. 2008. Faecal coliform dispersal by rain splash on slopes. *Agricultural and Forest Meteorology*, Vol.148 (8-9): 1395-1400.

Brion, G.M and Lingireddy, S. 2003. Artificial neural network modeling: a summary of successful applications relative to microbial water quality. *Water Science Technology*, Vol. 47: 235-240.

Brunder, W., Salam Khan, A., Hacker, J and Karch, H. 2001. A novel fimbrial gene cluster encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli O*157: H$^-$. *Infection and Immunity*, Vol. 69: 4447-4457.

Burkhardt III, W., Calci, K.R., Watkins, W.D., Rippey, S.R and Chirtel, S.J. 2007. Inactivation of indicator microorganisms in estuarine waters. *Water Research*, Vol. 34(8): 2207-2214

Carlos, C., Pires, M.M., Stoppe, N.C., Hachich, E.M., Sato, M.I.Z, Gomes, T.A.T, Amaral, L.A.A and Ottoboni, L.M.M. 2010. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of faecal contamination. *BMC Microbiology*, Vol. 10(161).

Centers for Disease Control and Prevention (CDC). 2011. Division of Bacterial and Mycotic Diseases.

Clark, A., Turner, T and Padma, D. 2003. Health hazards due to pollution of waters along the coast of Visakhapatnam, east coast of India. *Ecotoxicology and Environmental Safety*, Vol. 56: 390-397.

Clarke, S.C., Haigh R.D., Freestone, P.P and Williams, P.H. 2003. Virulence of enteropathogenic *Escherichia coli*: A global pathogen. *Clinical Microbiology Reviews*, Vol. 16(3): 365-378.



Collins, C.M and Falkow, S. 1990. Genetic analysis of *Escherichia coli* urease genes: evidence for two distinct loci. *Journal of Bacteriology*, Vol. 172(12): 7138-7144.

Craig, D.L., Fallowfield, H.J and Cromar, N.J. 2002. Enumeration of faecal coliforms from recreational coastal sites: evaluation of techniques for the separation of bacteria from sediments. *Applied Microbiology*, Vol. 93: 557-565.

Craig, D.L., Fallowfield, H.J and Cromar, N.J. 2004. Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements. *Applied Microbiology*, Vol. 96: 922-930.

Dallas, H.F and Day, J.A. 2004. *The Effect of Water Quality Variables on Aquatic Ecosystems: A Review*. WRC Report No. TT224/04.

Darkwa, I. 2006. *Post-occupancy evaluation of state-subsidized housing units in Khayamnandi, Stellenbosch.* Unpublished Magister Scientiae, University of Stellenbosch, Cape Town.

Daskin, J.H., Calci, K.R., William Burkhardt III and Carmichael, R.H. 2008. Use of N stable isotope and microbial analyses to define wastewater influence in Mobile Bay, Al. *Marine Pollution Bulletin*, Vol. 56: 860-868.

De La Cruz, F., Müller, D., Ortiz, J.M and Gober, W. 1980. Haemolysis determinant common to *Escherichia coli*: Haemolytic plasmids of different incompatible groups. *Journal of Bacteriology*, Vol. 143(2): 825-833.

De Mora, K., Joshi, N., Balint, B.L., Ward, F.B., Elfick, A and French, C.E. 2011. A pH-based biosensor for detection of arsenic in drinking water. *Analytical and Bioanalytical Chemistry*, Vol. 400: 1031-1039.

Department of Housing. 2004a. Western Cape Housing.

Department of Housing. 2004b. Programme Performance. General non-financial performance, Approved subsidies.

Department of Water Affairs and Forestry (DWAF). 1993. *Western Cape System Analysis. Water Quality*, Volume 1: General, Prepared by AJ Bath of Niham Shand Inc. in association with BKS inc. as part of the Western Cape System Analysis. DWAF Report No. P G000/00/2891. Cape Town.

Department of Water Affairs and Forestry. 1996a. South African water quality guidelines. Volume 1: Domestic use. DWAF, Pretoria.

Department of Water Affairs and Forestry. 1996b. South African water quality guidelines. Volume 7: Aquatic ecosystems. DWAF, Pretoria.

Department of Water Affairs and Forestry. 1996c. South African water quality guidelines. Volume 5: Agricultural use: Livestock watering. DWAF, Pretoria.

Department of Water Affairs and Forestry. 2001. Managing the water quality effects of settlements: The economic impacts of pollution in two towns.

Department of Water Affairs and Forestry. 2002. *National Microbial Monitoring Programme for Surface Water* (NMMP). *Implementation Manual.* Pretoria.

Department Of Water Affairs and Forestry. 2006. Resource Directed Management of Water Quality: Management Instruments. Volume 4.2. Guideline for Determining Resource Water Quality Objectives (RWQOs), Allocatable Water Quality and the Stress of the Water Resource. (2nd eds). Water Resource Planning Systems Series, Sub-Series No. WQP 1.7.2. Department of Water Affairs and Forestry, Pretoria, South Africa.

Department of Water Affairs and Forestry/National Microbial Monitoring Programme. 2004. Implementation in the Berg River water management area.

Diefenbach, A. 2004. Integrated GIS and microbial water quality modelling through use of network processing, Department of Water and Forestry/Resource Quality Services Resource Quality Information.

Do, T.N., Cu, P.H., Nguyen, H.X., Au, T.X, Vu, Q.N., Driesen, S.J., Townsend, K.M, Chin, J.J and Trott, D.J. 2006. Pathotypes and serogroups of enterotoxigenic *Escherichia coli* isolated from pre-weaning pigs in North Vietnam. *Journal of Medical Microbiology,* Vol. 55: 93-99.

Donnenberg, M.S. 2005. *Enterobacteriaceae*, In: Mandell G.L., Bennett, J.E., Dolin, R. Mandell, D., and Bannett's Principles and Practice of Infectious Diseases, Ed 6. Philadelphia: Elsevier Churchill Livingstone, Vol. 2: 2567-2586.

Donovan, E.P., Staskal, D.F., Unice, K.M., Roberts, J.D., Haws, L.C, Finley, B.L and Harris, M.A. 2008. Risk of gastrointestinal disease associated with exposure to pathogens in the sediments of the Lower Passaic River. *Applied and Environmental Microbiology,* Vol. 74: 1004-1018

Dorch, M.M., Cameron, A.S and Robinson, B.S. 1983. The epidemiology and control of primary amoebic meningoencephalitis with particular reference to South Australia. *Transaction of the Royal Society of Tropical Medicine and Hygiene,* Vol. 77: 372-377.

Eberspacher, B., Hugo, H and Bhakdi, S. 1998. Quantitative study of the binding and haemolytic efficiency of *Escherichia coli* haemolysin. *Infection and Immunity,* Vol. 57(3): 983-988.

Ehrenberg, J.P and Ault, S.K. 2005. Debate, neglected diseases of the neglected populations: Thinking to reshape the determinants of health in Latin America and the Caribbean. *Biomedcentral Public Health*, Vol. 5.

Erhard, A. 2000. Informelle Wirtchaft und informelle Siedlung- globale Phänomene und das Beispiel Südafrika. *GW-Unterricht*, Vol. 79: 29-41.

Eschbach, E., Scharsack, J.P., John, U and Medlin, K.L. 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. *Journal of Applied Toxicology*, Vol. 21: 513-519.

Farré, M., Brix, R and Barceló, D. 2005. Screening water for pollutants using biological techniques under European Union funding during the last 10 years. *Trends in Analytical Chemistry*, Vol. 24(6): 532-545.

Fatoki, O.S, Muyima, N.Y.O and Lujiza, N. 2001. Situation analysis of water quality in the Umtata River catchment. *Journal of Water South Africa*, Vol. 27(4): 467-474.

Field, K.G and Samadpour, M. 2007. Faecal source tracking, the indicator paradigm, and managing water quality. *Water Research*, Vol. 41: 3517-3538.

Finney, M., Smullen, J., Foster, H.A., Porokx, X and Storey, D.M. 2003. Evaluation of Chromocult coliform agar for detection and numeration of *Enterobacteriaceae* from faecal samples from health subjects. *Journal of Microbiological Methods*, Vol. 54: 353-358.

Fitzhugh, T.W and Richter, D.B. 2004. Quenching urban thirst: Growing cities and their impacts on freshwater ecosystems. *Bioscience*, Vol. 54(8): 741-754.

Fong, T.T and Lipp, E.K. 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews*, Vol. 69(2): 357-371.

Fong, T.T, Griffin, D.W and Lipp, E.K. 2005. Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. *Applied and Environmental Microbiology*, Vol. 71(4): 2070-2078.

Fratamico, P.M., Buchanan, R.L and Cooke, H.P. 1993. Virulence of an *Escherichia coli* O157:H7 sorbitol-positive mutant. *Applied and Environmental Microbiology*, Vol. 59: 4245-4252.

Frick, E.A and Gregory, M.B. 2001. Microbial indicators in streams in the Chattahoochee River national recreation area watershed, Metropolitan Atlanta, Georgia, in Hatcher, K.J. (ed.), Proceedings of the 2001 Georgia Water Resource Conference, Athens.

Friedrich, A.W., Nierhoff, K.V., Bielaszewska, M., Mellmann, A and Karch, H. 2004. Phylogeny, clinical associations, and diagnostic utility of the pilin subunit gene (*sfpA*) of sorbitol-fermenting, enterohemorrhagic *Escherichia coli* O157:H. *Journal of Clinical Microbiology*, Vol. 42(10): 4697-4701.

Friedrich, A.W., Zhang, W., Bielaszewska, M., Mellmann, A., Kock, R., Fruth, A., Tschape, H and Karch, H**.** 2007. Prevalence, virulence profiles, and clinical significance of Shiga toxin-negative variants of enterohaemorrhagic *Escherichia coli* O157 infection in humans. *Clinical Infectious Diseases*, Vol. 45: 39-45.

Galane, P.M and Le Roux, M. 2001. Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarrhoeal diseases. *Journal of Health, Population and Nutrition*, Vol. 19(1): 31-38.

Galkin, A., Kulakova, L., Sarikaya, E., Lim, K, Howard, A and Herzberg, O. 2004. Structural insight into arginine degradation by arginine deaminase, an antibacterial and parasite drug target. *The Journal of Biological Chemistry*, Vol. 279(14):14001–14008.

Gemmell, M.E and Schmidt, S. 2011. Microbiological assessment of river water used for the irrigation of fresh produce in a sub-urban community in Sobantu, South Africa. *Food Research International*, Vol. 47(2): 300–305.

Grabow, G.L., Spooner, J., Lombardo, L.A and Line, D.E. 1999. *Detecting water quality changes before and after BMP Implementation*: Use of a spreadsheet for statistical analysis. *NCSU water quality group newsletter*.

Grabow, W.O.K and Du Preez, M. 1979. Comparison of m-Endo LES, MacConkey, and Teepol Media for membrane filtration of total coliform in water. *Applied and Environmental Microbiology*, Vol. 30(3): 351-358.

Greenfield, R., Van Vuren, J.H.J and Wepener, V. 2010. Bacterial levels in the Nyl River system, Limpopo Province, South Africa. *African Journal of Aquatic Science*, Vol. 35(1): 55-59.

Griffin, P.M and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, and other enterohaemorrhagic *E. coli*, and the associated haemolytic uremic syndrome. *Epidemiologic Reviews*, Vol. 13: 60-98.

Guerrant, M.E and Steiner, T.S. 2005. Principles and syndromes of enteric infection. In: Mandell G.L., Bennett, J.E., Dolin, R. Mandell, D., and Bannett's Principles and Practice of Infectious Diseases, Ed 6. Philadelphia: Elsevier Churchill Livingstone, Vol 1:1215-1231. Guidelines (2nd ed). Volume 1: Domestic Use.

Guven, K., Togrul, S., Uyar, F., Ozant, S and Pomerai, D. 2003. A comparative study of bioassay based on enzyme biosynthesize in *Escherichia coli* and *Bacillus subtilis* exposed to heavy metals and organic pesticides. *Journal of Enzyme and Microbial Technology*, Vol. 32: 658-664.



Hall, B.G. 1982. Chromosomal mutation for citrate utilization by *Escherichia coli* K-12. *Journal of Bacteriology*, Vol. 151(1): 269-273.

Hallenbeck, P.C and Ghosh, D. 2009. Advances in fermentative biohydrogen production: the way forward? *Trends Biotechnology*, Vol. 27(5): 287-297.

Haller, L., Poté, J., Loizeau, J.L and Wildi, W. 2009. Distribution and survival of faecal indicator bacteria in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Ecological Indicators*, Vol. 9(3): 540-547.

Halpern, M., Shake, T., Pukall, R. and Schumann, P. 2009. *Leucobacter chironomi sp. nov*., a chromateresistant bacterium isolated from a chironomid egg mass. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 59, 665–670.

Hara-Kudo, Y., Miyahara, M and Kumagi, S. 2000. Loss of O157 O antigenicity of verotoxin-producing *Escherichia coli* O157:H7 Surviving under starvation conditions. *Applied and Environmental Microbiology*, Vol. 66(12): 5540-5543.

Hartel, P., Gates, K., Payne, K., McDonald, J., Rodgers, K., Hemmings, S., Fisher, J and Gentit, L. 2005. Target sampling to determine source of faecal contamination. *The Journal for Surface Water Quality Professionals, Stormwater,* Vol. 6(2): 46-53.

Heizmann, W., Döller, P.C., Gutbrod, B and Werner, H. 1998. Rapid identification of *Escherichia coli* by fluorocult media and positive indole reaction. *Journal of Clinical Microbiology,* Vol. 26(12): 2682-2684.

Hendricks, Y. 2003. *The Environmental health impacts associated with flooding and pollution of riverine environments: A case study of the Kuils and Eerste Rivers.* Unpublished Masters Degree, University of the Western Cape, Cape Town.

Holmes, B., Wilcox, W.R and Lapage, S.P. 1978. Identification of *Enterobacteriaceae* by API 20E system. *Journal of Chemical Pathology*, Vol. 31: 22-30.

Homendra, N. 2004. Turbidity studies on mixed surfactant system in hard water. A new method of estimating water hardness. *Indian Journal of Chemical Technology*, Vol 11(6): 783-786.

http://www.saexplorer.co.za/south-africa/map/stellenbosch_map.asp

Huang, C.J and Barrett, E.L. 1991. Sequence analysis and expression of the *Salmonella typhimurium asr* operon encoding production of hydrogen sulfide from sulfite. *Journal of Bacteriology,* Vol. 173(4): 1544-1553.

Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J and Stetzenbach, L.D. 2002. *Manual of Environmental Microbiology*, 2nd Ed. ASM Press, Washington, DC.

Hurter, E., Pool E.J and Van Wyk, J.H. 2002. Validation of an *ex vivo Xenopus* liver slice bioassay for environmental estrogens and estrogen mimics. *Ecotoxicology and Environmental Safety*, Vol. 53(1): 178–187.

Ishiguro, N., Oka, C., Hanzawa, Y and Sato, G. 1979. Plasmids in *Escherichia coli* controlling citrate-utilizing ability. *Applied and Environmental Microbiology*, Vol. 38(5): 956-964.

Ishii, S and Sadowsky, M.J. 2008. *Escherichia coli* in the environment: Implications for water quality and human health, mini-review. *Microbes and Environments*, Vol. 23(2): 101-108.

Iversen, C., Lehner, A., Mullane, N., Eva Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R and Jooste, H. 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus Cronobacter gen. nov. and descriptions of *Cronobacter sakazakii comb. nov. Cronobacter sakazakii subsp. sakazakii, comb. nov., Cronobacter sakazakii subsp. malonaticus subsp. nov., Cronobacter turicensis sp. nov., Cronobacter muytjensii sp. nov., Cronobacter dublinensis sp. nov.* and *Cronobacter* genomospecies 1. *Biomedcentral Evolutionary Biology*, Vol. 7(64).

Jackson, S.G., Goodbrand, R.B., Johnson, R.P., Odorico, V.G., Alves, D., Rahn, K., WIlson, J.B., Welch, M.K and Khakhria, S.K., *Escherichia coli* O157:H7 diarrhoea associated with well water and infected cattle on an Ontario farm *Epidemiology and Infections*. (1998), Vol. 120, 17-20.

Jackson, V.A., Paulse, A.N., Bester, A.A., Neethling, J.H., Khan, S and Khan, W. 2009. Bioremediation of metal contamination in the Plankenbrug River, Western Cape, South Africa. *International Biodeterioration & Biodegradation*, Vol. 63(5): 559-568.

Jaynes, D.B., Dinnes, D.L., Meek, D.W., Karlen, D.L., Cambardella, C.A and Colvin, T.S. 2004. Using the late spring nitrate test to reduce nitrate loss within a watershed. *Journal of Environmental Quality*, Vol. 33(2): 669-677.

Jeng, H.A.C., Englande, A.J., Bakeer, R.M and Bradford, H.B. 2005. Impact of urban stormwater runoff on estuarine environmental quality. *Estuarine, Coastal and Shelf Science*, Vol. 63: 513-526.

Jung-Kon, K., Kyungho, C., Il-Hyoung, C., Hyun-Seok, S and Kyung-Duk, Z. 2007. Application of a microtoxicity assay for monitoring treatment effectiveness of pentachlorophenol in water using UV photolysis and $TiO_2$ photocatalysis. *Journal of Harzardous Materials*, Vol. 149(1-2): 281-286.

Kanungo, S. 2009. A Simplified analysis of different Escherichia coli strains by using RAPD technique. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* Vol. 37(2): 257-260.

Kapaj, S., Peterson, H., Liber K and Bhattacharya, P. 2006. Human Health Effects From Chronic Arsenic Poisoning–A Review, *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering*, Vol. 41(10): 2399-2428.

Karakousis, P.C., Moore, R.D and Chaisson, R.E. 2004. *Mycobacterium avium* complex in patients with HIV infection in the era of highly active antiretroviral therapy. *Lancet Infectious Diseases*, Vol. 4: 557-565.

Karch, H and Bielaszewska, B. 2001. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H2 strains: Epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *Journal of Clinical Microbiology*, Vol. 39(6): 2043-2049.

Karch, H., Böhm, H., Schmidt, H., Gunzer, F., Aleksic, S and Heesemann, J. 1993. Clonal structure and pathogenicity of Shiga-like–toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H. *Journal of Clinical Microbiology,* Vol. 31: 1200-1205.



Karch, H., Wiß, R., Gloning, H, Emmrich, P., Aleksic, S and Bockemuhl, J. 1990. Haemolytic uremic syndrome in children caused by verotoxin-producing *Escherichia coli. Dtsch Med Wochenschr*, Vol. 115: 489-495.

Kazi, T.G., Arain, M.B., Jamali, M.K., Jalbani, N., Afridi, H.I., Sarfraz, R.A., Baig, J.A and Shah Q.A. 2009. Assessment of water quality of polluted lake using multivariate statistical techniques: A case study. *Ecotoxicology and Environmental Safety*, Vol. 72: 301– 309.

Kenner, B.A., Clark, H.F and Kabler, P.W. 1961. Faecal streptococci: Cultivation and enumeration of streptococci in surface waters. *Journal of Applied Microbiology,* Vol. 9(1): 15-20.

Ketchum, B.H. 1955. Distribution of coliform bacteria and other pollutants in tidal estuaries. *Sewage and Industrial. Wastes*, Vol. 27(11): 1288-1296.

Kimberley, L.A., Whitlock, J.E and Harwood, V.J. 2005. Persistence and differential survival of faecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology*, Vol. 71(6): 3041-3048.

Kirby-Smith, W.W and White, N.M. 2006. Bacterial contamination associated with estuarine shoreline development. *Journal of Applied Microbiology*, Vol. 100: 648-657.

Kistemann, T., Claßen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V and Exner, M. 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. *Applied and Environmental Microbiology*, Vol. 68(5): 2188-2197.

Kistemann, T., Dangendorf, F. and Exner, M. 2001, A Geographical information system (GIS) as a tool for microbial assessment in catchment areas of drinking water reservoirs. *International Journal of Hygiene and Environmental Health*, Vol. 203: 225-233.

Kohn, J. 1953. A Preliminary report of a new gelatin liquefaction method. *Journal of Clinical Pathology*, Vol. 6(249).

Kotlowsky, R., Bernstein, C.N., Sepehri, S and Krause, D.O. 2007. High prevalence of *E. coli* belonging to the V2+D phylogenetic group inflammatory bowel disease. *Gut and International Journal of Gastroenterology and Hepatology*, Vol. 56(5): 669-675.

Kuntz, R.I., Hartel, P.G., Godfrey, D.G., McDonald, J.L., Gates, K.W and Segars, W.R. 2003. Targeted sampling as prelude to bacterial source tracking with *Enterococcus faecalis*. *Journal of Environmental Quality*, Vol. 32: 2311-2318.

Lakhan, S.E and Kirchgessner, A. 2010. Gut inflammation in chronic fatigue syndrome. *Nutrition & Metabolism*, Vol. 7: 79-89.

Lapage, S.P., Efstratiou, A and Hill, L.R. 1973. The ortho-nitrophenol (ONPG) test and acid from lactose in Gram-negative genera. *Journal of Clinical Pathology*, Vol. 26: 821-825.

LeChevallier, M.W., Welch, N.J and Smith, D.B. 1996. Full-scale studies of factors related to coliform regrowth in drinking water. *Applied and Environmental Microbiology*, Vol. 62(7): 2201-2211.

Leclerc, H., Mossel, D.A.A., Edberg, S.C and Struijk, C.B. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annual Review Microbiology,* Vol. 55: 201-234.

LeClercq, A., Lambert, B., Pierard, D and Mahillon, J. 2001. Particular biochemical profiles for enterohemorrhagic *Escherichia coli* O157:H7 isolates on the ID 32E system. *Journal of Clinical Microbiology,* Vol. 39(3):1161–1164.

Lee, J.H and Choi, S.J. 2006. Isolation and characteristics of sorbitol-fermenting *Escherichia coli* O157 strains from cattle. *Microbes and Infection,* Vol. 8(8): 2021–2026.

Lee, C.M., Lin, T.Y., Ling, C.C., Kohbodi, G.A., Batt, A., Lee, R and Jay, J.A. 2006. Persistence of faecal indicator bacteria in Santa Monica Bay beach sediments. *Water Research,* Vol. 40: 2593-2602.

Li, Y.P and Zhang, M.F. 2004. Rapid pathotyping of Newcastle disease virus from allantoic fluid and organs of experimentally infected chickens using two novel probes: Brief report. *Archives of Virology*, Vol. 149: 1231-1243.



Lipp, E.L., Farrah, S.A and Rose, J.B. 2001. Assessment and impact of microbial faecal pollution and human enteric pathogens in a coastal community. *Marine Pollution Bulletin*, Vol. 42(4): 286-293.

Liu, Z., Hashim, N.B., Kingery, W.L and Huddleston, D.H. 2010. Faecal coliform modelling under two flow scenarios. *Journal of Environmental Science and Health Part A*, Vol. 45: 282-291.

MacDonell, M.T., Singleton, F.L and Hood, M.A. 1982. Diluent Composition for use of API 20E in characterizing marine and estuarine bacteria. *Applied and Environmental Microbiology*, Vol. 44(2): 423-427.

Magalhaes, M and Vance, M. 1977. Hydrogen sulphide-positive strains of *Escherichia coli* from swine. *Journal of Medical Microbiology*, Vol. 11: 211-214.

Manafi, M. 2000. New developments in chromogenic and fluorogenic culture media. *International Journal of Food Microbiology*, Vol. 60: 205-218.

Marker, M.D and Washington II, J.A. 1974. Hydrogen sulfide-producing variants of *Escherichia coli*. *Applied Microbiology*, Vol. 28(2): 303-305.

Marx, M.C., Wood, M and Jarvis, S.C. 2001. A microplate fluorimetric assay for the study of enzyme diversity in soil. *Soil Biology and Biochemistry*, Vol. 3: 1633-1640.

Mayer, H.B and Wanke, C.A. 1995. Enteroaggregative *Escherichia coli* as a possible cause of diarrhoea in an HIV-infected patient. *New England Journal of Medicine*, Vol. 332: 273-274.

McCarty, LS and Borgert, C.J. 2006. Review of the toxicity of chemical mixtures: Theory, policy, and regulatory practice. *Regulatory Toxicology and Pharmacology*, Vol. 45: 119-143

McCarty, LS. 2002. Issues at the interface between ecology and toxicology. *Toxicology*, Vol. 181-182: 497-503.

McCleery, D.R and Rowe, M.T. 2002. Development of a model meat system and investigation of the growth characteristics and genetic stability of *Escherichia coli* O157:H7, in the absence of meat microflora. *Journal of Microbiological Methods*, Vol. 49:135-145.

Mellmann, A., Bielaszewska, M., Köck, R., Friedrich, A.W., Fruth, A., Middendorf, B., Harmsen, D., Schmidt, M.A and Karch, H. 2008. Analysis of collection of hemolytic uremic syndrome–associated enterohaemorrhagic *Escherichia coli*, *Emerging Infectious Diseases*, Vol. 14(8): 1287–1290.

Merck KGaA. 2000. Chromocult® coliform agar presence/absence membrane filter test method for detection and identification of coliforms and *Escherichia coli* for finished waters. Darmstadt, Germany.

Mohamed, H., Abirosh, H and Sherin, V. 2008. Increased prevalence of indicator and pathogenic bacteria in the Kumarakam Lake: a function of salt water regulator in Vembanadu Lake, A Ramsar Site, along West coast of India. In Sengupta M, Dalwani R (eds) Proceedings of Taal 2007, the 12th World Lake Conference, pp. 250-256.

Monarca, S., Feretti, D., Collivingnarelli, C., Guzzella, L., Zerbini, I., Bertanza, G and Pedrazzani, R. 2000. The influence of different disinfectants on mutagenicity and toxicity of urban wastewater. *Water Research*, Vol. 34(17): 4261-4269.

Morada, M., Manzur, M., Lam, B., Tan, C., Tachezy, J., Rappelli, P., Dessi, D., Fiori, P.L and Yarlett, N. 2010. Arginine metabolism in *Trichomonas vaginalis* infected with *Mycoplasma hominis*. *Microbiology*, Vol. 156: 3734-3743.

Morgan, T.P., Guadagnolo, C.M., Grosell, M and Wood, C.M. 2005. Effects of water hardness on the physiological responses to chronic waterborne silver exposure in early life stages of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, Vol. 74(4): 333-350.

Moses, A.E., Garbati, M.A., Egwu, G.O and Ameh, J.A. 2006. Detection of *E. coli* O157 and O26 serogroups in human immunodeficiency virus-infected patients with clinical manifestation of diarrhoea in Maiduguri, Nigeria. *Research Journal of Medicine and Medical Science,* Vol. 1(4): 140-145.

Moyo, S., Wright, J, Ndamba, J and Gundry, S. 2004. Realizing the maximum health from water quality improvements in the home: A case from Zaka district, Zimbabwe. *Physics and Chemistry of the Earth*, Vol. 29: 1295-1299.

Mugg, P and Hill, A. 1981. Comparison of the Microbact-12E and 24E systems and the API-20E system for the identification of *Enterobacteriaceae*. *Journal of Hygiene, Cambridge,* Vol.87: 287-298.

Müller, E.E, Grabow, W.O.K and Ehlers, M.M. 2003. Immunomagentic separation of *Escherichia coli* O15H:7 from environmental and wastewater in South Africa. *Journal of Water South Africa*, Vol. 29(4): 431-439.

Müller, E.E., Ehlers, M.M and Grabow, W.O.K. 2001. The occurrence of *E. coli* O157:H7 in South African sources intended for direct and indirect human consumption. *Water Research*, Vol. 35(13): 3085-3088.

Moniruzzaman, M., Lai, X., York, S.W and Ingram, L.O. 1997. Extracellular melibiose and fructose are intermediates in raffinose catabolism during fermentation to ethanol by engineered enteric bacteria. *Journal of Bacteriology*, Vol. 179(6): 1880-1886.

Nagvenkar, G.S and Ramatah, N. 2009. Abundance of sewage-pollution indicator and human pathogenic bacteria in a tropical estuarine complex. *Environmental Monitoring and Assessment*, Vol. 155: 245-256.

Neely, T and Campbell, L. 2005. A modified assay to determine haemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae*, Vol. 5(5): 592-598.

Ngwenya, F. 2006. *Water quality trends in the Eerste River, Western Cape, 1990-2005*. Unpublished Magister Scientiae, University of the Western Cape, South Africa.

Njemanze, P.C., Anozie, J., Ihenacho, J.O., Russell, M.J and Uwaeziozi, A.B. 1999. Application of risk analysis and geographic information system technologies to the prevention of diarrhoeal disease in Nigeria. *American Journal of Tropical Medicine and Hygiene*, Vol. 61: 356-360.



Nleya, N. 2005. *Institutional overlaps in water management in the Eerste River catchment.* Unpublished Magister Scientiae, University of the Western Cape, South Africa.

Nomquphu, W. 2005. Overview of the situation and challenges for water quality monitoring and reporting in South Africa. Work session on water statistics, Vienna 20-22 June. Available [on line] Download date: 14 October 2007.

Nucera, D., Maddox, C.W., Hoien-Dalen, P and Weigel, R.M. 2006 .Comparison of API 20E and invA PCR for identification of *Salmonella enterica* isolates from swine production units. *Journal of Clinical Microbiology*, Vol. 44(9): 3388–3390.

O'Hara, C.M., Rhoden, D.L and Miller, J.M. 1992. Re-evaluation of the API 20E identification system versus conventional biochemicals for identification of members of the family *Enterobacteriaceae*: A new look at an old product. *Journal of Clinical Microbiology*, Vol. 30(1): 123-125.

Oberholtzer, P.J and Ashton, P.J. 2008. State of the nation report: An overview of the current status of water quality and eutrophication in South African rivers and reservoirs. Parliamentary grant deliverable.

Orsi RH, Stoppe NC, Sato MIZ, Gomes TAT, Prado PI, Manfio GP, Ottoboni LMM. 2007. Genetic variability and pathogenicity potential of *Escherichia coli* isolated from recreational water reservoirs. *Research in Microbiology*,Vol. 158:420–427.

Owen, M., Willis, C and Lamph, D. 2010. Evaluation of the TEMPO® most probable number technique for the enumeration of *Enterobacteriaceae* in food and dairy products. *Journal of Applied Microbiology*, Vol. 109(5): 1810-1816.

Owusu-Asante, Y and Ndiritu, J. 2009. The simple modelling method for storm- and grey-water quality management applied to Alexandra settlement. *Water South Africa*, Vol. 35(5): 615-625.

Palmer, C.G, Berold, R.S and Muller, W.J. 2004. Environmental water quality in water resources management. WRC Report No TT 217/04, Water Research Commission, Pretoria, South Africa.

Park, S.Y., Choi, J.H., Wang, S and Park, S.S. 2006. Design of a water quality monitoring network in a large river system using genetic algorithm. *Ecological Modelling*, Vol. 199: 280-297.

Peele, D., Bradfield, J., Pryor, W and Vore, S. 1997. Comparison of identifications of human and animal source Gram-negative bacteria by API 20E and Crystal E/NF systems. *Journal of Clinical Microbiology*, Vol. 35(1): 213-216.

Pérez, F., Tryland, I., Mascini, M and Fiksdal, L. 2001. Rapid detection of *Escherichia coli* in water by a culture-based amperometric method. *Analytica Chimica Acta*, Vol. 427: 149-154.

Pollock, K.G.J., Locking, M.E., Beattie, T.J., Maxwell, H., Ramage, I., Hughes, D., Cowieson, J., Allison, L., Hanson, M and Cowden, J.M. 2010. Sorbitol-fermenting *Escherichia coli* O157, Scotland . *Emerging Infectious Diseases*, Vol. 16(5): 881–882.

Pos, K.M., Dimroth, P and Bott, M. 1998. The *Escherichia coli* citrate carrier CitT: A member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts. *Journal of Bacteriology*, Vol. 180(16): 4160-4165.

Poté, J., Goldscheider, N., Haller, L., Zopfi, J., Khajehnouri, F and Wildi, W. 2008. Origin and special-temporal distribution of faecal bacteria in a bay of Lake Geneva, Switzerland. *Environmental Monitoring and Assessment*. Doi 10.1007/s10661-008-0401-8.

Powers, E.M and Latt, T.G. 1977. Simplified 48-hour IMViC tests: An agar plate method. *Journal of Applied and Environmental Microbiology*, Vol. 34(3): 274-279.

Prager, R., Fruth, A., Busch, U and Tietze, E. 2011. Comparative analysis of virulence genes, genetic diversity, and phylogeny of Shiga toxin 2g and heat-stable enterotoxin STIa encoding *Escherichia coli* isolates from humans, animals, and environmental sources. *International Journal of Medical Microbiology,* Vol. 301: 181-191.

Prats, G., Mirelis, B., Miró, E., Navarro, F., Llovet, T., Johnson, J.R., Camps, N., Dominguez, A and Salleras, L. 2003. Cephalosporin-resistant *Escherichia coli* among summer camp attendants with salmonellosis. *Emerging Infectious Diseases,* Vol. 9(10): 1273-1280.



Puzon, J.G., Lancaster, J.A., Wylie, J.T and Plumb, J.J. 2009. Rapid Detection of *Naegleria fowleri* in water distribution pipeline biofilms and drinking water samples. *Environmental Science and Technology*, Vol. 43: 6691-6696.

Queensland Water Recycling Strategy (QWRS). 2000. Wastewater recycling health effects scoping study, Queensland Health Scientific Services, National Research Centre for Environmental Toxicology and Envirotest, Queensland Government.

Raji, M. A., Minga, U and Machangu, R. 2006. Medical Progress: Current epidemiological status of enterohaemorrhagic *Escherichia coli* O157: H7 in Africa. *Chinese Medical Journal*, Vol. 119(3): 217-222.

Ralph, E.T., Guest, J.R and Green, J. 1998. Altering the anaerobic transcription factor FNR confers a haemolytic phenotype on *Escherichia coli* K12. *Proceeding National Academy of Science*, Vol. 95(18): 10449-10452.

Ratnam, S., March, S.B., Ahmed, R., Bezanson, G.S and Kasatiya, S. 1988. Characterization of *Escherichia coli* serotype 0157:H7. *Journal of Clinical Microbiology*, Vol. 26(10): 2006-2012.

Richards, R.P and Grabow, G.L. 2003. Detecting reductions in sediment loads associated with Ohio's Conservation Reserve Enhancement Program. *Journal of the American Water Resources Association*, Vol. 39(5): 1261-1268.

River Health Programme (RHP). 2005. State of rivers report: Greater Cape Town. Pretoria.

Robinson, A., McCarter, Y.S and Tetreault, J. 1995. Comparison of Crystal Enteric/Nonfermenter system, API 20E system, and Vitek automicrobic system for identification of Gram-negative bacilli. *Journal of Clinical Microbiology*, Vol. 33(2): 364-370.

Rodrigues, V., Ramaiah, N., Kakti, S and Samant, D. 2010. Long-term variations in abundance and distribution of sewage pollution indicator and human pathogenic bacteria along the central west coast of India. *Ecological Indicators*, Vol. 11: 318–327.

Rodriguez, A.I, Hariharan, H and Nimrod, S. 2011. Occurrence and antimicrobial drug resistance of potential bacterial pathogens from shellfish, including Queen Conchs (*Strombus Gigas*) and Whelks (*Cittarium pica*) in Grenada. *WebmedCentral Microbiology*, Vol. 2(5):WMC001943.

Rose, S. 2007. The effects of urbanization on hydrochemistry of base flow within the Chattahoochee river basin, (Georgia, USA). *Journal of Hydrology*, Vol. 341: 42-54.

Rossouw, A. 2004. *Assessment of the required competencies for the senior management service in South Africa public service*. Unpublished Masters Degree, University of Stellenbosch, South Africa.

Sanders, B.F., Arega, F and Sutula, M. 2005. Modelling the dry weather tidal cycling of faecal indicators bacteria in surface water of intertidal wetland. *Water Research*, Vol. 39: 3394-3408.

Sasaki-Imamura, T., Yano, A and Yoshida, Y. 2010. Production of indole from L-tryptophan and effects of these compounds on biofilm formation by *Fusobacterium nucleatum* ATCC 25586. *Applied and Environmental Microbiology*, Vol. 76(13): 4260-4268.

Schleifer, K.H and Stackebrandt, E. 1983. Molecular systematics of prokaryotes. *Annual Review of Microbiology*, Vol. 37: 143-187.

Shukla, V.B., Zhou, S., Yomano, L.P., Shanmugam, K.T., Preston, J.F and Ingram, L.O. 2004. Production of D(−)-lactate from sucrose and molasses. *Biotechnology Letters*, Vol. 26: 689–693.

Smith, H.W., Parsell, Z and Green, P. 1978. Thermosensitive Hl plasmids determining citrate utilization. *Journal of General Microbiology*, Vol. 109: 305-311.

Smith, P.B., Tomfohrde, K.M., Rhoden, D.L and Balows, A. 1972. API system: A multitube micromethod for identification of *Enterobacteriaceae*. *Applied Microbiology*, Vol. 24(3): 449-452.

Sotomayor-Ramírez, D., Alameda, M., Martínez, G.A., Pérez-Alegría, L and Corvera-Gomringer, R. 2006. Microbiological surface-water quality of the Rio Grande de Añasco watershed, Puerto Rico. *Caribbean Journal of Science*, Vol. 42(2): 151-163.

Soule, M., Kuhn, E., Loge, F., Gay, J and Call, D.R. 2006. Using DNA Microarrays to identify library-independent markers for bacterial source tracking. *Applied and Environmental Microbiology*, Vol. 72(3): 1843-1851.



Souza, V., Rocha, M., Valera, A and Eguiarte, L.E. 1999. Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Applied and Environmental Microbiology*, Vol. 65(8): 3373-3385.

Spyropoulou, K.E., Chorianopoulos, N.G., Skandamis, P.N and Nychas, G.J.E. 2001. Survival of *Escherichia coli* O157:H7 during the fermentation of Spanish-style green table olives (*Conservolea* variety) supplemented with different carbon sources. *International Journal of Food Microbiology*, Vol. 66: 3-11.

Steiner, T.S., Nataro, J.P., Poteet-Smith, C.E., Smith, J.A and Guerrant, R.L. 2000. Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *The Journal of Clinical Investigation*, Vol. 105(12): 1769-1777.

Steyert, R.S., Rasko, D.A and Baker, J.B. 2011. Functional and phylogenetic analysis of *ureD* in Shiga toxin-producing *Escherichia coli*. *Journal of Bacteriology*, Vol. 193(4): 875-886.

Stumpf, C.H., Piehler, M.F., Thompson, S and Noble, R.T. 2010. Loading of faecal indicator bacteria in North Carolina tidal creek headwaters: Hydrographic patterns and terrestrial runoff relationships. *Water Research*, Vol. 44: 4704-4715.

*The Constitution of the Republic of South Africa*, 1996. *Act 108, Section 27.*

Treleaven, B.E., Diallo, A.A and Renshaw Jr., E.C. 1980. Spurious hydrogen sulfide production by *Providencia* and *Escherichia coli* species. *Journal of Clinical Microbiology*, Vol. 11(6): 750-752.

Trinh, C.T., Unrean, P and Srienc, F. 2008. Minimal *Escherichia coli* cell for the most efficient production of ethanol from hexoses and pentoses. *Applied and Environmental Microbiology*, Vol. 74(12):3634-3643.

Tyagi, V.K., Chopra, K., Kazmi, A.A and Kumar, A. 2006. Alternative microbial indicators of faecal pollution: Current perspective. *Iranian Journal of Environmental Health, Sciences and Engineering*, Vol. 3(3): 205-216.



Ullah, R, Malik, R.N and Qadir, A. 2009. Assessment of groundwater contamination in an industrial city, Sialkot, Pakistan. African Journal of Environmental Science and Technology, Vol. 3: 429-446.

United Nations Educational, Scientific and Cultural Organization (UNESCO). 2003. Water for people water for life. The United Nations World Water Development Report; UNESCO and Berghahn Books.

U.S. Environmental Protection Agency. 1991. *National Primary Drinking Water Regulations*. Consumer Factsheet on: NITRATES/NITRITES, Office of Research and Development, United States Environmental Protection Agency, Cincinnati.

U.S. Environmental Protection Agency. 2000a. The quality of our nation's water EPA841-S-00-0011-20., U.S. Environmental Protection Agency, Washington, DC.

U.S. Environmental Protection Agency (USEPA). 2000b. List of drinking water contaminants & maximum contamination levels, EPA816-F-02-013 July 2002, U.S. Environmental Protection Agency, Washington, DC.

Vaughn, R.H., Osborne,J.T., Wedding, G.T., Tabachnick,J., Beisel, C.G and Braxton, T., 1950, The utilization of citrate by *Escherichia coli*, *Journal of Bacteriology*, Vol. 60(2):119-129.

Vaz, T.M.I., Irino, K., Kato, M.A.M.F., Dias, A.M.G., Gomes, T.A.T., Medeiros, M.I.C., Rocha, M.M.M and Guth, B.E.C. Virulence properties and characteristics of shiga toxin-producing *Escherichia coli* in São Paulo, Brazil, from 1976 through 1999. *Journal of Clinical Microbiology*, Vol. 42(2): 903-905.

Venugopal, V and Nadkarni, G.B. 1977. Regulation of the arginine dihydrolase pathway in *Clostridium sporogenes*. *Journal of Bacteriology*, Vol. 131(2): 693-695.

Wang, D and Fiessel, W. 2007. Evaluation of media for simultaneous enumeration of total coliform and *Escherichia coli* in drinking water supplies by membrane filtration techniques. *Journal of Environmental Sciences*, Vol. 20: 273-277.



Wang, D., Ding, X and Rather, P.N. 2001. Indole can act as an extracellular signal in *Escherichia coli*. *Journal of Bacteriology*, Vol. 183(14): 4210-4216.

Water Research Council (WRC). 2000. National Eutrophication Monitoring Programme. Implementation Manual. Pretoria.

Werber, D., Bielaszewska, M., Frank, C., Stark, K and Karch, H. 2011. Watch out for the even eviler cousin—sorbitol-fermenting *E coli* O157. *The Lancet*, Vol. 377(9762): 298 – 299.

Wheeler, A.L., Hartel, P.G., Godfrey, D.G., Hill, J.L and Segars, W.I. 2002. Potential of *Enterococcus faecalis* as a human faecal indicator for microbial source tracking. *Journal Environmental Quality*, Vol. 31(4): 1286-1293.

Whiley, H., Keegan, A., Giglio, S and Bentham, R. 2012. *Mycobacterium avium* complex- the role of potable water in disease transmission. *Journal of Applied Microbiology*, doi:10.1111/j.1365-2672.2012.05298.x.

Wilkes, G., Edge, T., Gannon, V., Jokinen, C, Lyautey, E., Medeiros, D., Neumann, N., Rueckerg, N., Topp, E., and Lapen, D.R. 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. *Water Research*, Vol. 43: 2209-2223.

Wohlhieter, J.A., Lazere, J.R., Snellings, N.J., Johnson, E.M., Synenki, R.M and L S Baron, L.S. 1975. Characterization of transmissible genetic elements from sucrose-fermenting Salmonella strains. *Journal of Bacteriology*, Vol. 122(2): 401-406.

World Health Organization, 2nd Edition. 2002, Guidelines for drinking-water quality: Addendum microbial agents in drinking water, Geneva.

World Health Organization. 2002. Guidelines for safe recreational-water environments, swimming pools, spas, and similar recreational-water environments, Vol. 2, Geneva.

World Health Organization/United Nations Educational, Scientific and Cultural Organization. 2000. Global water supply and sanitation assessment 2000 report. USA: World Health Organization and United Nations Children's Fund; 2000. http://www.who.int/water_sanitation_health/monitoring/jmp2000.pdf.



www.kznhealth.gov.za/cholerareport1.pdf, 2001

Yanez, M.A., C. Valor, C and Catalan, V. 2006. A simple and cost-effective method for the quantification of total coliforms and *Escherichia coli* in potable water. *Journal of Microbiological Methods*, Vol. 65: 608-611.

Yoon, J.W and Hovde, C.J. 2008. All blood, No stool: enterohaemorrhagic *Escherichia coli* O157:H7 infection. *Journal of Veterinary Science*, Vol. 9(3): 219-231.

Yoshida, T., Yamauchi, H and Sun, G.F. 2004. Chronic health effects in people exposed to arsenic via the drinking water: dose–response relationships in review *Toxicology and Applied Pharmacology*, Vol. 198: 243– 252.

Zhu, F., Wang, S and Zhou, P. 2003. *Flavobacterium xinjiangense sp. nov.* and *Flavobacterium omnivorum sp. nov*., novel psychrophiles from the China No. 1 glacier. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 53: 853-857.

Zhu, P., Shelton, D.R., Karns, J.S., Sundaram, A., Li, S., Amstutz, P and Tang, C. 2005. Detection of water-borne *E. coli* O157 using the integrating waveguide biosensor. *Biosensors and Bioelectronics*, Vol. 21: 678-783.

Zingoni, E., Love, D., Magadza, C., Moyce, W and Musiwa, K. 2005. Effects of a semi-formal urban settlement on groundwater quality, Epworth (Zimbabwe): Case study and groundwater quality zoning. *Physics and Chemistry of the Earth,* Vol. 30: 680-688.

Zinnah, M.A., Bari, M.R., Islam, M.T., Hossain, M.T., Rahman, M.T., Haque, M.H., Babu, S.A.M., Ruma, R.P and Islam, M.A. 2007. Characterization of *Escherichia coli* isolated from samples of different biological and environmental sources. *Bangladesh Journal of Veterinary Medicine,* Vol. 5(1): 25-32.