Assessment of microbial levels in the Plankenburg and Eerste Rivers and subsequent carry-over to fresh produce using source tracking as indicator

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Nicola Huisamen **Date**

ABSTRACT

The agricultural sector of South Africa is currently facing a serious water crisis. The decreased availability of water as a result of climate change and the constantly growing population has left many farmers increasingly dependant on surface water as primary source of irrigation. Urbanisation along with out-dated and insufficient wastewater treatment works have all contributed to polluting large volumes of these resources. Consequently, many farmers have been forced to use irrigation water, not only of poor quality, but often water which has been polluted with untreated sewage. As a result, this project aimed at investigating the link between the quality of irrigation water and the impact on the safety of fresh produce.

A base-line of the microbial load at three sites along the Plankenburg and Eerste Rivers was established using standard microbial methods for the detection of indicator organisms such as total and faecal coliforms, *Escherichia coli* and Enterococci as well as potential pathogens that included *Salmonella*, *Listeria*, *Staphylococcus*, endosporeformers and aerobic colony counts. Chemical parameters such as pH, alkalinity, conductivity and chemical oxygen demand (COD) were also monitored, but were not correlated to microbial pollution levels in the rivers. High faecal coliform and *E. coli* concentrations, ranging from 310 to 7 x 10^6 cfu.100 mL^{-1} and 230 to 7 x 10^6 cfu.100 mL^{-1}, respectively, were detected. The recommended irrigation water guidelines of ≤1 000 (WHO, 1989) and ≤4 000 cfu.100 mL^{-1} (DWAF, 2008) for faecal coliforms and *E. coli* were exceeded, indicating faecal pollution and thus a high health risk. This health risk was confirmed when potential pathogens such as *Aerococcus viridans*, *Klebsiella*, *Listeria monocytogenes* and *Salmonella typhimurium* were detected at all three sites.

The carryover of organisms from rivers to produce (green beans and grapes) was investigated by comparing the microbial population of the Plankenburg and Eerste Rivers to the population recovered from irrigation water and the surface of fresh produce. Faecal coliforms, *E. coli*, *Aerococcus viridans*, *Enterobacter aerogenes*, *Klebsiella*, *L. innocua*, *L. grayi*, *L. monocytogenes* and *Staphylococcus aureus* were detected in all three sample types, indicating a similarity between the microbial populations found in the river, the irrigation water and produce. Thus, the transfer of potential pathogens from the rivers to produce is a strong possibility. The build-up of organisms on the surface of green beans as a result of multiple irrigations was also confirmed by an increase in faecal coliform

concentrations from initial concentrations of none detected to 44 000 cfu.100 mL^{-1} over a 10 day irrigation period.

Finally, microbial source-tracking techniques including multi-antibiotic resistance (MAR) profiling, and the API 20E classification system were used to determine genotypic and phenotypic characteristics of 92 faecal isolates (from irrigation water and produce) and 13 reference strains. Numerical classification systems was used to classify the 105 faecal isolates according to the degree of similarity between the genotypic and phenotypic characteristics of the 105 isolates. A high degree of similarity indicates a high probability that isolates originate from the same strain and therefore from the same source, thereby confirming the transfer of organisms

Faecal isolates (93 and 98%, respectively) were found to be resistant to Vancomycin at both the 5 and 30 µg concentrations. The majority of isolates presented some resistance to Erythromycin (15 µg) and Ampicillin (25 µg), with 82% of isolates presenting an inhibition zone ≤4 mm. Isolates were sensitive towards Ciprofloxacin (1 and 5 µg), Ofloxacin (15 µg), Ceftriaxone (30 µg) and Cefotaxime (5 µg), which were able to inhibit the growth of 79.8, 93.3, 79.8, 88.5 and 71.2% of the isolates, respectively.

The 13 medical reference strains all presented different genotypic and phenotypic characteristics and thereby indicated a high degree of variability between isolates from the same species. Finally, 35% of the isolates could be grouped together based on similar genotypic and phenotypic characteristics, therefore, more than a third of the faecal isolates obtained from the surface of the fresh produce was as a result of faecal contaminants in the irrigation water.

It could therefore be concluded that a health risk is associated with the water from the Plankenburg and to a lesser extent, Eerste River when used as source of irrigation, thereby risking the transfer of potentially harmful organisms, present in the rivers as result of faecal pollution, to the surface of fresh produce.

UITTREKSEL

Suid-Afrika stuur tans af op ‘n dreigende water krisis. Klimaatsverandering tesame met ‘n spoedig groeiende bevolking het gelei tot ‘n aansienlike vermindering in die land se varswaterbronne terwyl veranderende reënvalpatrone daartoe bygedra het dat talle boere al hoe meer afhanklik geword het van oppervlakvarswaterbronne as hul hoof-besproeïngsbron. Verstedeliking, armoede asook verouderde en onvoldoende infrastrukture het egter bygedra tot die besoedeling van baie van hierdie oppervlakvarswaterbronne. Gevolglik is meeste boere genoodsaak om klaar te kom met besproeïngswater van, nie net onaanvaarbare mikrobiese kwaliteit nie, maar dikwels water wat gekontamineer is met onbehandelde riool. Hierdie studie was gevolglik daarop gemik om die impak van die mikrobiologiese kwaliteit van besproeïngswater op die veiligheid van vars groente en vrugte te bepaal.

Standaard mikrobiologiese metodes vir die bepaling van indikator organismes soos totale en fekale kolivorms, *E. coli* en enterococci asook potensiële patogene wat *Salmonella*, *Listeria* en *Staphylococcus* insluit, was gebruik om die mikrobiese lading by drie verskillende punte (P_1, P_2 en P_3) in die Plankenburg en Eerste Rivier te bepaal. Chemiese parameters soos pH, alkaliniteit, conduktiwiteit an Chemiese Suurstof Behoefte was ook bepaal maar geen korrelasie kon tussen hierdie eienskappe en die mikrobiese besoedelingsvlakke getref word nie. Hoë konsentrasies fekale kolivorms en *E. coli* wat onderskeidelik vanaf 3.1×10^2 tot 7×10^6 kve.100 mL^{-1} en 2.3×10^2 tot 7×10^6 kve.100 mL^{-1} gestrek het en gereeld die voorgeskrewe riglyne van onderskeidelik ≤1 000 (WHO, 1989) en ≤4 000 kve.100 mL^{-1} (DWAF, 2008) oorskry het, was by al drie punte gevind. Hierdie resultate het gedui op fekale besoedeling wat gevolglik met ‘n hoë gesondheidsrisiko geassosieer kon word. Hierdie risiko was bevestig deur die teenwoordigheid van talle potensiële patogene, soos *Aerococcus viridans*, *Klebsiella*, *Listeria monocytogenes* en *Salmonella typhimurium*, wat vanaf al drie punte geïsoleer was.

Die oordrag van organismes vanaf die besoedelde riviere na vars vrugte en groente (groen bone en druiwe) was bepaal deur die mikrobiese lading in die Plankenburg en Eerste Rivier te vergelyk met dié verkry vanuit die besproeïngswater en vanaf groen bone wat besproei was met hierdie water. Fekale kolivorms, *E. coli*, *Aerococcus viridans*, *Enterobacter aerogenes*, *Klebsiella*, *L. innocua*, *L. grayi*, *L. monocytogenes* en *Staphylococcus aureus* was vanaf al drie die monster tipes geïsoleer. Hierdie resultate het gedui op eenderse mikrobiese populasies in al drie bronne en het daarom die

moontlike oordrag van patogene bevestig. Die opbou van organismes as gevolg van veelvuldige besproeïngsessies aan die oppervlak van groen bone was bevestig deur die toename in fekale kolivorm konsentrasie vanaf ‘n begin telling van nul tot ‘n maksimum konsentrasie van 44 000 kve.100 mL^{-1}.

Laastens was mikrobiologiese bron naspeurbaarheidstegnieke soos multi-antibiotika weerstandbiedende profiele en die API 20E klassifikasie sisteem gebruik om individuele genotipe en fenotipe profiele van die 105 fekale isolate saam te stel. Numeriese klassifikasie sisteme was gebruik om die isolate op grond van ooreenkomste tussen hul individuele fenotipiese en genotipiese karaktereienskappe te groeppeer. ‘n Hoë mate van ooreenkomstigheid sal dan daarop dui dat isolate van dieselfde besoedlingsbron afkomstig is en gevolglik die oordrag van organismes vanaf besproeïngswater na vrugte en groente bevestig.

Onderskeidelik 93 en 98% van die fekale isolate het daarop gedui om weerstandbiedend te wees teen beide 5 en 30 µg Vancomycin. Die meerderheid isolate (82%) het ook ‘n mate van weerstand teenoor Erythromycin (15 µg) en Ampicillin (25 µg) getoon met inhibisie sones ≤4 mm. Isolate was ook sensitief teenoor Ciprofloxacin (1 en 5 µg), Ofloxacin (15 µg), Ceftriaxone (30 µg) en Cefotaxime (5 µg). Hierdie antibiotikums was in staat om die groei van onderskeidelik 79.8, 93.3, 79.8, 88.5 en 71.2 % van die isolate te inhibeer.

Alhoewel resultate ‘n hoë mate van variasie tussen isolate van dieselfde spesie getoon het was dit nogtans moontlik om 35% van die isolate saam te groeppeer op grond van ooreenstemmende genotipe en fenotipe profiele. Meer as ‘n derde van die fekale isolate wat vanaf die oppervlakte van die groente en vrugte geïsoleer was, was afkomstig vanaf fekale besmetting in die besproeïngswater. Die oordrag van potensieël patogene organismes vanaf besoedelde riviere na vars vrugte en groente tydens besproeïng was sodoende bevestig.

‘n Hoë gesondheidsrisiko was gevolglik gekoppel aan die gebruik van water vanaf die Plankenburg Rivier, en in ‘n minder mate die Eerste Rivier, as bron van besproeïngswater.

Dedicated to my parents, grandparents & husband

Thank you for your unconditional love and support

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CONTENTS

The language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

The weather conditions in South Africa play an important role in determining the amount of available fresh water resources. South Africa's average annual rainfall of 500 mm (Wentzel, 2009) is influenced predominantly by the cold Benguela and warm Mozambique ocean currents. Together with the restricted air movement as a result of the intense high pressure belt across the central parts of the country, rainfall is often scattered and erratic. Consequently, water is a scarce and sought after resource, with most parts of the country prone to droughts during seasons of low rainfall.

The continuously growing population, urbanisation, industrial effluent and out-dated, or in some cases, non-existent waste water treatment plants (WWTP), all contribute to aggravate an already water-stressed situation (Paton, 2008). Insufficient upgrading and maintenance has left WWTP out-dated and incapable of delivering adequately treated water. Precarious conditions in informal settlements due to the lack of adequate sanitation and waste disposal systems have forced many to use near-by rivers as means for disposing of both household and human waste. Ultimately, rivers are polluted with high concentrations of potential pathogenic organisms and highly infectious viruses (Barnes *et al*., 2004).

Faecal pollution caused by surface run-off, wild and domestic animals, flooding and sewerage treatment plants functioning well over capacity, have all contributed to severely polluted rivers and dams (Brackett, 1999; Schultz-Fademrecht *et al*., 2008; Warner *et al*., 2008). These rivers include some of our most valuable fresh water resources such as the Vaal River which is regarded as the principle source of water to the economic heartland of Gauteng (Paton, 2008), the Crocodile River catchment in Eastern Gauteng that supports some of the largest irrigation areas in South Africa (Ashton *et al*., 1995) and the Lourens River used for many agricultural purposes in the Western Cape (Thiere & Schulz, 2004).

Agriculture is a key contributor to the stability of South Africa's economy, with exports to the European Union estimated at R27 billion per year (Paton, 2008). These exports not only generate much needed income through both international and local trade, but also create thousands of job opportunities and provide a livelihood for many

subsistence farmers. Agriculture is unavoidably dependant on the availability of either sufficient rainfall or large volumes of clean irrigation water from rivers and dams. Therefore, as the most important limiting factor to agriculture, the restricted availability of clean water and extensive pollution is of great concern. With almost 50% of South Africa's water resources applied to irrigate an estimated 1.3 million hectares of land (Schreiner & Naidoo, 2009), and the scarcity of water regarded as a fundamental development constraint (Turton, 2008), managing and protecting our water resources in order to guarantee a sustainable supply of fresh water in future is of utmost importance. Especially in view of the fact that almost 98% of South Africa's fresh water resources are already allocated for usage.

Climate change has caused changes in weather patterns which have resulted in decreased rainfall patterns, thereby further aggravating this water-stressed problem (Turton, 2008). As a result, farmers have become even more dependant on irrigation water from rivers and dams and have little control over the extent to which this water is polluted. The polluted state of many rivers and dams posed the question of how this would impact the health of the thousands of people who rely on these water resources on a daily basis (G. Backeberg, personal communication, 2008). Other concerns referred to the possible carryover of potential pathogens to produce irrigated with polluted water and the impact there-of on the safety of fresh fruit and vegetables.

The potential threat to the health of humans as a result of the likely transfer of pathogenic organisms from the polluted rivers to the produce has consequently become a major concern (Abadias *et al*., 2008; Goss & Richards, 2008). The potential of pathogenic organisms being transferred repeatedly onto the surface of fresh produce during multiple irrigation sessions, along with their ability to survive for several months in these unfavourable conditions (Brackett, 1999; Goss & Richards, 2008) presents the scenario where consumers unknowingly face a high risk of being infected with harmful organisms when consuming fresh produce. Internationally, a sharp increase in foodborne outbreaks related to the consumption of fresh or minimally processed food (Abadias *et al*., 2008; Warner *et al*., 2008) has been observed and South Africa is most likely to follow.

Outbreaks related to the consumption of contaminated produce such as cantaloupe, tomatoes, lettuce and alfalfa sprouts (Brackett, 1999) include 10 000 people infected with *E. coli* O157:H7 as a result of contaminated radish sprouts. In the USA, between 1990 and 2002, 56 outbreaks which included 6 762 cases were linked to the consumption of contaminated fresh produce (Siro *et al*., 2005). Contaminated tomatoes have been implicated in numerous *Salmonella* outbreaks in the USA (Steele & Odumeru, 2004; Stine

et al., 2005). A large scale *E. coli* O157:H7 outbreak in the USA during September of 2006 resulted in 200 cases (including three fatalities) of Hemolytic Uremic Syndrome (Abadias *et al*., 2008). Irrigation water has also been implicated in outbreaks of *E. coli* O157:H7 infections in the past. *Escherichia coli* detected on cabbage seedlings irrigated with sewage-contaminated water implicated the polluted irrigation water as most likely source of contamination as none were found on seedlings in an adjacent field irrigated with municipal water (Steele & Odumeru, 2004). Ultimately, South Africa is not only facing a water-demand crisis, but the health implications of such polluted rivers and dams are far reaching, not to mention potentially life-threatening.

On account of associated health risks, authorities (EU) have issued warnings claiming that unless the quality of rivers in the Western Cape improves drastically, export licenses could be annulled (Anon., 2005). This would result in an economic disaster, should the export of fresh fruit and vegetables be terminated, trust in the local market will decline and thousands of people would be left jobless. Ultimately, immense financial losses, devastating health implications and damage to the country's economic well-being could be the result of this serious water pollution crisis.

This research project was therefore initiated with the main objective of establishing the link between the water quality and the microbial quality of fresh produce by means of standard microbial methods for the detection of indicator and potential pathogenic organisms. This will be achieved firstly, by establishing a base-line of the microbial levels found in the Plankenburg and Eerste Rivers, secondly, comparing microbial loads obtained from the surface of produce to the loads detected in the rivers and irrigation water. Finally, microbial source tracking (MST) techniques including multi-antibiotic resistance (MAR) profiling and API (Biomerieux) will be incorporated in identifying and grouping different isolates based on their unique genotypic and phenotypic characteristics. The carryover of organisms from rivers to the produce via irrigation water will then be confirmed based on the degree of similarity between the genotypic and phenotypic characteristics of the different isolates using numerical clustering techniques.

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CHAPTER 2
LITERATURE REVIEW

A. BACKGROUND

In recent years the whole of South Africa, especially the Western Cape, has experienced various crises indicative of the country's failing infrastructure. These crises included the recent electricity crisis, failing water and sewage treatment plants, acid mine drainage and now also the threatening water crisis. Most of these problems have been caused by a lack of foresight, planning and shortage of skilled personnel (Turton, 2008), and an all-round increase in demand for fresh water caused by the continuous growth of the world population (Barnes, 2006). The reckless and irresponsible past-behaviour of the entire population have finally caught up with us.

Natural resources are being depleted at a pace faster than they can be restored and manufacturing and production processes are incorporated without giving enough thought to the waste that will be generated. Sustainability has become one of mankind's biggest obstacles. The overuse of natural resources such as fossil fuel, minerals and water along with the inability to recycle or reuse the vast amount of waste and by-products that are generated on a daily basis have all contributed to a scenario that could be compared to an engine running on maximum capacity.

Processes and systems that humans rely on daily, such as electricity, food, water, sanitation and transportation are dependant on the availability of the sufficient supply of natural resources: coal for generating energy; clean water for irrigation, drinking and sanitation; and fossil fuel for transportation. These resource have, however, been depleted to such an extent that they are no longer available in abundance, in fact, if the remaining resources are not managed properly, there will soon be nothing left (Turton, 2008).

Water is one resource essential to all life sustaining processes that is becoming a cause of great concern (Steele & Odumeru, 2004). The available fresh water resources are restricted and it is becoming increasingly difficult to supply the constantly growing demand needed for the production of food and sanitation, not to mention compensating for the vast amounts that are being polluted and consequently unacceptable for further use.

The focus of this literature study will therefore be to review the factors contributing to the pollution of fresh water resources and review some literature on the

impact of the deteriorating water quality on the safety of fresh produce when used to irrigate crops intended to be consumed raw or in a minimally processed state.

B. FACTORS CONTRIBUTING TO THE DIRE SITUATION

The current state of South Africa's water resources cannot be blamed on a single cause. Various factors contributing to the declining water quality of South Africa can be identified: the most important being South Africa's failing infrastructure; the constant and rapid growing population of South Africa and urbanisation (Barnes, 2006).

South Africa's infrastructure has been severely neglected over the last decade. According to Dr Anthony Turton (2008) this country has just passed the "Uhuru" decade. "Uhuru" refers to the ability of an infrastructure to function adequately for a certain period of time, usually ten years, due to the success of previous maintenance, before collapsing completely. It is caused by the insufficient maintenance and upgrading of our country's infrastructure, short-sightedness and the lack of planning (Turton, 2008). Short-comings of the outdated infrastructure was exacerbated by the electricity crisis. Sewage treatment plants have become increasingly ineffective due the continuous power-surges which cause sewerage to pass through the treatment works without being treated.

Other important factors contributing to the deteriorating water quality and the limited availability of fresh water resources is the rapidly growing population along with urbanisation (Paulse *et al*., 2009). With the South African population estimated at about 50 million people, the continuous increase of individuals and the subsequent growing demand for food, housing and sanitation as well as the additional waste that is generated have all negatively impacted the current water quality.

Urbanisation, driven by poverty and the hope of a better future added strain on the existing infrastructure due to the sudden increase of population in and around cities. This lack of infrastructure force many people to survive on the bare minimum and consequently brought about the development of various informal settlements around the cities. In most cases these informal settlements have little, if any, sanitation facilities and people are often forced to live in harsh conditions. Here, the lack of sanitation, domestic water supply and basic services, such as waste removal, along with insufficient wastewater treatment and sewage plants lead to an uncontrollable situation whereby large amounts of waste are generated and none removed or treated (Paulse *et al*., 2009). Consequently, the accumulation of toxic waste caused by flooding of

houses, streets and common areas where large groups of people generally dwell has occurred (Barnes, 2003). This not only caused the contamination of soil, but also generated severely polluted surface run-off which is discharged into storm water drainage pipes, flowing into near-by rivers and groundwater resources, thereby polluting these resources and making it unsafe for human consumption (Paulse *et al*., 2009).

A shortage of funds, the crucial need for qualified personnel and poverty are more key contributors to the current state of affairs. Municipalities are unable to afford the essential upgrading of equipment, not to mention conducting routine maintenance of existing facilities and are unable to source qualified personnel (Turton, 2008). According former senior water researcher from the Council for Scientific and Industrial Research (CSIR), Turton (2008), the serious shortage of skilled individuals and the lack of research are crucial factors contributing to the dilemma that South Africans are now finding themselves in. The crucial shortage of a qualified workforce has caused important tasks to be assigned to incompetent people; consequently these tasks are not performed properly or even at all.

South Africa is therefore faced with a devastating problem. The constant deterioration of the water quality along with the gradual depletion of the remaining useful water resources could lead to numerous devastating repercussions affecting not only agriculture, but also the sustainability of all forms of life. Severe water shortages are becoming a serious concern for South Africa (Paulse *et al*., 2009) and badly polluted surface water is causing the seeping of contaminated water from various sources such as rivers and dams to the underground ground water resources, thereby endangering these future water resources. Seeing that no nation would be able to function without water and restoring these groundwater resources could take years, South Africa is facing a devastating water crisis. In the words of Turton (2008): “When people are denied access to clean drinking water, social instability will grow and South Africa will slowly slide into anarchy and chaos”.

C. IMPLICATIONS OF THE CURRENT STATE OF AFFAIRS

Water is one of our most important, if not the most important, natural resource, essential to all forms of life (Wenhold & Faber, 2009). Water is used during most of our daily tasks such as cooking, cleaning, and practicing personal hygiene. The survival of both humans and animals is dependant upon sufficient, good quality water (Wenhold & Faber, 2009). The production of food would not be possible without it and the intake of

sufficient good quality water is directly linked to optimal nutrition (Wenhold & Faber, 2009).

South Africa receives an average annual rainfall of about 500 mm per year which is distributed unevenly across the country. The 400 mm line, which can be drawn across the interior parts, divides the country in a wetter Eastern region, receiving up to 1 000 mm per year (Fig. 1), and a drier Western region which receives as little as 100 mm annually (Fig. 1). Many farmers can therefore not rely exclusively on the limited amount of rainfall for irrigation and are consequently largely dependant on rivers and boreholes as primary source of irrigation water.

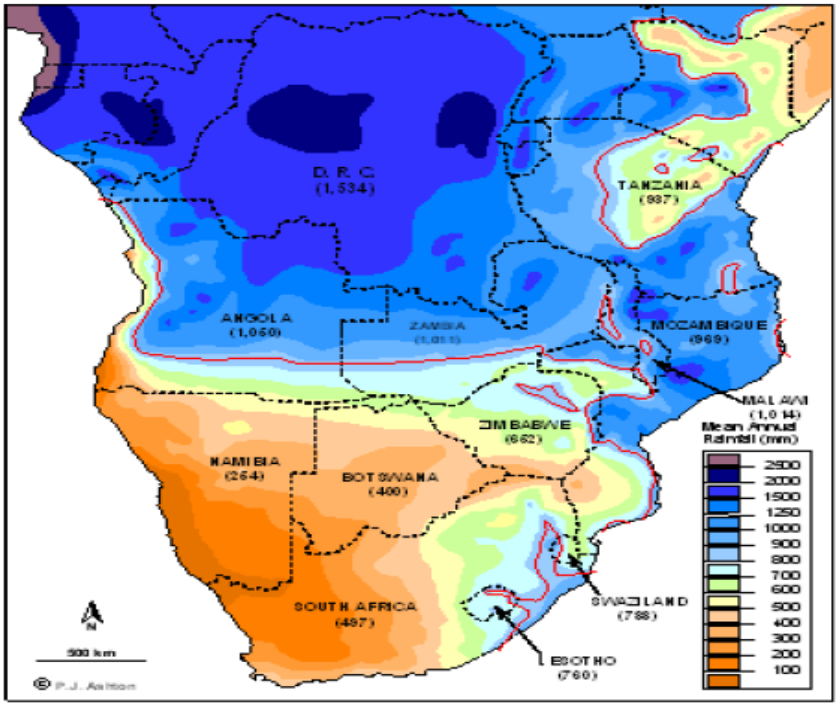

Figure 1 Mean annual precipitation map of the Southern African region (Turton, 2008)

The potential devastating effects of climate change have added to concerns on the limited availability of fresh water resources. Predictions of a drying western half of the country with a considerably shorter rainfall season in the Western Cape and increased rainfall patterns along the Eastern parts describe a completely different situation to the current one. Decreased rainfall will bring about the availability of even less fresh water with the possible changes regarding the distribution of water to wreak havoc with the ecosystem. Decreased river flows caused by the lack of rain have already resulted in rivers becoming even more polluted (Turton, 2008) and

consequently, farmers have been forced to make do with the polluted rivers as their main source of irrigation.

Faecal pollution increases the nutrient, sodium and phosphorous levels in water. This ultimately leads to lake eutrophication, algae blooms and elevated levels of harmful bacteria, viruses and protozoa (Moussa & Massengale, 2008). Numerous South African rivers are contaminated with harmful pathogenic organisms such as *E. coli*, *Salmonella*, *Klebsiella* and *Listeria* (Ashton, 1995; Taylor *et al*., 2001; Barnes, 2003; Barnes & Taylor, 2004; Thiere & Schulz, 2004; Jagals *et al*., 2006; Jackson *et al*., 2009; Nleya & Jonker, 2009), which are all well-known causes of a wide spectrum of food borne diseases (Moussa & Massengale, 2008). These organisms are known to be excellent vectors for the spread of infectious diseases (Chale-Matsau, 2005), therefore the high frequency of infections and outbreaks can be related to contaminated water (Islam *et al*., 2004).

In conditions of poor sanitation, under-nourishment and, often including people with compromised immune systems, infections will spread quickly and soon lead to a full-blown outbreak. Immune-deficiency disorders such as TB (Tuberculosis) and HIV/AIDS (Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome) are causing people to become increasingly vulnerable and susceptible to contracting infections and diseases (Barnes, 2006). Whilst living in close proximity to one another without the necessary sanitation facilities and proper nutrition provides the ideal environment that promotes the spread of highly contagious diseases.

Patients infected with contagious diseases are also regarded as merely the primary vector of disease transmission. As living incubators of potentially fatal viruses and bacteria, organisms are able to multiply within the host and excreted in high concentrations (Fong *et al*., 2005). Therefore, if infections are not treated adequately, these organisms will ultimately end up in the rivers and dams where they will be able to survive and potentially infect even more people. An example of such an outbreak is the recent Cholera outbreak in Zimbabwe (Anon., 2008). Cholera is a ruthless diarrhoeal illness caused by the bacterium *Vibrio Cholerae*. It is a highly infectious disease, able to spread via contaminated water. This deadly outbreak crossed South African borders via the contaminated waters of the Limpopo River (Anon., 2008). People dependant on water from this river was therefore in danger of being infected with this harmful pathogen. It is thus clear that favourable conditions for the spread of waterborne infections exist in South Africa.

The primary transmission of diseases due to the direct contact with contaminated water is, however, no longer the only concern, farmers are also faced with increased pressure to produce larger crops to meet the increased demand for fresh produce. The increased demand is not only as a result of the growing population, but can also be attributed to the changing trends amongst consumers. In recent years a sharp increase in the consumption of fresh fruit and vegetables has been widely published (Beuchat, 1995; Matthews, 2006), a tendency which could be as a result of the consumer's ever changing needs.

The increased consumption of fresh fruit and vegetables could be ascribed to consumers becoming more educated concerning the benefits of a healthy life-style (Abadias *et al*., 2008) along with the many dangers associated with an unbalanced diet. The increased risk of cancer and cardiovascular diseases along with the growing number of individuals who are currently overweight and obese, have led to a dramatic change of life-style amongst many consumers. Governments have also established campaigns such as 5-a-day and 9-a-day which promote the consumption of more fresh fruit and vegetables (Heaton & Jones, 2007). Ultimately, increased awareness amongst consumers, the availability of a wider variety of fresh fruit and vegetables and the willingness to pay extra for premium quality have caused consumers to purchase more fresh fruit and vegetables and ultimately led to increased strain on the South African agricultural community to answer to this drastic increase in demand.

The inevitable dependency of agriculture on the availability of fresh water compels farmers to make do with irrigation water, not only of poor quality, but often water which has been polluted with untreated sewage. In cases where badly polluted rivers are the only source of irrigation water and used to irrigate crops intended to be consumed raw or in a minimally processed state, the possible transfer of potential pathogenic organisms to produce could pose a threat to the health of the consumer (Abadias *et al*., 2008).

The production of top-quality fruit and vegetables, and more importantly, produce safe for human consumption is determined, not only by the quantity, but also the quality of water which is used for irrigation. According to research conducted by Steele & Odumeru (2004), contaminated water containing pathogenic organisms presents a definite health hazard to humans when used as sources of irrigation due to the possible transfer of these organisms to the crops during irrigation (Lu *et al*., 2004). The importance of using good quality irrigation water was emphasised even further by the

identification of *E. coli* throughout the tissue of lettuce irrigated with contaminated water. These findings suggested the possibility that bacteria could be absorbed through the root systems of crops (Steele & Odumeru, 2004).

A sharp increase in the number of foodborne illnesses related to the consumption of fresh or minimally processed fruit and vegetables (Beuchat, 1995; Abadias *et al*., 2008), of which some severe cases have even resulted in death, have been reported. Although various routes of fruit and vegetable contamination exist (Johannessen *et al*., 2002), the link between poor quality irrigation water and outbreaks of Salmonellosis, Listeriosis and Cholera, to name a few, cannot be ignored.

In 2005, the European Union (EU) started raising concerns regarding the microbial quality of some rivers in the Western Cape (Cape Times, 2005). During this time the media reported on warnings issued by the EU to various export farmers who use water, specifically from the Berg River, for irrigational purposes. Along with the benefits of trading produce on international markets come a number of standards and specifications to which exporters must adhere. The ≤1 000 faecal coliforms per 100 ml irrigation water guideline (WHO, 1989) is one of the more serious stipulations. Results of studies conducted previously on the microbial quality of water from the Berg River indicated exceptionally high *Escherichia coli* levels in this river. These concentrations exceeded requirements of the WHO (1989) by up to 2 400 times and caused great concern to the EU (Cape Times, 2005). Farmers were then warned to either clean the irrigation water from the Berg River or possibly face their export products being rejected (Cape Times, 2005).

The impact on the South African agricultural community could therefore be devastating. The credibility of South African produce will be lost, both on the international and local market and once the fear of illness or death is associated with local produce consumers will be hesitant to purchase and exports could plummet.

D. ROLE OF AGRICULTURE

Farming in South Africa consists of an active dual agricultural community, comprising both commercial as well as subsistence farming. The availability of an adequate water supply is regarded as the most significant limiting factor due to the limited supply of fresh water resources and relatively poor rainfall. South Africa is rated amongst the 20 most water-scarce countries in the world (Woodford *et al*., 2009), and considering that 98% of its fresh water resources are already assigned to various agricultural, domestic

and industrial purposes and that the demand of fresh water resources are expected to exceed supply by 2025 (Die Burger, October 2008) it is understandable why concerns are being expressed about the sustainability South Africa's ground water resources.

With an estimated 1.3 million hectares of land under irrigation, farming is mostly reliant on surface water that includes rivers and dams as sources of irrigation. These resources are constantly replenished by limited rainfall and melting snow and are estimated to contribute to about 50% of the annual water resources used for agricultural purposes (Department of Agriculture, 2009). Surface water resources are therefore important and these restricted resources will have to be managed correctly in order to be sustainable.

South Africa reaps the benefits of its prime location, as it not only enjoys the counter-seasonality to Europe, but also contains three deep-water ports, international airports and a well-established road and railway network. These key competitive advantages makes South Africa one of the major exporters in Africa and amid the top five exporters in the world of amongst other things, pears, table grapes and meat products.

As one of the primary pillars on which the South African economy is based, primary agriculture contributes about 2.6% to the gross domestic product (GDP) (Department of Agriculture, 2009). Income generated from exports to trading partners such as the EU, United States of America (USA) and sub-Saharan countries serve as crucial contributors to the success of the South African economy. The Western Cape alone accounts for between 55 and 60% of South Africa's total agricultural exports (WESGRO, 2003), estimated to the worth of R7 billion per year. Whilst stimulating economic growth, with an annual growth rate of 5%, agriculture in the Western Cape contributes substantially to generate essential job opportunities which include 8 500 commercial and 2 500 subsistence farmers and around 220 000 farm workers (WESGRO, 2006).

When taking into consideration the vital contributions of agriculture in South Africa, its high demand for sufficient, accessible and clean water should not be taken lightly. Without an adequate supply of clean water the production of good quality agricultural products and products that are safe for human consumption would not be possible. Subsequently, trade agreements could be lost.

E. MICROBIAL INDICATORS OF WATER QUALITY

The microbial quality of water is a critical element which should be considered due to the public health risks involved (Carroll *et al*., 2009). Although the microbial quality of water does not indicate the actual ability of water to cause disease (Jagals *et al*., 2006) the quality is determined in order to asses the potential of pathogenic organisms present in the water to cause disease (Hunter *et al*., 2003).

Microbial contamination of water is of importance as pathogenic organisms in water could cause harm or illness and even result in death to any person who came into contact or consumed the contaminated water (Field & Samadpour, 2007). Linking water quality to health is very complicated as the degree of risk presented by a certain concentration of pathogenic organism is influenced by variables such as the infectivity and invasiveness of the pathogen (Campos, 2008), size of the excreted load, the duration of the latency period before the pathogenic organism become infectious, and its ability to survive and multiply in the environment (Steele & Odumeru, 2004).

The microbial population of a particular water source could consist of many different types of organisms, all which may be present in varying concentrations and identified with different kinds of often, complex and expensive techniques (Campos, 2008). It is therefore impractical to identify every organism present in a particular water sample as the determination of the possible contamination or quality of water could result in a time consuming and costly process (Field & Samadpour, 2007; Wilkes *et al.*, 2009). The direct monitoring of pathogenic organisms has also been proven to be impractical as these organisms are often present at very low concentrations, difficult to culture and patchy in distribution, yet highly infectious, even at low concentrations (Field & Samadpour, 2007; Yan & Sadowsky, 2007). Therefore, the detection focus shifted to the determination of a specific set of organisms classified as “indicator” or “index” organisms (Campos, 2008). Levels of faecal pollution are quantified by the concentration of indicator organisms such as *E. coli* and Enterococci (Fong *et al*., 2004; Moussa & Massengale, 2008). “Indicator” organisms are not necessarily pathogenic or harmful to humans and should not occur naturally in the environment, but serve as indicators of the success of treatment processes and possible faecal pollution (Campos, 2008). Index organisms in contrast refer to specific species or groups of organisms that indicate the presence of pathogenic organisms (Campos, 2008; Parajuli *et al*., 2009).

General microbial indicators are those which indicate the efficiency of a process, the concentration of total coliforms being an example of the efficiency of chlorine to act

as disinfectant (Campos, 2008), whilst faecal indicators, such as *E. coli* serve as an indicator of faecal pollution as well as insufficient hygienic procedures (Siro *et al*., 2005).

Several criteria exist to which indicator organisms should conform and include:

- should consistently be present in faeces;
- must be unable to multiply outside the intestinal tract (Field & Samadpour, 2007);
- must at least be as resistant to environmental conditions and disinfectants as pathogenic organisms;
- must have a definite correlation with the occurrence of pathogenic organisms (Field & Samadpour, 2007);
- must be easily detectable with the help of reliable detection methods (Savichtcheva & Okabe, 2006);
- must not be harmful to humans and thus safe to work with;
- must be present in concentrations indicative of the extent of pollution;
- must be present in concentrations higher than that of the pathogenic organisms; and
- must not be present in unpolluted water (Chale-Matsau, 2005).

It is important to emphasise that there is no single organism that complies with all of these requirements. Even though a good indicator organism should be present in the presence of pathogenic organisms and be easily detectable with rapid and inexpensive methods (Yan & Sadowsky, 2007; Wilkes *et al*., 2009) each indicator organism has its own set of strengths and weaknesses (Table 1). The disadvantages of using indicator organisms to predict water quality include: faecal coliforms occur in both human and animal faecal matter thereby hampering the identification of the source of pollution; coliform standards could fail to predict the presence of waterborne human pathogenic organisms; and traditional bacterial indicators die off quickly in comparison to their pathogenic counterparts (Fong *et al*., 2005). As a result a combination of indicator organisms are often used (Chale-Matsau, 2005).

Indicator organisms are either identified and enumerated or simply detected as present or absent, depending on the limitations of the specific detection methods. These results are then compared to the relevant standards and guidelines such as the South African Water Quality Guidelines (DWAF, 2002) and World Health Organisation

(WHO) Drinking Water Guidelines (1989) to determine the associated health risks (Jagals *et al*., 2006). In the past, the total coliform bacteria group was frequently used as an indicator of faecal pollution. This has, however, proved to be inaccurate as total coliform bacteria could also originate from non-faecal sources such as water and soil, thereby lowering the value of total coliforms as indicators of faecal pollution (Johannessen *et al*., 2002). Today, *E. coli* is the most commonly used indicator organism and regarded as a more definite indicator of faecal contamination (Hurst *et al*., 2007) due to its strong association with faecal contamination (Johannessen *et al*., 2002; Yan & Sadowsky, 2007).

E. coli originate in the gut of warm-blooded animals and humans (Jagals *et al*., 2006) and discharged in their faeces (Chandran & Hatha, 2005). The presence of *E. coli* is commonly used as indicator of faecal contamination (Theron & Cloete, 2004) and the possible presence of pathogenic organisms (Jagals *et al*., 2006). Total coliforms, faecal coliforms, *E. coli*, faecal *streptococci* and nematode eggs were used as indicators of microbial quality during the formulations of these standards (Steele & Odumeru, 2004) (Table 1).

The selection of the specific combinations of indicator organisms, the frequency of analyses and deciding upon methods that will be incorporated differ from one scenario to the next. These issues are influenced by various factors unique to each situation, such as the risk of infection, possible sources of contamination, as well as the availability of financial resources, testing facilities and equipment and trained personnel (Chale-Matsau, 2005).

F. STANDARDS AND GUIDELINES USED TO DETERMINE WATER QUALITY AND PRODUCT ACCEPTABILITY

The growing demand for clean water along with the contamination of existing resources has caused the availability of water to become an increasingly critical problem (Gerba, 2006). In South Africa, contamination caused by sewage discharge from informal settlements and overloaded or inadequately functioning sewage treatment plants have led to the rapid deterioration of river water quality.

Water standards and regulations are requirements which have been established to enforce a minimum standard to which water resources must conform to. These requirements have been formulated with a specific application in mind, such as drinking

water, irrigation water and recreational water, there-by ensuring the safety of everyone who comes in contact or makes use of the water (Fong *et al*., 2005).

Water quality guidelines, on the other hand, act as a source of reference or recommendation which could be used for the development of national standards (Campos, 2008). The word "guideline" is used intentionally since these are not official standards to which one must comply (Campos, 2008), but rather specifications based on scientific research to encourage the formulation and enforcement of standards and the ultimate improvement of the general quality of water all over the world.

Table 1 The most commonly used indicator organisms (Chale-Matsau, 2005)

Indicators	Classification characteristics or appearance	Specific use or indication	Limitations
Total coliforms	Colonies with a metallic sheen on L-EMB agar after incubation of 20-24 h at 35˚C	General sanitary condition of water	Presence of non-faecal and lactose-negative coliform bacteria limits the success of this group as indicator of faecal contamination
Faecal coliforms	Blue colonies on m-FC agar after 20-24 h incubation at 44.5˚C	Secondary indicator of probable faecal pollution	Some faecal coliforms are not necessarily of faecal origin
Escherichia coli	Possess the enzymes β-galactosidase and β-glucuronidase. Grows at 44 to 45˚C with the fermentation of lactose and mannitol to produce acid and gas	Evaluate the probable faecal origin of total and faecal coliforms	Some strains of *E. coli* do occur naturally in the environment
Enterococci (faecal streptococci)	Reddish colonies on m-Enterococcus after incubation of 48 h at 35˚C. Possess the Lancefield group antigen	Used as additional indicator to evaluate the success of treatment processes and the subsequent safety of recreational waters	Not all Enterococci are of faecal origin and comprise a subgroup of faecal streptococci

Water quality standards and guidelines differ significantly between countries, for the various water resources such as surface water, ground water, drinking water and human wastewater (Steele & Odumeru, 2004) as well as for the different uses of water. Differences in standards between countries are usually as a result of to the economic status of the specific country as well as the general uncertainty regarding the risks associated with the different types of pathogenic organisms present in contaminated water (Steel & Odumeru, 2004) and contribute significantly to the heightened risk for outbreaks of human gastroenteritis as a result of contaminated fresh produce (Beuchat, 1995).

Although water of outstanding quality is always desired, the poor economic state of a country along with the extremely high costs associated with water treatment processes, methods of detection and the development and maintenance of the necessary infrastructure makes it impossible to achieve the same standards in different countries (Steele & Odumeru, 2004). Uncertainty also exists regarding the effectiveness of organisms such as *E. coli* as indicator of faecal pollution, thereby causing doubt regarding the risk of disease transmission. Even though these differences in standards and guidelines might seem confusing, it is very important to be familiar with them when wanting to export and be part of the international market.

According to standards required by the USEPA, no faecal coliforms may be present in wastewater intended to be used as irrigation source for crops destined to be consumed in a minimally processed state. A concentration not greater than 200 faecal coliforms per 100 ml is allowed for irrigating crops intended to be used as feed for animals (Steele & Odumeru, 2004).

The Canadian Water Quality Guidelines for the protection of agricultural water uses require total and faecal coliform concentrations ≤1 000 and ≤100 cfu.100 ml^{-1} of irrigation water, respectively (Steele & Odumeru, 2004). Guidelines formulated by the WHO (1989) state that a faecal coliform concentration of up to 100 000 cfu.100 ml^{-1} is allowed for irrigating crops which will be processed post-harvest (Steele & Odumeru, 2004), whilst a limit of 1 000 faecal coliforms per 100 ml is set for all the remaining irrigation water (Shuval *et al*., 1997). Other recommendations of the WHO (1989) include guidelines for the use of biologically treated effluent as irrigation source. In the case of crops being consumed uncooked the coliform concentration in irrigation water should not exceed 100 cfu.100 ml^{-1} water in at least 80% of all samples tested (WHO, 1989). In contrast, DWAF (2002) set that same standard at ≤4 000 cfu.100 ml^{-1} (Table

2), a level four times higher than that of the WHO and USEPA. According to DWAF, faecal concentrations exceeding this guideline are associated with a high risk when irrigating crops intended to be consumed raw (DWAF, 2002).

There are many post-harvest factors, such as washing and handling of fresh fruit and vegetables, which influences the final microbial quality of products. For this reason the South African Department of Health (2006) compiled specific guidelines applicable to producers for fresh fruit and vegetables at point of sale (Table 3). According to these guidelines no *E. coli* and *Listeria monocytogenes* should be detected per gram of product and no *Salmonella* may be present in at least 25 g of product. Other requirements state a coliform concentration ≤200 cfu.g^{-1} of product and ≤100 000 cfu.g^{-1} of yeast and moulds (Table 3).

Table 2 Guidelines for assessing the potential health risk for the four recommended water uses (DWAF, 2002)

Faecal coliforms.100 ml^{-1}					
Low	**High**		**Sensitive Water Use**	**Narrative Identifier**	**Abbreviated Identifier**
1	10	1	Drinking untreated water	Guideline: >10 = high risk when drinking untreated water	Untreated
600	2000	2	Full or partial contact	Guideline: >2 000 = high risk from full or partial contact	Contact
1000	4000	3	Irrigation of crops eaten raw	Guideline: >4 000 = high risk when irrigating crops that are to be eaten raw	Irrigation
2000	20000	4	Drinking after limited treatment	Guideline: >20 000 = high risk when drinking after only limited treatment	Limited treatment

Several South African retail organisations have additionally established their own and much stricter set of microbial specifications which stipulate the exact requirements to which their products must conform (Table 4). These specifications were formulated

with the consumers' health and the finest quality as main priority and adapted from the British Retail Consortium's set of standards (Carstensen, 2007). The retail organisations perform their own microbial testing on all products upon arrival at their distribution centres in order to establish whether or not it meets the necessary requirements (Carstensen, 2007). A specified number of samples (n) for each incoming batch has to be analysed and the data can then be evaluated according to specific minimum (m) and maximum (M) values with only a certain number of samples allowed to vary between the maximum and minimum (m-M) category. All products exceeding the acceptable allowed microbial limits (class s) are then rejected. Based on these results products will either be accepted and distributed to the various stores or rejected and returned to the supplier.

The retailers insist on having the presence of *E. coli* and *Staphylococcus aureus* evaluated daily, *Clostridium perfringens* weekly and *Listeria monocytogenes* monthly and require levels ≤100 cfu.g^{-1}, ≤1 000 cfu.g^{-1}, ≤10 000 cfu.g^{-1} and ≤100 cfu.g^{-1}, respectively (Table 4).

Table 3 Guidelines formulated by the Food Control Directorate of the Department of Health (2006) for the interpretation of data based on the microbial analysis of food

Food type	Analysis	Limits
Raw vegetables and raw fruits, including fresh fruit salad, salad dressing, peanut butter and cheese	Coliform count	<200.g^{-1}
	Yeast and mould count	<100 000.g^{-1}
	E. coli	0.g^{-1}
	Salmonella spp.	0.25 g^{-1}
	L. monocytogenes	0/g^{-1}

G. CURRENT CONDITION OF THE SOUTH AFRICA'S WATER RESOURCES

Numerous studies (Ashton, 1995; Taylor *et al*., 2001; Barnes, 2003; Barnes & Taylor, 2004; Thiere & Schulz, 2004; Jagals *et al*., 2006; Jackson *et al*., 2009; Nleya & Jonker,

2009) on the quality of South Africa's water resources have been conducted in the past and the results obtained by the various institutions such as the CSIR, University of Stellenbosch, NNR (National Nuclear Regulator) and DWAF ultimately all pointed to one important fact: South Africa is currently facing a life-threatening water crisis as a result of the polluted rivers. Although problems related to the national water crisis differ significantly across the country, the North Eastern parts focussing more on chemical pollution and the Western Cape struggling predominantly with faecal pollution, corrective action has to be taken urgently.

Table 4 Microbial standards for fresh produce as required by certain South African retailers (Carstensen, 2007)

Organism	Test frequency	n	c	Limit per ml or gram			
				m	m-M	M	S
E. coli	Daily	5	2	<20	20 – $<10^2$	$\geq 10^2$	N/A
S. aureus	Daily	5	1	<20	20 – $<10^2$	10^2–10^3	$\geq 10^3$
Bacillus cereus	Weekly	5	1	$<10^3$	10^3– $<10^4$	10^4-$<10^5$	$\geq 10^5$
Salmonella spp.	Daily	10	0	ND in 25 g	-	-	Detected in 25 g
Clostridium perfringens	Weekly	5	0	<20	20 – $<10^2$	10^2–10^4	$\geq 10^4$
E. coli 0157*	Daily	5	0	ND in 25 g	-	-	Detected in 25 g
Listeria monocytogenes	Monthly	5	2	<20	20 - $<10^2$	N/A	$\geq 10^2$

n- number of samples that have to be tested; c- number of samples allowed to fall in the m-M category; m- minimum limit; M- maximum limit; s- unacceptable microbial load, product rejected

In 1998, Barnes and Taylor (2004) initiated a research project based on the impact of formal and informal urban developments on the water quality and subsequent health risk of the Plankenburg River (Boland, Western Cape). Faecal coliform and *E. coli* concentrations from eight sites distributed along the entire length of the river were

monitored every six weeks to investigate the effect of seasonal variation on the pollution levels found in the river (Barnes, 2003). Results of this study, as summarised in Tables 5 and 6, indicated unacceptably high levels of faecal coliforms for most of the 6 years (1998 to 2003) with the exception of the sampling point "Before Kayamandi" which is situated upstream from the informal settlement and unaffected by the unsanitary practises downstream. Pollution levels were more often than not dangerously higher than either the ≤1 000 cfu.100 ml^{-1} guideline as required by the WHO (1989) or ≤4 000 cfu.100 ml^{-1} as stipulated for faecal coliforms by DWAF (2002). The data summarised in Table 5 also indicate, in some instances, lower faecal coliform concentrations during the winter months, most probably as a result of the lower river temperatures and increased winter rainfall during this period (Barnes, 2003).

Table 5 Faecal coliform concentrations (cfu.100 ml^{-1}) detected at four sampling points in the Plankenburg River for a period of five years (Barnes, 2003)

Date	Sampling Points			
	Before Kayamandi	Below Kayamandi	Adam Tas Bridge	Die Boord
May 1998	12 000	16 000	17 000	11 000
Aug 1998	329	172 300	3 290	4 930
Dec 1998	6 310	792 000	17 240	10 860
Jan 1999	347	493 000	792 000	4 930
Jun 1999	10 860	49 300	22 120	10 860
Dec 1999	329	4 930 000	490 000	172 400
Jan 2000	130	17 420 000	944	10 860
Jun 2000	493	2 640	6 700	1 406
Dec 2000	493	3 290 000	10 860	10 860
Jan 2001	3 290	3 290 000	79 200	12 990
Jul 2001	278	32 900	16 600	9 200
Dec 2001	221	69 900	49 300	264 000
Jan 2002	493	17 500	9 440	3 290
Jun 2002	3 454	493 000	3 290	14 060
Oct 2002	1 300	129 000	7 000	2 310

The results given in Table 6 indicate high *E. coli* concentrations at all four sites over the five year sampling period. High levels of *E. coli*, the well-known indicator

organism, gives an indication of the faecal pollution of these water resources, and therefore the presence of other pathogenic organisms (Barnes, 2003) in this river.

Untreated sewage discharge and polluted surface runoff from informal settlements were identified as the leading contributing factors to this pollution problem (Barnes, 2003).

Table 6 *Escherichia coli* concentrations (cfu.100 ml^{-1}) detected at four sampling points in the Plankenburg River over a period of five years (Barnes, 2003)

Date	Sampling Points			
	Before Kayamandi	Below Kayamandi	Adam Tas Bridge	The Boord
May 1998	6 300	16 000	11 000	7 000
Aug 1998	329	172 300	3 290	3 290
Dec 1998	6 310	792 000	10 860	7 920
Jan 1999	347	493 000	129 900	4 930
Jun 1999	10 860	49 300	14 060	10 860
Dec 1999	329	4 930 000	490 000	108 600
Jan 2000	130	12 990 000	944	10 860
Jun 2000	221	1 660	6 700	631
Dec 2000	493	2 310 000	7 000	10 860
Jan 2001	3 290	2 310 000	79 200	12 990
Jul 2001	278	23 100	6 800	4 930
Dec 2001	221	69 900	49 300	264 000
Jan 2002	493	9 440	9 440	3 290
Jun 2002	3 454	493 000	3 290	10 080
Oct 2002	1 300	129 000	4 560	2 310

Additional research conducted on the quality of the Plankenburg and Eerste Rivers by monitoring chemical parameters such as pH, conductivity, alkalinity and COD (Ngwenya, 2006) as well as metal contamination (Jackson *et al*., 2009) emphasised the deteriorating water quality of both rivers. An overall trend of an increasing pH was observed over the study period (1990-2005) with pH values in the Eerste River increasing from 6.78 (upstream) to 7.6 downstream to where the Plankenburg and Eerste River merge (Ngwenya, 2006). Conductivity measurements of water obtained from the Eerste River also increased significantly from 10.5 $mS.m^{-1}$ (upstream) to 40 $mS.m^{-1}$ downstream where the Plankenburg and Eerste River merge (Ngwenya, 2006). Nitrate and nitrite concentrations, phosphates, and the COD increased systematically

with concentrations of 0.5 – 4.0 mg.L^{-1}, 0.1 – 3.1 mg.L^{-1} and 15 – 42 mg.L^{-1}, respectively (Ngwenya, 2006). According to these results, the water quality in the Eerste River deteriorates along with the distance downstream, implicating human practices such as fish farming as possible sources of pollution (Ngwenya, 2006).

Metal contamination of water from the Plankenburg River in 2009 showed very high aluminium and iron concentrations ranging up to 48 mg.l^{-1} and 14 363 mg.l^{-1}, respectively. These results are worrying as organisms may use these metals for a variety of growth related functions and their presence could have harmful long-term effects on human health (Jackson *et al*., 2009).

In 2007, the Water Research Commission (WRC) initiated a research project that investigated the transfer of potential pathogenic organisms to fresh produce (Lö tter, 2010. Microbiological analysis was done on water from the Mosselbank River (Boland, Western Cape) and produce (cabbage and lettuce) irrigated with water from this river. Results obtained during this study are presented in Table 7 (Lőtter, 2010).

Table 7 Faecal coliform and *E. coli* concentrations detected during the microbial analyses of irrigation water and irrigated produce (Lőtter, 2010)

	Mosselbank irrigation water		**Lettuce**		**Cabbage**	
Date	**Faecal coliforms**	***E. coli***	**Faecal coliforms**	***E. coli***	**Faecal coliforms**	***E. coli***
March 2008	ND	ND	ND	ND		
			ND	ND		
April 2008	ND	ND	ND	ND		
			4.5	TG		
May 2008	33	TG	4.5	TG		
			330	TG		
May 2008	49	ND			1.8	ND
					1.8	ND
June 2008	7.8	TG			2	ND
					ND	ND
July 2008	32	TG			ND	ND
					1 600	TG
Sept 2008	490 000	TG			ND	ND
					7.8	TG

ND – None detected; TG – Typical growth

The results presented in Table 7 indicated that the faecal coliform concentrations in the Mosselbank River rarely exceeded the ≤1 000 cfu.100 ml^{-1} WHO (1989) guideline. In July 2008 a faecal coliform concentration of 1 600 cfu.100 ml^{-1} was recovered from cabbage with much lower concentrations (32 cfu.100 ml^{-1}) detected in the corresponding irrigation water. Results obtained in April 2008 presented positive results for the detection of *E. coli* on lettuce, but none detected in the irrigation water. Although these irregular occurrences could possibly be justified by the possible build-up of organisms due to repeated irrigation sessions, the need for more extensive research and the investigation of a possible correlation between the extent of transfer of pathogenic organisms from irrigation water to produce and the extent of pollution in the river water needs to be investigated further. The low faecal coliform concentrations detected in the river water could also possibly be ascribed to lower average water temperatures and increased rainfall during the autumn and winter months.

The potential of agricultural run-off and especially pesticides, to pollute fresh water resources and impact the aquatic macro-invertebrate fauna was investigated by routine analyses conducted on water samples from the Lourens River, Western Cape (Thiere & Schulz, 2004). Water samples were obtained 24 h after each rainfall event from November 2001 to January 2002. Results indicated the first sampling site, situated upstream from all agricultural practices, to be free of pesticides. Further downstream, however, results indicated increased levels of pesticides with 0.05 $\mu g.L^{-1}$ azinphos-methyl (AZP) and 0.02 $\mu g.L^{-1}$ malathion (MLT) detected respectively, insecticide levels in this range of concentrations reported to affect aquatic invertebrates (Thiere & Schulz, 2004). Consequently, seven out of 17 taxa (*Demoreptus* sp., *Castanophlebia* sp., *Aphanicera* sp., *Petrothrincus* sp., *Leptoceridae*, the Helodidae larvae and water mites were significantly less abundant downstream. Therefore, based on the differences in the macro-invertebrate community at the two sampling sites, it was concluded that pesticide run-off caused a significant impact on the deteriorating water quality of the Lourens River (Thiere & Schulz, 2004). Co-workers

In Gauteng, South Africa, Taylor and colleagues (2001) used sensitive HAV-and HAstV-specific RT-PCR-oligonucleotide probe assays in conjunction with cell culture amplification for the routine detection of hepatitis A virus (HAV) and human astrovirus (HAstV) in surface water samples obtained from the Klip River and Vaal Dam. These surface water resources are used for domestic and recreational purposes and the presence of HAV and HAstV, highly infectious viruses associated respectively with

hepatitis A outbreaks and gastroenteritis in hospitalised patients, therefore present a definite health risk to especially young children and the elderly (Taylor *et al*., 2001). A total of 51 weekly samples were obtained from both the river as well as the dam from June 1997 to May 1998. Results indicated the presence of HAV in 35.3 and 37.3% of the river and dam water samples, respectively, whilst HAstV was detected in 21.6% of the river and 5.9% of the dam water samples. Both these viruses are excreted in human faeces, therefore results not only indicated the possible health threat as direct result of the presence of HAV and HAstV, but also the faecal pollution of these resources and therefore the presence of other potential pathogenic organisms.

The water quality of another crucial water resource, the Crocodile River catchment in Eastern Gauteng, which supports an assortment of land uses, amongst others the largest irrigation areas in South Africa, was investigated in a study conducted by Ashton *et al.* (1995). These researchers combined geographical data such as geology, soil and climatic patterns with water quality data (Ashton *et al*., 1995). Acceptable water quality in the upper reaches, some deterioration in the middle region and poor water quality, particularly during dry seasons in the lower parts of the river indicated a progressive decrease of water quality with distance along the river (Ashton *et al*., 1995). Levels of total dissolved solids such as electrical conductivity, chloride and ammonium, frequently exceeded proposed limits required to maintain adequate water quality standards (CSIR, 1994), thereby indicating pollution caused by domestic land uses (Ashton *et al*., 1995). Discharge of sewage effluent and surface run-off from informal settlements were identified as the major contributing factors to cause these elevated ammonium levels.

It is thus clear that the water from the Western Cape is severely polluted. This pollution varies between faecal and agricultural, both of which presents serious health implications to both humans and animals.

H. OUTBREAKS RELATED TO POLLUTED IRRIGATION WATER AND CONTAMINATED PRODUCE

Water can act as vehicle of transmission for infective agents such as bacteria, viruses and parasites. These can cause infections like *Salmonella* typhoid, cholera, hepatitis A, rotavirus, giardiasis and hookworm infections (Wenhold & Faber, 2009). Outbreaks related to polluted water can either be linked directly or indirectly. The consumption of polluted water that results in a waterborne infection is an example of a directly linked

incident. In 2000, the WHO estimated 1.5 billion people to suffer from some sort of waterborne disease at any given moment and calculated about 3.4 million deaths per annum which can be related to the direct or indirect consumption of contaminated water.

The severity of infections, number of cells to cause infections, fatality rate (Table 8), and symptoms varies per pathogen. Diarrhoea-causing infections are classified amongst the most important cause of global childhood mortality and morbidity and are estimated to cause 2.2×10^6 of the 3.4×10^6 deaths per year (Wenhold & Faber, 2009). According to the WHO Global Health Statistics 77 344 cases of diarrhoea per 100 000 people occurred internationally in 1990, causing 2.9×10^6 deaths of which mostly were children under 5 years of age (WHO, 2000).

Table 8 Characteristics of infections caused by pathogenic organisms that may originate from polluted water (Goss & Richards, 2008)

Pathogen	Disease severity	Fatality case rate (%)	No. of cells to cause infection
E. coli O157:H7	Moderate-severe	2	$<10^3$-10^9 CFU
Salmonella	Mild-severe	0.1	1-10^9 CFU
Campylobacter	Mild-moderate	0.05	500 CFU
Cryptosporidium	Mild-severe	-	10-30 Oocysts
Giardia	Mild-moderate	-	10 Cysts

The largest waterborne disease outbreak in Canada took place during May 2000 in Walkerton, Ontario. Although the actual pathway between the pollution sources and ground water resources could never be confirmed, the presence of pathogenic organisms (originating from manure) in water were identified as the cause of 2 300 cases of gastroenteritis and seven deaths (Goss & Richards, 2008).

Nowadays, the focus has however shifted towards the indirect consequences of using contaminated water during the production of food-related products. A prime example being the application of polluted water onto the surface of fresh produce during irrigation sessions. Fresh produce contaminated with pathogenic organisms could lead to the infection of many people with harmful organisms, especially if consumed raw or in a minimally processed state (Beuchat, 1995). The impact of polluted irrigation water on the safety of fresh produce is therefore a critical problem. Every type of fruit or

vegetable can be contaminated, for this reason many different pathogenic organisms have been isolated from various commodities in the past (Table 9).

Internationally, foodborne illnesses related to the consumption of contaminated fresh produce occur frequently and are well documented, but in South Africa, however, such incidences are uncommon (Barnes, 2003). This can be ascribed to the lack of an established data basis for the documentation of food-related illnesses as well as misinformed consumers. In general, consumers have certain misconceptions regarding food infections; they are inclined to automatically associate the cause of their illness with some sort of protein-related food, ultimately causing most sources of outbreaks to be overlooked.

Table 9 Fresh produce from which pathogenic organisms have been isolated (Brackett, 1998)

Product	**Pathogen**
Alfalfa sprouts	*Aeromonas*, *E.coli* O157:H7
Asparagus	*Aeromonas*
Bean sprouts	*L. monocytogenes*, *Salmonella*
Beet leaves	*Salmonella*
Broccoli	*Aeromonas*
Cabbage	*E. coli* O157:H7, *L. monocytogenes*, *V. cholerae*, *Salmonella*
Carrots	*Staphylococcus*
Cauliflower	*Aeromonas*, *Salmonella*
Celery	*Aeromonas*, *E. coli* O157:H7
Cilantro	*E. coli* O157:H7
Coriander	*E. coli* O157:H7
Cucumber	*L. monocytogenes*
Green onion	*Shigella*
Lettuce	*Salmonella*, *Staphylococcus*, *Aeromonas*, *Shigella*, *E. coli* O157:H7
Mushrooms	*Salmonella*
Radish	*Staphylococcus*, *L. monocytogenes*
Salad greens	*Salmonella*, *S. aureus*
Salad vegetables	*Shigella*, *S. aureus*, *L. monocytogenes*, *Yersinia enterocolitica*
Spinach	*Aeromonas*, *Salmonella*
Tomato	*L. monocytogenes*, *Salmonella*
Watermelon	*Salmonella*

Large volumes of untreated sewage and other wastewater that are pumped into rivers and dams in South Africa contribute to the substantial increase in concentrations of potentially pathogenic organisms in water from these resources. According to previous research (Barnes, 2003; Barnes & Taylor, 2004; Jagals *et al*., 2006; Jackson *et al*., 2009) these harmful organisms occur in concentrations much higher than the required guidelines and could subsequently contributed towards a higher rate of foodborne incidences associated with the consumption of fresh fruit and vegetables. In these cases infections range from mild diarrhoea to more serious cases such as hepatitis A and cholera and could in severe cases even result in death. The implications of foodborne outbreaks are thus much more far reaching than one might expect. According to a study published in 1999, pathogenic organisms were thought to cause around 76 million food-related illnesses per year in the USA alone of which 325 000 cases involved hospitalisation and nearly 5 000 cases resulted in death (Mead *et al*., 1999). All of these incidences involve great discomfort and financial losses to the patient, a decrease in productivity as a result of sick-leave and consequently financial losses to the patient's employer, and most important of all, damage to public trust.

A high risk of infection is associated with the consumption of fresh fruit and vegetables since they are most often consumed raw or in a minimally processed state. Hurdles such as washing and rinsing of vegetables have been proven to be insufficient (Brackett, 1998; Steele & Odumeru, 2004; Warner *et al*., 2008) and do little to decrease the risk of pathogen transmission. According to Zhang *et al*. (1995) and Zhang & Faber (1996), sanitizers and surfactants were found to be inadequate in reducing concentrations of *Listeria monocytogenes* and *Salmonella*. Researchers therefore concluded that sanitizers can not guarantee complete elimination of pathogens from produce.

Many sources promoting or contributing to the contamination of fresh produce during the cultivation there-of have been identified (Fig. 3). These include faecal pollution from human, domestic and wild animals, and ineffective sewage treatment works (Beuchat, 1995; Duffy *et al*., 2004), fertiliser which has not been properly composted (Heaton & Jones, 2007), contaminated equipment (Gomes da Cruz *et al*., 2005), unhygienic practices of food handlers and contaminated irrigation water (Ibekwe *et al*., 2004; Siro *et al*., 2005).

In 2005, however, Stein *et al*. identified irrigation water as the leading cause of contaminated produce when it was established that about 50% of all incidences of fresh produce contamination with faecal pollution were connected to the irrigation water.

Outbreaks related to the consumption of fresh or minimally processed fruit and vegetables include widespread Salmonellosis (Siro *et al*., 2005), a Cholera epidemic which occurred in 1970 in Jerusalem that pointed towards the illegal use of untreated wastewater for the irrigation of vegetables (Shuval *et al*., 1997) and *E. coli* O157:H7 outbreaks in Montana and Connecticut, USA, linked to the consumption of lettuce and green salad (Steele & Odumeru, 2004).

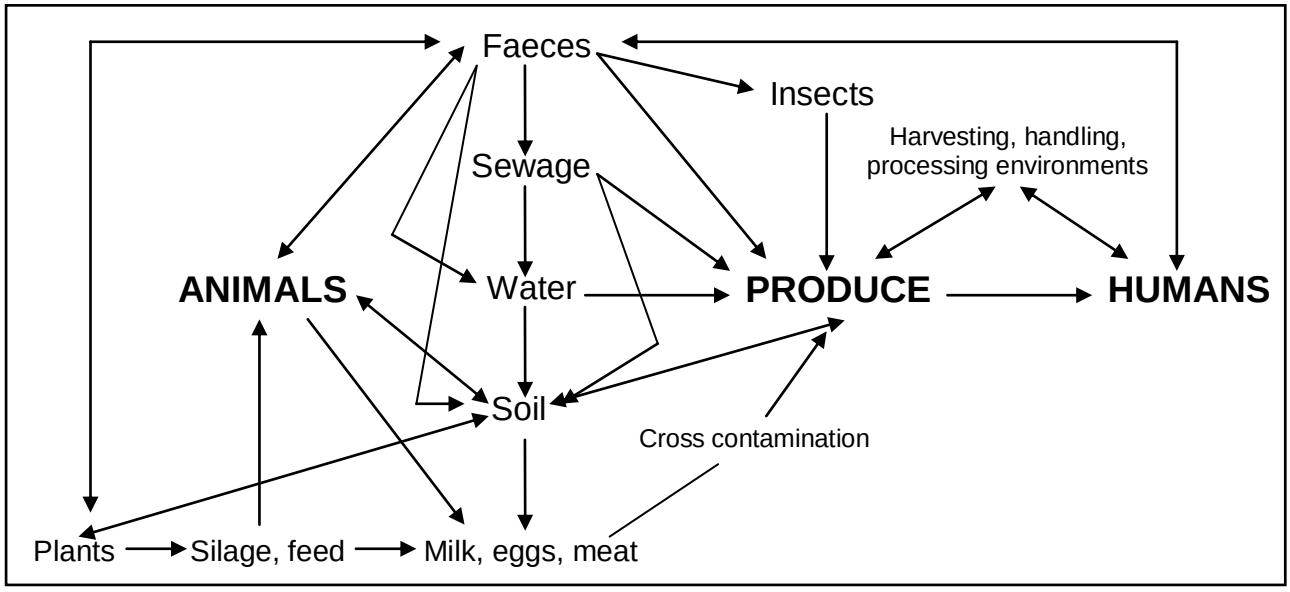

Figure 3 Mechanisms by which produce can become contaminated and then serve as vehicle of human disease (Beuchat, 1995)

In another case, iceberg lettuce contaminated with *Shigella sonnei* caused a serious outbreak of food infections in several European countries, of which 110 confirmed cases occurred in Norway and in the same year, 11 students from the University of Texas tested positive for *E. coli* O157:H7 infections after eating at a local salad bar (Beuchat, 1995).

The same scenarios most likely exist in South Africa, ignorance is just preventing the truth from being revealed. There is thus an urgent need to setup a reliable system where foodborne illnesses can be reported, documented and monitored.

I. MICROBIAL SOURCE TRACKING

When taking into account the serious threat that high levels of pathogenic organisms present to the health of humans and animals, the identification of the specific source of faecal pollution is of utmost importance in order to successfully control the problem (Lu *et al*., 2004; Field & Samadpour, 2007; Robinson *et al*., 2007; Parajuli *et al*., 2009).

The identification and elimination of faecal pollution sources by means of microbial source tracking techniques is therefore both useful and of great value as this would protect and prevent further damage to the environment and promote food safety (Lu *et al*., 2004) by contributing significantly towards the production of microbiologically safe fruit and vegetables (Lu *et al*., 2004).

Microbial source tracking (MST) refers to the use of various genotypic, phenotypic and chemical tests with the purpose of identifying the source of pollution (Graves *et al*., 2009; Stapleton *et al*., 2007; Hartel *et al*., 2008; Parajuli *et al*., 2009) and differentiate between the different contributors of a diffuse source of pollution (Stapleton *et al*., 2007). Although the microbiological analyses for the detection of indicator organisms can be used to determine the magnitude of a pollution problem, microbial results gives no indication of the source of the pollution (Stapleton *et al*., 2007; du Preez *et al*., 2008; Moussa & Massegale, 2008).

MST is based on the concept that different sources of contamination possess fundamental component, of both chemical and microbial origin. Unique characteristics of these components can be used to differentiate between the different sources of pollution (Field & Samadpour, 2007; Moussa & Massengale, 2008). The choice of a particular tracking component is determined by both the type and discriminating power of the analytical method used (Yan & Sadowsky, 2007). Chemical tracking components include caffeine, corprostanol and optical brighteners (Yan & Sadowsky, 2007), whilst microbial tracking components refer to microbiological species associated with a particular source of contamination (Graves *et al*., 2009). Microbial source tracking methods can be divided into two groups based on the specific type of microbial tracking component used. Host-specific methods include the tracking of organisms such as *Enterococcus columbae* that only occur in the intestines of pigeons (Graves *et al*., 2009). Serotypes of F+RNA coliphages are unique to human and animal faeces (Yan & Sadowsky, 2007) and used during population-specific methods. In contrast, species-specific methods are regarded as the most discriminatory as this method is capable of identifying host sources at animal population levels (Yan & Sadowsky, 2007).

Numerous chemical, biochemical and molecular techniques, although not new, have in the recent past been used to develop MST techniques (Stapleton *et al*., 2007; Moussa & Massengale, 2008). These can be classified as either library-dependant or library-independent techniques. Library-dependant techniques include carbon-source utilisation profiling (CUP), DNA fingerprinting, fatty acid methyl ester (FAME) and antibiotic resistance profiling (ARP). These techniques rely on an already established library consisting of characteristic profiles of isolates of known origin. Libraries may consist of thousands of profiles, as the source predictability increases with the number of profiles. It is important that the library represents each likely source of pollution evenly in order to prevent biased predictions. Libraries should be frequently updated by the addition of new profiles as great variance exist between isolates originating from the same species.

Library dependant source tracking techniques involves the characterisation of profiles of known and unknown isolates using specific statistical analyses. These include techniques such as Discriminant Analysis (DA), Jacknife Analysis (JA) and Cluster Analysis (Moussa & Massengale, 2008) to predict the source of indicator organisms. DA is used to classify unknown isolates into source groups based on a 'rule' developed from a calibration data set (Robinson *et al*., 2007). This classification 'rule' is based on the distribution distances between library fingerprints of unknown isolates and the centroid of each group to predict the likelihood an isolate belonging to a particular group (Robinson *et al*., 2007; Moussa & Massengale, 2008). It is, however, important to note that disproportionate libraries may create unstable or biased source estimates (Robinson *et al*., 2007). Furthermore, the accuracy and representativeness of the source tracking technique can be determined based on the average rates of correct classification (ARCC) (Moussa & Massengale, 2008).

Library-independent genotypic techniques aimed at 'fingerprinting' genetic characteristics of micro-organisms by identifying genetic markers specific to a particular host (du Preez *et al*., 2008) include ribotyping, REP-PCR (repetitive extragenic palindromic polymerase chain reaction), PFGE (pulsed-field gel electrophoresis) and DGGE (denatured gradient gel electrophoresis) (Field & Samadpour, 2007; Leach *et al*., 2008).

The choice of MST techniques is influenced by factors such as the specific aim of the study, the amount of detail required, the accessibility of resources and equipment, number of sources as well as the costs involved (Stapleton *et al*., 2007).

Carbon-source utilisation profiling

CUP uses the Biolog Micrologll Identification System and Biolog GN2 microplates to generate unique profiles of known and unknown isolates based on their carbon-source utilization ability. This system allows the simultaneous identification and profile generation of isolates using Biolog GN2 which are coated with 95 different carbon sources coupled to tetrazolium dye (Moussa & Maasengale, 2008). Isolates are classified according to their ability to utilise these carbon sources as illustrated by a colour change of which the turbidity is measured using a plate reader. In 2003, Hagedorn *et al*. obtained a 92.7% ARCC (average rate of correct classification) for CUP when they identified the source of various isolates obtained from water samples. The CUP technique is relatively quick (24 h) and simple to use, but also very costly, therefore limited research has been conducted using this technique.

DNA fingerprinting - Polymerase chain reaction

PCR is regarded as a very promising option, but once again limited research has been conducted on this technique due to the shortage of ideal markers and low output (Yan & Sadowsky, 2007). This technique is based on the degree of genetic relatedness between different isolates determined by amplified fragment length polymorphism (AFLP) fingerprinting (du Preez *et al*., 2008). In 2004, however, Robinson conducted a study on increased faecal coliform levels in a coastal watershed in Mississippi using rep-PCR as source tracking technique. Three potential sources of contamination; dog, gull and sewage were identified. Various shortcomings of this technique such as the variable rate of isolation, confirmation and the selection of unique fingerprint patterns by removing identical fingerprints from the same sample led to “disproportional” representation amongst the different source candidates. This raised concerns of possible biased identifications.

In another study the genetic relatedness of various *E. coli* isolates obtained from in-house water storage containers, drinking cups, hand-swabs, cattle dung and spring water were determined using AFLP to identify the most likely origin or place of introduction of *E. coli* (du Preez *et al*., 2008). AFLP is based on the selective amplification of a subset of DNA fragments in order to discriminate between different strains of bacterial species (du Preez *et al*., 2008). Results indicated similarity scores >85% and therefore a high frequency of identical *E. coli* genetic patterns at various points within the domestic pathway. Although AFLP proved to have high discriminatory

powers, universal applicability, good reproducibility and capable of determining genetic relatedness, the high genetic diversity for *E. coli* bacteria isolated from different sources hampered the identification of post collection contamination sources (du Preez *et al*., 2008).

Pulsed-field gel electrophoresis

PFGE is based on comparisons of the genetic relatedness of different indicator isolates. A high degree of similarity indicates a high probability that the organisms originate from the same source. Previous studies, however, indicated significant diversity amongst the genotypes of isolates within a subset of *E. coli*. This ultimately creates doubt regarding the credibility of this technique (Lu *et al*., 2004). Overall, the biochemical source tracking methods are expensive, time-consuming, and tedious to prepare for routine analyses (Castro *et al*., 1991).

Multi-antibiotic resistance profiling

Multi-antibiotic resistance (MAR) profiling, a species-specific phenotypic method (Yan & Sadowsky, 2007), is based on the resistance of faecal isolates, such as *E. coli*, to a selection of antibiotics at different concentrations, for the generation of resistance profiles that are unique to a specific isolate (Carroll *et al*., 2009). MAR is regarded as the best-analysed MST method (Moussa & Massengale, 2008) and has the advantage of being a reliable, rapid, inexpensive and straightforward test with a high output capacity (Yan & Sadowsky, 2007). This can easily be used to replace the more time-consuming and expensive biochemical tests (Castro *et al*., 1991).

MAR is classified as a library-dependant technique (Field & Samadpour, 2007) as it relies on a previously set-up library consisting of unique antibiotic resistance profiles determined by the genetic ‘fingerprints’ from isolates of known origin (Robinson *et al*., 2007; Carroll *et al*., 2009; Moussa & Massengale, 2008). These isolates with their unique resistance profiles are grouped into different classes based on the source of pollution from which they originated. An example of these classes are environmental, human and animal sources of pollution. Based on similar genetic characteristics, profiles of isolates originating from unknown pollution sources can be compared to the library of known sources to attain a percentage of probability of the most likely source of contamination for that particular isolate (Yan & Sadowsky, 2007; Carroll *et al*., 2009; Moussa & Massengale, 2008). In order to obtain sufficient discrimination between

different source isolates the reference library has to be compiled to contain the maximum number of profiles of known isolates, preferably a few hundred (Carroll *et al*., 2009). The correct statistical algorithm should then be used to prevent biased predictions whilst also ensuring that each source candidate is well-represented (Robinson *et al*., 2007). This library should also be constantly updated and extended in order to ensure that it continues to maintain a sufficient predictive ability (Carroll *et al*., 2009). The construction of known-source libraries are, however, often limited by the availability of samples and the ability to correctly collect and process these samples. This could lead to disproportional representation amongst source candidates and consequently biased source classification (Robinson *et al*., 2007).

The specific combination of antibiotics used during an investigation can vary according to the number, type and concentration of antibiotics that are used. Whilst previous research studies only between 8 to 9 different types of antibiotics (Carroll *et al*., 2009; Parajuli *et al*., 2009) others have included as many as 13 (Peters *et al*., 2003) and even 15 different types (Olaniran *et al*., 2009). Antibiotics can be classified in different classes based on their structure and mode of action (Table 10).

Antibiotics generally administered to humans and domesticated animals are most often chosen for MAR due to the generation of very distinctive profiles. Isolates from human and animal origin will most likely indicate a relatively higher degree of resistance towards specific antibiotics than isolates originating from environmental sources (Carroll *et al*., 2009). Research conducted by Castro *et al*. (1991) also indicated a strong correlation between the resistance profiles of related strains, thereby confirming the use of multi-antibiotic resistance patterns as a good source tracking technique.

In the recent past, an increased level of antibiotic resistance of several pathogens have come to light (Campos, 2008; Olaniran & Naicker, 2009). The resistance which bacteria present towards specific antibiotics can either be natural or acquired. Some organisms are naturally able to withstand the inhibitory effect of selected antibiotics as they (antibiotics) tend to vary both in structure and mechanism of action (Miranda *et al*., 2009) and are therefore not effective in inhibiting every type of pathogenic organism.

Organisms do acquire an increased resistance to some antibiotics. This could be as a result of antibiotic abuse as well as the exposure of organisms to environmental changes and pollutants which cause selective pressure (Miranda *et al*., 2009; Olaniran & Naicker, 2009). Consequently, antibiotic treatments are becoming increasingly

Table 10 Types and concentrations of antibiotics that have been reported in the literature (Castro *et al*., 1991; Peters *et al*., 2003; Edge & Hill, 2007; Carroll *et al*., 2009; Olaniran & Naicker, 2009; Parajuli *et al*., 2009)

Class	Antibiotic	Concentration ($\mu g.L^{-1}$)
β-lactam	Ampicillin	5-32
Cephalosporin	Cephalothin	5-32
	Chlortetracycline	20-80
Phenicol	Cloramphenicol	5-32
	Erythromycin	25-100
	Irgasan	0.01-0.5
	Kanamycin	1-16
	Oxytetracycline	1-16
	Penicillin	25-100
Aminoglycoside	Streptomycin	1-16
	Sulfamethoxazole	50-512
Tertacycline	Tetracycline	1-16
	Amoxicillin	5-20
Cephalosporin	Cephalothin	10-100
	Erythromycin	20-200
Aminoglycoside	Gentamicin	20-80
	Ofloxacin	5-20
	Chlortetracycline	20-80
Tertacycline	Tetracycline	20-80
	Moxalactam	5-20
Fluoroquinolone	Ciprofloxacin	5
Quinolone	Nalidixic acid	30
Cephalosporin	Cefuroxime	30
Cephalosporin	Cefotaxime	30
Cephalosporin	Ceftriaxone	30
Cephalosporin	Cefoxitin	30
Aminoglycoside	Tobramycin	10
Aminoglycoside	Amikacin	30
Aminocoumarin	Novobiocin	30
	Carbencillin	100
	Cephalosporin	30
	Ceftazidine	30
	Clindamycin	2
	Colistine	10
	Ticarcillin	75

ineffective in fighting bacterial infections and even resulting in some antibiotics such as novobiocin becoming completely ineffective, leaving humans and animals unprotected against some harmful pathogenic organisms.

A library dependant technique such as MAR has its own set of disadvantages, specifically in terms of structure complexity and high costs involved when establishing a library. One advantage is that library-based techniques can be used in instances were genetic markers have not yet been identified (Edge & Hill, 2007).

In 1991, Castro *et al*. used MAR to distinguish between several *Bacillus* species that were thought to be of great biotechnological interest. Twenty different antibiotics at varying concentrations were used to generate individual resistance profiles. Based on the results, Castro *et al*. were able to classify the unknown *Bacillus* strains into five different groups: *B. circulans*; *B. subtilis*; *B. polymyxa*; and *B. amyloliquefaciens*, thereby replacing traditional tedious test methods.

In another study a library consisting of antibiotic resistance patterns of 1 005 *E. coli* isolates originating from both human and non-human sources was established (Carroll *et al*., 2009) to aid in the identification of faecal pollution sources. Eight different types at four concentrations of antibiotics were used. Resistance profiles were generated for a total of 199 unknown *E. coli* isolates and the results analysed statistically using DA with StatisiXL software. Correct classification rates of >80% were achieved during this trial. Although most of the unknown isolates were classified as non-human isolates, the percentage of human isolates were, however, seen to increase at sites downstream of urbanised areas. Based on the results of these, as well as many other studies (Peters *et al*., 2003; Olaniran & Naicker, 2009; Parajuli *et al*., 2009), the use of ARP for identifying various sources of faecal contamination have shown to produce reliable results.

Composite source tracking techniques

Every microbial source tracking technique has its unique combination of strengths and weaknesses. The use of a combination of source tracking techniques to increase the predictive potential and accuracy has therefore been suggested (Mousse & Massengale, 2008).

The efficacy of using multiple source tracking techniques was investigated in 2005 when Genthner *et al*. used MAR and repPCR fingerprinting to source track Enterococci found in the shoreline marine waters of Pensacola Beach, Florida. Results

indicated a higher confidence and predictive accuracy when using the combined MAR and repPCR as opposed to the MAR alone.

The comparison of library dependant, library independent or a combination of both was done by Ahmed *et al*. in 2007 in a study to identify human related faecal pollution in three non-sewage catchments. The library dependant methods included existing metabolic fingerprinting libraries of *E. coli* and Enterococci and carbon-source utilization processes whilst library-independent techniques comprised of PCR to detect host-specific markers. A total of 550 *E. coli* isolates and 700 Enterococci isolates obtained from 18 water samples were biochemically fingerprinted and compared to metabolic fingerprint libraries of 4 508 *E. coli* and *4 833* Enterococci isolates. Based on the *E. coli* data, human biochemical phenotypes were identified in 50% of the water samples whilst 56% of the water samples were linked to faecal pollution based on the Enterococci data. Results showed library dependant and library independent source tracking to compliment each other by respectively providing additional information when the other failed to detect human faecal pollution (Ahmed *et al*., 2007).

In 2008, Moussa and Massengale conducted a similar study on *E. coli* isolates using MAR and CUP source tracking techniques. This was done by generating individual MAR and CUP datasets as well as a combined MAR-CUP dataset for *E. coli* isolates obtained from known faecal sources. According to results, 77 of the 95 carbon sources contributed to the differences amongst isolates originating from different animals, therefore the remaining 23 carbon sources were excluded from the remainder of the study. The CUP dataset could only be used to present an ARCC of 86.7% in comparison to the much higher ARCC of 96.7% generated by the composite MAR-CUP dataset. The average frequency of misclassification (AFM) for each method (MAR, CUP and combined) were also determined by dividing the number of isolates classified incorrectly into the total number of isolates of a specific category. Results not only revealed that the MAR-CUP had the lowest AFM, but also the highest predicted accuracy of all three test methods. It was subsequently concluded that combining datasets to create a composite set for analyses provided better discrimination between different source groups (Moussa & Massengale, 2008). Therefore, a combination of methods is recommended to be used to identify the source of faecal pollution (Ahmed *et al*., 2007).

Further research on the different MST techniques would therefore be of great value to develop established and reliable methods for the determination of specific sources of pollution.

DISCUSSION AND CONCLUSION

The existence of a national water crisis in South Africa is undeniable, not only regarding the availability, but especially the quality of the water resources. High levels of indicator organisms indicating faecal pollution and the possible presence of pathogenic organisms have been confirmed. Additionally, the serious health risk associated with the direct contact and the consumption of contaminated water as well as the indirect link with the polluted water when used as irrigation source or during food processing is a serious problem.

From the literature it is clear that although traditional microbiological detection methods have been used to quantify the pollution problem, standards and guidelines have achieved very little in identifying the sources of the pollution. This is a field that needs to be evaluated in further studies. In spite of all the benefits of source tracking techniques in general, MST does not provide the solution which will end the pollution crises. Probably one of the biggest drawbacks of MST is that it presents no indication of the extent of pollution (Stapleton *et al*., 2007). MST techniques are therefore in no way able to replace the traditional microbiological techniques, but can rather be used in combination with them in order to quantify the extent as well as identify the different sources contributing to the pollution crisis (Stapleton *et al*., 2007).

It is important that an environmental forensic technique is able to demonstrate its ability to accurately represent the true state of the environmental contaminants. As a result, most source tracking techniques have not yet been approved as a qualified forensic tool, however, continued development of these methods and further studies would increase the confidence of MST in future (Field & Samadpour, 2007; Stapleton *et al*., 2007). This information will ultimately enable authorities to prioritize the different health hazards and formulate corrective action procedures accordingly that will not only alleviate the pollution in the rivers, but also eliminate the source of pollution (Robinson *et al*., 2007). Once these procedures have been established MST techniques could be used to monitor the success of the new treatment processes. It is therefore advisable to incorporate various MST techniques, such as MAR, in order to identify the various sources of pollution in order to ultimately eliminate them.

As stated before, little is known on a national and international level regarding the contribution of irrigation water and the associated, potentially contaminated raw produce to the burden of disease. A clearer understanding of the problem is urgently required to make inputs for policy formulation and regulation to reduce contamination of irrigation water. It is thus essential to evaluate the extent of the contamination of irrigation water and how serious the link between the water and irrigated produce is and thus to provide recommendations on the way forward in terms of treatment of irrigation water.

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CHAPTER 3

BASELINE STUDY OF THE MICROBIAL LOAD IN THE PLANKENBURG AND EERSTE RIVERS (FEB 2008 – 2009)

ABSTRACT

The potential threat presented by rivers such as the Plankenburg and the Eerste Rivers (Boland, Western Cape) was assessed by determining the microbial quality of these two irrigation sources in an effort to quantify the extent of pollution. This objective was achieved by monitoring four sampling sites allocated along the banks of these two rivers and incorporating several standard microbial methods for the detection of potential pathogenic organisms such as *Salmonella*, *Listeria*, Enterococci, *Staphylococcus aureus* as well as faecal indicator organisms which included total and faecal coliforms and *E. coli*. Although chemical analyses provided contradictory results, microbial results obtained during this study indicated especially high concentrations of faecal indicator organisms in the Plankenburg, and to a lesser extent, the Eerste River. Faecal coliforms and *E. coli* concentrations exceeded the ≤4 000 cfu.100 mL^{-1} guideline (DWAF, 2002) on 67% of the sampling occasions at sites P_1 and P_3, not once at site P_2 and were detected at concentrations as high as 7 x 10^6 cfu.100 mL^{-1}. Enterococci concentrations as high as 6 993 (site P_1) and 2 760 cfu.100 mL^{-1} (site P_3) were also detected. Faecal contaminants isolated from these rivers indicated faecal pollution possibly as a result of the informal settlement and other human activities upstream of the river sampling points. The presence of these indicator organisms did, however, not only indicate unsanitary processes, but also the possible presence of potential pathogenic organisms. Several potential pathogens such as *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Salmonella* were isolated from all three sites. Based on these results the microbial quality of these rivers was found to be of unacceptable standard and it could be concluded that a high health risk was associated with water from these two rivers, especially the Plankenburg River, when used as irrigation sources.

INTRODUCTION

The availability of South Africa's fresh water resources has decreased dramatically over the past decades, influenced primarily by overexploitation and pollution. The increased demand of the growing population for clean potable water along with urbanisation, poverty

and insufficient upgrading and maintenance of water treatment plants have all contributed to the problem (Nleya & Jonker, 2009). Consequently, many of South Africa's most relied on fresh water resources have been polluted to such an extent, making them unsafe for use.

The Plankenburg and Eerste Rivers are two important sources of irrigation water to many farmers in the Boland (Nleya & Jonker, 2009). Due to the collective effect of the poorly serviced Kayamandi Informal Settlement and pollution from wineries and agricultural activities, water quality in this river has deteriorated noticeably (Nleya & Jonker, 2009; Paulse *et al*., 2009). In 2003, Barnes detected *Escherichia coli* concentrations of up to 12.9 x 10^6 cfu.100 mL^{-1} in this river system, in 2009 Paulse *et al*. detected faecal coliforms and *E. coli* at 3.5 x 10^6 cfu.100 mL^{-1}, respectively, whilst Jackson *et al*. (2009) frequently detected metal contaminants that exceeded the recommended water quality guidelines. In 2007, Paulse and colleagues isolated various members of the *Enterobacteriaceae* group including *Serratia* sp., *Citrobacter* sp., *Yersinia ruckeri* and *Enterobacter* sp. from the water of the Plankenburg River. These organisms not only confirmed faecal contamination of this water, but also included *Yersinia* which is regarded as one of the major human pathogens and possibly the causative agent of plague (Paulse *et al*., 2007; Paulse *et al*., 2009). Based on these results, industrial and residential areas were identified as most likely sources of pollution.

Usually characterised as having near pristine water, the water quality from the Eerste River was also found to deteriorate significantly with distance downstream. Conductivity measurements of water samples obtained from this river increased from 10.5 (upstream) to 40 $mS.m^{-1}$ downstream from where the Plankenburg and Eerste River merge. Nitrate, nitrite, phosphates and COD levels increased systematically with concentrations of 0.5 – 4.0 $mg.L^{-1}$, 0.1 – 3.1 $mg.L^{-1}$ and 15 – 42 $mg.L^{-1}$ being recorded (Ngwenya, 2006). Faecal coliform concentrations of up to 3.5 x 10^7 cfu.100 mL^{-1} were detected in the Berg River (Paulse *et al*., 2007). This was mainly ascribed to human activities, fish farming, polluted water from the Plankenburg River and the Stellenbosch Wastewater Treatment Works (Ngwenya, 2006; Paulse et al., 2009).

The use of such polluted water does, however, not only present a health threat when consumed directly, but could also result in a high health risk associated with the consumption of fresh fruit and vegetables as a result of the possible transfer of pathogenic organisms from the polluted irrigation water to the produce (Brackett, 1999; Abadias *et al*., 2008; Goss & Richards, 2008). As a leading African exporter of agricultural products such

as crops, grapes and deciduous fruits, the availability of sustainable fresh water resources is of utmost importance.

The aim of this study was to quantify the microbial pollution levels in the Plankenburg and Eerste Rivers by doing a base-line microbial study of water from selected sites over a period of 13 months. The presence of index and indicator organisms such as coliforms, faecal coliforms, *E. coli*, *Staphylococcus*, *Salmonella* and Enterococci would not only be detected, the concentrations of some organisms was also quantified to give insight into the type and extent of pollution that exist within these rivers.

MATERIALS AND METHODS

Sampling points

During the period of February 2008 to February 2009, monthly water samples were obtained from four sampling sites (sites P_0, P_1, P_2 and P_3) (Fig. 1) in the Plankenburg and Eerste Rivers (sites P_4 and P_5 will be discussed in Chapter 4).

Site P_1 is situated in the Plankenburg River just downstream from the Kayamandi Informal Settlement and the Plankenbrug industrial area on the outskirts of Stellenbosch. This site was chosen as a site indicative of pollution caused by small industries and informal settlements.

Site P_2, in the Eerste River which originates in the Jonkershoek mountains, was situated 200 m upstream from where the Plankenburg and Eerste Rivers merge. This site represents river water flowing through a small town.

The third site (site P_3) was identified at the point where the two rivers (Plankenburg and Eerste Rivers) merge, whilst sites P_4 and P_5 were situated further down the Eerste River as part of an irrigation system.

River sampling site P_0 was added at the end of the sampling period. It was situated upstream of the Kayamandi Informal Settlement and used as a site representing a normal river. This site was also used to determine the impact of the inadequate and in some cases, non-existent sewerage systems in the downstream informal settlements on the water quality of the river.

Sample collection

The sampling procedure was formulated with safety as the main priority. Suitable protective gear including water-proof waders, latex gloves, artificial insemination gloves

and masks were used to minimise the potential danger of exposure to polluted water. Samples were obtained according to the South African Bureau of Standards guidelines (SANS 5667-6, 2006). Samples were collected at each site using sterile 1 L Schott bottles (autoclaved for 20 min at 121°C). Once sterilised, the bottles remained sealed and were only opened beneath the surface during sampling.

The water samples were collected mid-stream and about 30 cm below the surface. In the case were the water depth was less than 30 cm, samples were taken halfway between the water surface and the riverbed as recommended by Barnes & Taylor (2004). All samples were transported in a laboratory container, on ice, and analysed within 60 min of arrival.

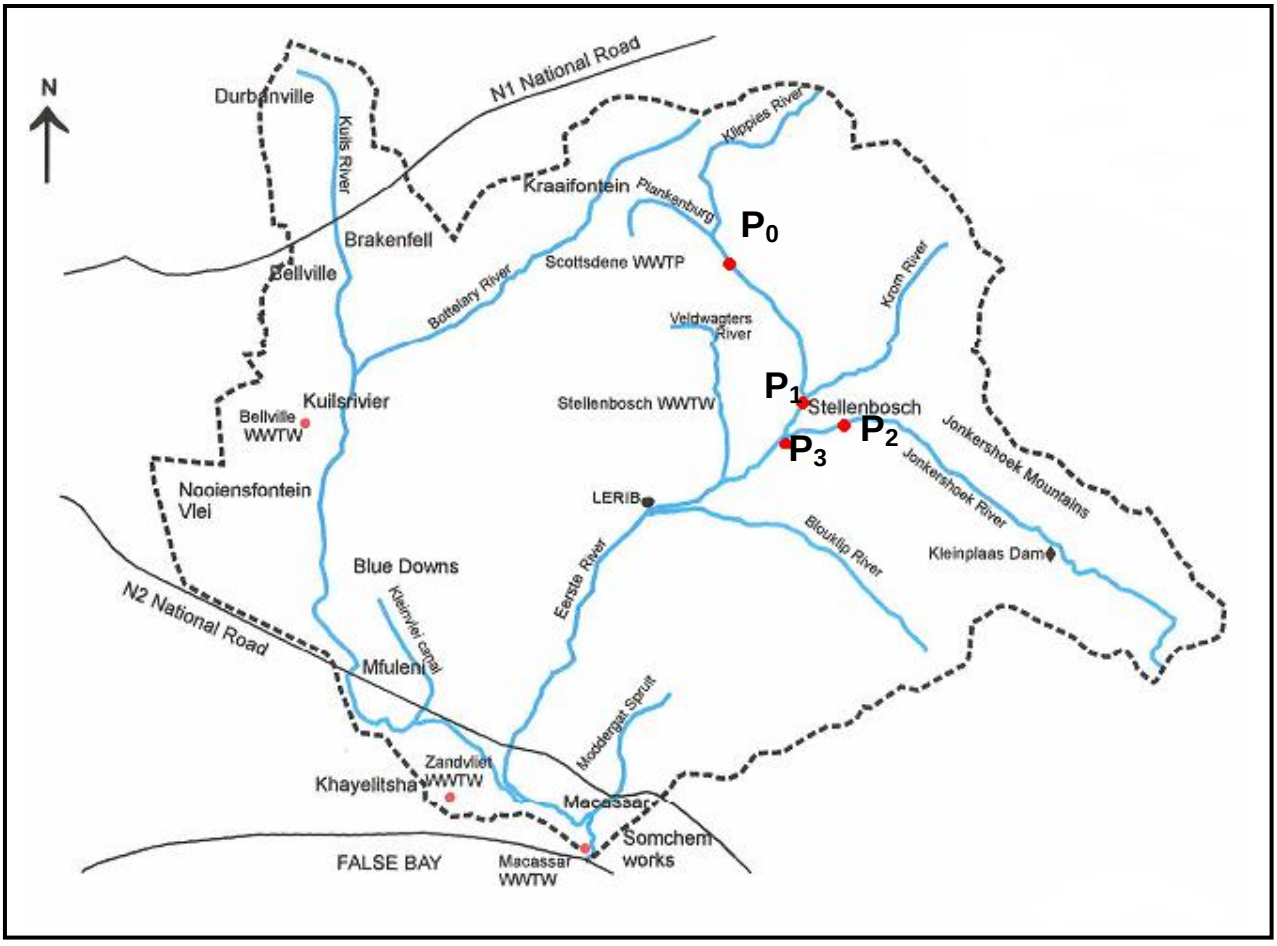

Figure 1 Sampling sites in the Plankenburg and Eerste Rivers (map as given by Ngwenya, 2006)

Environmental parameters

Environmental parameters included the water temperature, river flow and turbidity, and foul smells. Water temperature was measured using a portable battery-operated

thermometer (Crison Thermometer 638Pt) by immersing the probe in the water and allowing the reading to stabilise.

Chemical analyses

pH

The pH of all water samples were measured according to the directions in the instruction manual of the WTW pH 320/SET Microprocessor pH-meter.

Alkalinity

The alkalinity of water samples was determined according to method 2320B given in Standard Methods (APHA, 1998) by titrating 20 mL of sample with 0.1 N H_2SO_4 until the pH of the sample reached 4.3.

Conductivity

A Multi-range Portable Conductivity Meter (Hanna Instruments, HI 8633) was used to measure the ion concentration of the water samples according to the instruction manual.

Chemical Oxygen Demand

Analysis for the determination of the Chemical Oxygen Demand (COD) were performed in duplicate and included a blank sample according to the instructions stipulated in the standard method for the determination of COD 5220D Standard Methods (APHA, 1998).

Microbiological testing

Microbial tests were performed on the water samples to obtain a baseline indication of the microbial loads present at the five sampling points. Standard microbial methods, as approved by the SABS (South African Bureau of Standards), SANS and Canadian Health standards were used during the microbial testing of all the water samples.

Staphylococcus aureus and other species – SANS-6888-1 (1999)

The horizontal method for the enumeration of coagulase-positive staphylococci (SANS-6888-1, 1999) was used for the detection of *Staphylococcus aureus* and other species. This was done by preparing and sterilising Baird-Parker agar (BPA) (Merck) according to the manufacturer's specifications. The agar was allowed to cool to approximately 50°C

before adding 50 mL of the Egg-Yolk Tellurite Emulsion (Oxoid) and finally poured into petri dishes (approximately 25 mL per petri dish) and allowed to set.

A dilution series (10^{-1} - 10^{-8}) was prepared of each sample and 0.1 mL of each dilution transferred to the already prepared petri dishes containing the, now solid, BP agar. Spread plates were prepared, the plates were allowed to dry and finally incubated at 35°C for 48 h. All plates were inspected after the first 24 h of incubation and all typical colonies marked.

After the incubation period all plates were inspected for typical or atypical growth and plates containing between 30 and 300 colonies were counted and the data recorded. Atypical growth could include dark brown or greyish colonies, most likely to be representative of the *Bacillus* species, as well as black colonies, resembling oil drops surrounded by a clear zone, most likely *Staphylococcus aureus*.

Suspect colonies were identified by means of the coagulase test as well as the API Staph test kit (Biomerieux, South Africa). All colonies testing positive for these tests were counted, the dilution factor of the particular plate was factored in and the concentration of cfu.mL^{-1} of sample determined.

Salmonella – SABS-6579 (2004)

The process of detecting different *Salmonella* species was done in accordance to the standard method (SABS-6579, 2004). This method was based on four crucial steps: a pre-enrichment step in a non-selective liquid medium; an enrichment step in selective liquid media; plating out on solid selective media; and the detection as well as final confirmation by means of various serological tests and the API test kit (Biomerieux).

Firstly, 225 mL Buffered Peptone water was prepared according to the manufacturer's instructions (Oxoid). It was then autoclaved for 15 min at 121°C after which 25 mL of the test sample was added and then incubated for 16 – 20 h at 35°C.

Two separate enrichment broths, 100 mL Selenite Cystine (Merck) and 10 mL Rappaport and Vassiliadis broth (RVS broth)(Merck) were then prepared. After the 16 h incubation period of the pre-enrichment step, 10 mL and 0.1 mL of the pre-enrichment solution were, respectively, transferred to the 100 mL Selenite Cystine and 10 mL RVS solutions. Both solutions were then incubated for 24 h, the Selenite Cystine at 35°C and the RVS at 42°C, respectively.

Pre-dried XLD plates (prepared in advance) were then inoculated with the Selenite Cystine enrichment solution by means of a loop wire. This process was repeated for the

RVS solution and plates were incubated inverted at 35°C for 20 – 24 h. Both the Selenite Cystine and RVS solution were again incubated for 24 h as before. After the second incubation period, both enrichment solutions were again streaked out on pre-dried XLD plates and incubated at 35°C.

Once the incubation periods had passed, all plates were inspected for possible growth. Plates containing slight or no typical colonies (typical colonies appearing black surrounded by a clear zone) were incubated for a further 24 h whilst typical colonies from all the other plates were streaked out individually on marked Nutrient agar (NA) plates and incubated at 35°C for 18 – 24 h. Colonies ultimately obtained from the NA plates were then be identified by means of API 20E (Biomerieux, South Africa).

Intestinal Enterococci – SANS 7899-2 (2004)

This method was based on the principle of filtering a specific volume of water through a membrane filter with a pore size adequate to retain bacteria present in the sample. The filter containing the bacteria was placed on a solid selective media to allow the growth of colonies which can reduce 2,3,5-tripenyltetrazolium chloride, (a colourless dye), to red formazan (SANS 7899-2, 2004).

Slanetz & Bartley agar (SBA)(Merck) was prepared according to the manufacturer's specifications. The agar was sterilised and allowed to cool to approximately 55°C. One gram of 2,3,5-triphenyltetrazolium chloride (TTC)(Merck) was suspended in 100 mL of distilled water and filtered through a 0.2 µm filter (Merck) to sterilise the solution.

When the SBA had cooled down, 10 mL of the TTC solution was added and mixed well by inversion. Approximately 15 mL of agar was then poured into small petridishes and allowed to set. Once the petri plates containing the Slanetz & Bartley agar had been prepared 100 mL of the sample water was filtered through a sterile 0.45 µm membrane filter (Merck) and transferred to the surface of a clearly marked (date and site number) petridish containing the SBA. The plates were then incubated at 36°C for 44 h. After the incubation period, all typical colonies which included any colonies that appeared red, maroon or pink, in the centre or throughout the entire colony, were counted and the results recorded.

BEA was prepared according to the manufacturer's (Merck) specifications, sterilised and cooled to approximately 55°C. It was then poured into small petridishes (approximately 15 mL per petridish) and allowed to set. The membrane filter was transferred from the SBA to a clearly marked pre-heated (44°C) BEA petridish and incubated at 44°C for 2 h after

which all typical colonies (colonies having a tan or black colour) were counted as intestinal Enterococci and recorded as cfu.100 mL^{-1} of the test sample.

Listeria monocytogenes – SABS 11290-1 (1996)

The process for the isolation and identification of *Listeria monocytogenes* from water samples comprises of four crucial steps, the first being the primary enrichment step. This step is performed using a selective liquid enrichment medium that contains a reduced amount of selective agents. The second step is a secondary enrichment step, using a liquid enrichment medium with full concentration of selective agents. Whilst the third and fourth steps include plating out on Oxford and Palcam selective media, and confirmation of all typical colonies isolated from the inoculated plates (SABS 11290-1, 1996).

Half-strength Fraser selective enrichment medium was prepared according to the manufacturer's specifications, sterilised and allowed to cool down. The contents of one vial of Fraser Selective Supplement (Oxoid) was then reconstituted and added to the aseptic Fraser solution.

Full-strength Fraser was prepared according to the manufacturer's (Oxoid) specifications. One vial of Fraser Selective Supplement was reconstituted and added to the aseptic solution.

The cooled half-strength Fraser selective enrichment medium (10 mL) was transferred aseptically to a sterile test tube. One millilitre of the water sample was added to the clearly marked (date and site number) test tube and incubated at 30°C for 24 h.

Oxford *Listeria* Selective agar (Oxoid) and PALCAM agar (Oxoid) were both prepared according the manufacturer's stipulations. Both agars were autoclaved for 15 min at 121°C and cooled to approximately 50°C in a pre-heated waterbath. One vial of Listeria Selective Supplement (Oxoid) was reconstituted and added to the OLS agar. The agar was then poured into petri dishes, approximately 25 mL per dish and allowed to set.

The content of one reconstituted vial of PALCAM Selective Supplement (Oxoid) was added to the PALCAM agar. The agar was poured into petri dishes (about 25 mL per plate) and allowed to set.

After the incubation period of the test tube containing the half-strength Fraser supplement, the surface of a clearly marked Oxford and PALCAM plate was inoculated with the suspension from the test tube by means of a sterile loop wire. The inoculated plates were incubated micro-aerobically at 35°C for 24 h. If no growth was detected after the first incubation period, a second incubation period of about 18 – 24 h was allowed.

After the respective incubation periods had passed all typical colonies were selected and streaked out on Tryptone Soya (TS) agar (Oxoid) to obtain isolated colonies which could be used during further confirmation tests.

Meanwhile, 1 mL of the first suspension (10 mL half-strength Fraser and 1 mL water sample) was transferred to a second sterile test tube containing 10 mL of full-strength Fraser Selective Medium and incubated at 35°C for 48 h. After the incubation period, Oxford and PALCAM plates were again inoculated with the suspension in the test tube. Inoculated plates were incubated micro-aerobically at 35°C for 24 h. The plates were incubated for a further 18-24 h if no growth was observed after the initial incubation period. Afterwards, all typical colonies were selected and streaked on TS agar to obtain well-isolated colonies which could be used during further confirmation tests. Pure colonies obtained from the TS agar were then identified by means of API *Listeria* (Biomerieux, South Africa).

Aerobic colony count technique – SABS 4833 (2007)

The purpose of conducting an aerobic colony count was to obtain an indication of the number of micro-organisms present in 1 mL of sample (SABS 4833, 2007).

This test was done by preparing Plate Count agar (PCA)(Merck) and Bacteriological agar (BA)(Merck) according to the manufacturer's specifications, sterilizing both suspensions and allowing it to cool to about 50°C in a pre-heated water bath.

A dilution series (10^{-1} to 10^{-8}) was prepared from the sample and starting at the highest dilution (10^{-8}), 1 mL of each dilution was transferred to an individual petri dish which had been clearly marked with the date, sample number and corresponding dilution. Approximately 18-20 mL of PCA was then poured into each petri dish and mixed by rotating the dishes.

Once the PCA had set, a thin layer of BA was poured over the PCA and once again allowed to set. Finally, all plates were incubated at 30°C for 72 h after which all plates containing 30–300 colonies were counted and the data recorded.

Aerobic and Anaerobic Endosporeformers – MFLP-44 (1998)

The determination of the aerobic and anaerobic endosporeformers present in a sample was performed in order to attain an indication of the number of viable endosporeforming organisms present in a particular sample (MFLP-44, 1998).

Tryptone Soya agar (TSA) was prepared according to the manufacturer's specifications and sterilised for 15 min at 121°C.

Sterile test tubes containing 9 and 18 mL PSS, respectively, were prepared in advance by autoclaving (sealed with test tube caps) at 121°C for 15 min. After the test tubes had cooled, 2 mL of the sample was added to the test tube containing the 18 mL of sterile PSS. The test tubes were then placed into a pre- heated waterbath (75°C) for 20 min.

A dilution series (10^{-1} to 10^{-3}) was prepared using the test tube containing 9 mL sterile PSS. Starting at the highest dilution, 1 mL of each dilution was transferred aseptically to clearly marked (site number, date and dilution) petri dishes and approximately 25 mL of TSA added to each. Dishes were swirled to ensure proper mixing of contents and allowed to set. This step was done in duplicate and clearly marked as either aerobic or anaerobic.

All aerobic dishes were incubated inverted at 35°C for 48 h, whilst the anaerobic dishes were incubated anaerobically at 35°C for 48 h. After the incubation period all dishes containing between 30 – 300 colonies were counted and the results recorded.

Coliforms, faecal coliforms and E. coli – Health Canada (2002)

The Multiple Tube Fermentation (MTF) method (Health Canada, 2002) is based on the ability of organisms to ferment lactose present in Lauryl Sulphate Tryptose (LST) broth (Oxoid) and consequently produce gas as by-product. Gas production from the LST broth serves as confirmation of coliforms, whilst the production of gas from Brilliant Green Bile broth (BGLB)(Oxoid) is an indication of the presence of faecal coliforms. *E. coli* is detected by gas production in EC broth (Oxoid) and confirmed on Levine Eosin Methylene Blue (L-EMB) agar (Oxoid) as dark centred or black colonies with a metallic sheen. These colonies can then be identified further by means of other biochemical tests such as API 20E.

Double-strength LST broth was prepared according to the manufacturer's instructions and dispensed in units of 10 mL into separate test tubes each containing a single Durham tube (Merck). Four hundred millilitres of LST broth was also prepared according to the manufacturer's specifications. Durham tubes and 10 mL single strength broth were dispensed into 40 test tubes after which they were all capped and sterilised at 121°C for 15 min. All tubes were thoroughly inspected after sterilisation to ensure that they did not contain any gas bubbles trapped in the Durham tubes. The five tubes

containing the double-strength LST broth and the first row (first 5 tubes) containing SS (Single-Strength) LST broth were each inoculated with 10 mL of sample water to create the DS (Double-Strength) and 10^0 dilutions. A dilution series ranging from 10^{-1} to 10^{-7} was prepared. The remaining 35 tubes each containing 10 mL single strength LST broth, were arranged into seven rows, each row containing five tubes. Each row was inoculated with a different dilution by adding 1 mL of the 10^{-1} dilution to all 5 tubes from the first row, 1 mL of the 10^{-2} dilution to all the tubes in the second row and continuing until the 10^{-7} dilution. All DS and SS tubes were incubated at 35°C for 24 h after which all positive tubes were transferred to BGLB as specified. The remaining LST tubes were incubated for a further 24 h, all positive tubes transferred to BGLB and all negative tubes (those that did not contain any gas bubbles in the Durham tube), were discarded.

BGLB broth was prepared according to the manufacturer's (Oxoid) specifications and dispensed into 100 test tubes, each containing 10 mL BGLB and a Durham tube. Tubes were capped and sterilised at 121°C for 15 min.

BGLB tubes were inoculated with positive LST tubes. The inoculated BGLB tubes were incubated for 24 h at 35°C and examined for the formation of gas bubbles in the Durham tubes. All tubes containing bubbles indicated a positive result and transferred to sterile EC tubes. The remaining BGLB tubes were incubated for a further 24 h at 35°C after which positive tube were transferred to EC and the remaining tubes (negative tubes) discarded.

EC broth was prepared by adding 37 g of EC powder (Oxoid) to 1 L of distilled water. One vial of MUG supplement (Oxoid) was reconstituted by adding 2 mL of sterile distilled water and mixing with a Vortex mixer (model number VM 1000). The contents of the vial was added to the sterile EC broth and dispensed, 10 mL per test tube, into 100 test tubes each containing a Durham tube. Test tubes were capped and sterilised at 121°C for 15 min.

Sterile EC tubes were inoculated with positive BGLB tubes and incubated at 44.5°C for 24 h after which they were examined under a ultra-violet light. Tubes that both fluoresced and contained gas bubbles indicated positive reactions. Positive EC tubes were streaked onto L-EMB agar, incubated at 35°C for 18 – 24 h and examined for typical growth.

Plates containing dark colonies with a metallic sheen were regarded as positive for *E. coli.* Further confirmation was obtained by identification using the API 20E Web database (Biomerieux).

Finally, the five tube MPN (Most Probable Number) De Mans Index Table (Health Canada, 2002) along with the number and position of positive LST, BGLB and EC tubes were used to calculate the number of coliforms, faecal coliforms and *E. coli* present in the original test sample.

RESULTS AND DISCUSSION

Sampling frequency

Rivers form part of a constantly changing ecological system, influenced by many external factors which make it difficult to obtain samples indicative of the true state of the ever-changing conditions in rivers. During this study both the sampling size and frequency were a concern as it was impossible to increase the frequency of sampling and sample size indefinitely in order to establish the constant changing levels of pollution. Sampling was conducted according to SANS (SANS 5667-6, 2006) on a monthly basis by collecting units consisting of 1 L each over a period of 13 months.

Environmental parameters

Environmental descriptors including water temperature, river flow, turbidity and odour, were recorded for each site for every sampling occasion.

Colour and turbidity

The colour and turbidity of the water remained constant during the entire sampling period, irrespective of the different seasons. The water colour of site P_1 was usually dark greenish brown and murky (Fig. 2A) with a urine-like stench. Site P_2 was much more serene with clear water (Fig. 2B) and no foul odours. Site P_3 had a very distinct separation line (Fig. 2C) caused at the merging point of the Plankenburg and Eerste Rivers. Sampling was done more to the left (the Plankenburg side) and was therefore associated with a cloudy, brown colour and urine smelling odour.

Water temperature and rainfall

The data in Table 1 shows water temperatures to decrease during the colder winter months with variations from 21.8°C during the months of December, January, February and March to temperatures as low as 10.1 °C (August 2008) recorded during the winter months.

Figure 2 The sampling sites, P_1 (A), P_2 (B) and P_3 (C) showing the strong river flow (left) during winter months and the regular river flow (right) during summer

The Western Cape is a winter rainfall region as indicated by the increased rainfall recorded during the winter months (Table 1). The capability of organisms to survive in nature and their reliance on various environmental factors such as pH, moisture content, sunlight and organic material content is well-known (Islam *et al*., 2004), but the relationship between the survival of organisms and temperature are however unique. The impact of environmental conditions, such as water temperature, on the microbial load in water was monitored by comparing ACC of each site (P_1, P_2 and P_3) to the corresponding water temperatures as shown in Fig. 3.

Although high temperatures are often used in the food industry to reduce microbial numbers and low temperatures to inhibit microbial growth, optimum temperatures specific to each type of organism, are known to promote the growth and survival of organisms. Therefore, temperate conditions in summer were expected to promote microbial growth.

Higher microbial concentrations were consequently anticipated during summer months when temperatures tend to be closer to the optimum growth conditions. Lower microbial concentrations were expected in winter.

It can be seen from the data in Fig. 3 that temperature differences up to 11°C inhibited microbial growth as ACC varied between lower numbers in winter (June to August) and higher numbers (2.7 x 10^6, 7.6 x 10^6 and 400 000 cfu.mL^{-1} at site P_1, P_2 and P_3, respectively) during warmer summer months (December to February). Rainfall also impacted the microbial concentrations as a result of the dilution of the contaminated water and flushing the rivers clean during winter.

The overall high ACC detected over the 13 month sampling period as well as the increase of microbial load just as the rainy season had passed, indicated the possibility of the continuous addition of pollutants to the rivers. This constantly added to the microbial load and consequently prevents the rivers from being permanently cleansed during winter months.

Table 1 Monthly rainfall (Wentzel, 2009) and water temperatures recorded at sites P_1, P_2 and P_3

	Site P_1	**Site P_2**	**Site P_3**	
Date	**Temp °C**	**Temp °C**	**Temp °C**	**Rainfall (mm)**
Feb 2008	20.8	21.8	21.6	45.5
March 2008	15.1	20.6	21.1	24.1
Apr 2008	16.3	15.1	14.8	23.4
May 2008	17.7	17.7	17.5	102
Jun 2008	14	14	14.3	115.6
Jul 2008	Excessive rainfall			260.1
Aug 2008	11.8	10.1	10.4	114.9
Sep 2008	12.6	11.5	Flooding	144.3
Oct 2008	16.6	16.5	16.5	18.6
Nov 2008	18.1	19.1	17.7	47.3
Dec 2008	20.6	21.1	21.1	34
Jan 2009	20.8	21.7	21.8	7.8
Feb 2009	18.1	19.3	18.2	15.4

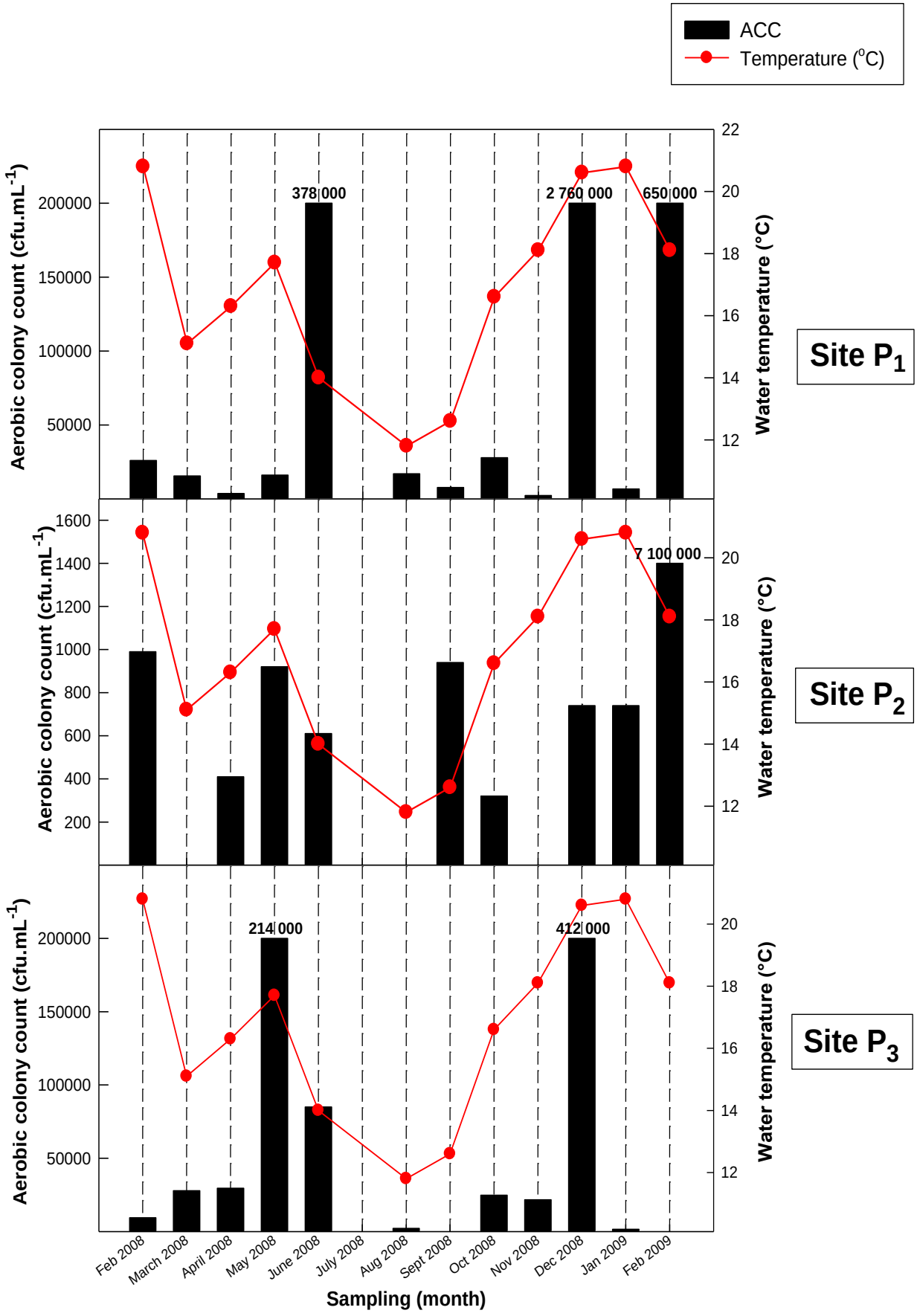

Figure 3 Aerobic colony counts (ACC) and water temperatures of sites P_1, P_2 and P_3. Counts ranging above 200 000 cfu.mL^{-1} were excluded (but the values given) to show fluctuations in the lower ranges

Chemical analyses

pH

The pH at sites P_1, P_2 and P_3 are presented in Table 2. Missing values were as a result of excessive rainfall which prevented sampling as the sampling sites were unsafe.

Table 2 pH and aerobic colony counts (ACC) for the period Feb 2008 to March 2009 for sites P_1, P_2 and P_3

	Site P_1		Site P_2		Site P_3	
Date	**pH**	**ACC** cfu.mL^{-1}	**pH**	**ACC** cfu.mL^{-1}	**pH**	**ACC** cfu.mL^{-1}
Feb 2008	6.80	25 900	6.47	990	6.85	9 400
March 2008	6.38	15 500	6.10	ND	6.31	27 900
April 2008	6.54	3 600	5.72	410	6.95	29 600
May 2008	6.61	16 100	6.05	920	5.78	214 000
June 2008	6.84	378 000	5.86	610	7.24	85 000
July 2008	Excessive rainfall					
Aug 2008	5.64	17 000	6.05	ND	6.52	2130
Sept 2008	7.20	7 700	6.60	940	Flooding	
Oct 2008	6.58	27 800	6.74	320	6.67	24 800
Nov 2008	7.16	2 230	7.05	ND	7.09	21 700
Dec 2008	6.89	2 760 000	6.97	740	6.72	412 000
Jan 2009	6.40	6 700	6.55	740	6.43	1 560
Feb 2009	6.84	650 000	6.59	7 100 000	6.92	ND

*Red values indicate pH values classified as class 2 according to DWAF (1996)
ND – None detected

The pH of water is an important parameter which has to be monitored constantly as irrigation water with a high pH could result in reduced plant growth and consequently a severe reduction in yield. The pH values for the three sites over the 13 month sampling period ranged between 5.64 and 7.24 (Table 2). According to DWAF (1996), the quality of irrigation water can be grouped into three classes based on pH. Class 1 is the most desirable: water with a pH <6.5. Class 2 is water with a pH between 6.5 and 8.4 and Class 3 is water with a pH >8.4. The lower pH of the water at the three sampling sites suggests

that the values of the soil are nearer to natural acidity and will ultimately lead to a lower water pH.

According to the data presented in Table 2, 34% of the results can be classified as Class 1 and 66% as Class 2 irrigation water. A total of 75% of the samples from site P_1 can be classified as Class 2 irrigation water, 50% from site P_2 and 67% from site P_3. Water from site P_1 which appears to be severely affected by the sanitary conditions in the Kayamandi Informal Settlement was expected to have the poorest water quality.

The Eerste River which originates in the Jonkershoek Mountains is less affected by human practices and therefore water from this site (P_2) was expected to exhibit a much better quality, in terms of pH, in comparison to that from site P_1. Data from sampling site P_3, which consists of water from both site P_1 and P_2 showed the dilution effect caused by the less polluted water from the upper Eerste River. Fewer samples from site P_3 were classified as Class 2 irrigation water (67%) in comparison to the 75% of site P_1.

No link could be identified between the two parameters when comparing the pH with the corresponding ACC as an indication of the microbial load present. An example of this was during December 2008 when the ACC of 2.7×10^6 and 740 $cfu.mL^{-1}$ were detected at sites P_1 and P_2, respectively.

In spite of the difference in microbial quantity between the two sites, irrigation water from both these sites were grouped as Class 2 irrigation water based on the corresponding pH values of 6.89 (P_1) and 6.97 (P_2). It was thus concluded that the pH of water as found in this study appears to be a poor indicator of the microbiological quality of the water.

Alkalinity

Alkalinity (as $mg.L^{-1}$ $CaCO_3$) is an indicator of the buffering capacity of water and the water's ability to neutralize acids. Alkalinity was determined for the three sites during every sampling session and the respective averages presented in Fig. 4.

Alkalinity readings varied between 14 and 1 125 $mg.L^{-1}$ $CaCO_3$ over the 13 month sampling period with average readings of 165.25, 55.1 and 72.5 $mg.L^{-1}$ $CaCO_3$ detected at sites P_1, P_2 and P_3, respectively. According to Bailey & Bilderback (2009), horticulture specialists from North Carolina State University, levels of 200 $mg.L^{-1}$ $CaCO_3$ (depicted by the gray line in Fig. 4) are regarded as safe for irrigation of most crops and only when the alkalinity exceed 200 $mg.L^{-1}$ should corrective action be considered. The alkalinity readings at the three sites never exceeded the maximum recommended value, apart from

one exception (1 125 mg.L^{-1} $CaCO_3$) during March 2008 at site P_1. It could therefore be concluded that, in terms of alkalinity, the irrigation water is of good quality.

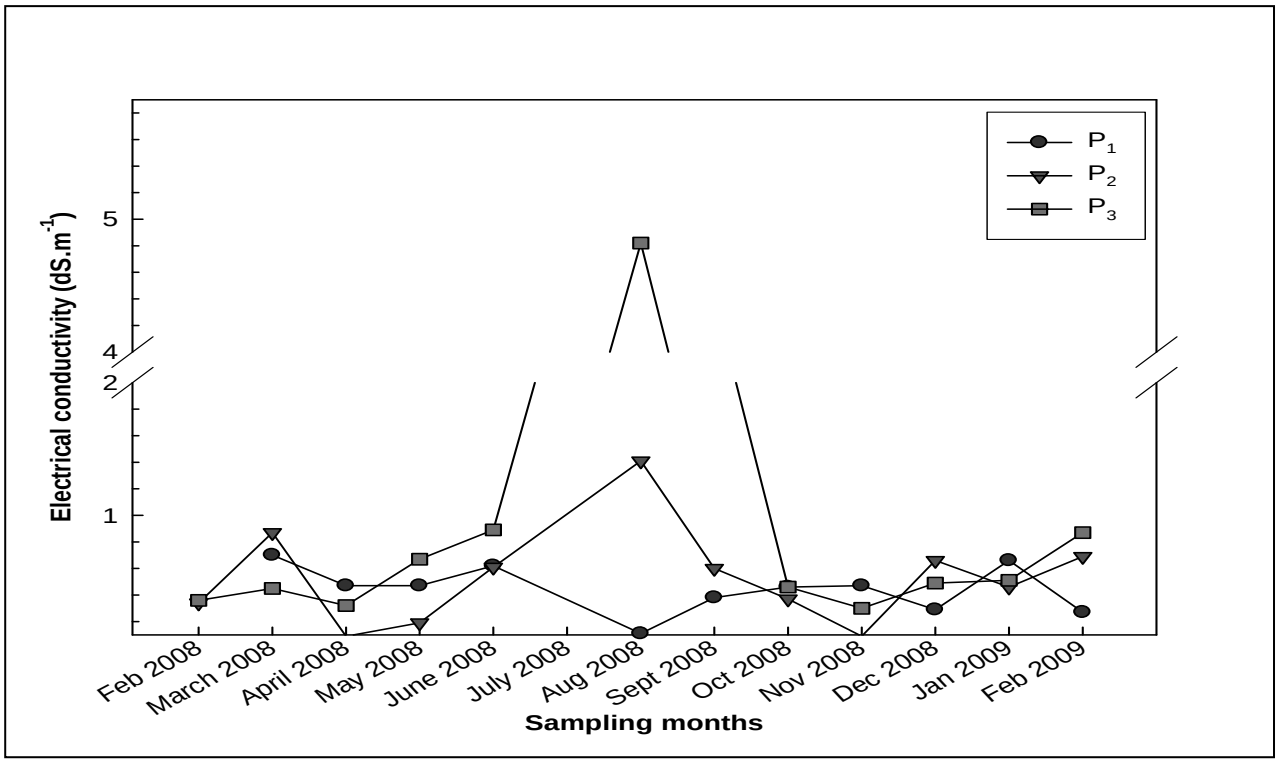

Figure 4 The average (n = 13) alkalinity measurements (mg.L^{-1} $CaCO_3$) for sites P_1, P_2 and P_3. Bars represent the minimum and maximum values. The grey line represents the maximum allowed alkalinity of 200 mg.L^{-1} (Bailey & Bilderback, 2009).

Conductivity

Conductivity, determined by the salinity of water, is a measure of the ion concentration, and regarded as the most important chemical guideline of irrigation water quality (Bauder *et al*., 2009). According to Bauder *et al*. (2009), irrigation water with a conductivity reading ≤0.25 dS.m^{-1} is rated as excellent whilst readings of >3.00 dS.m^{-1} are regarded as unacceptable (Table 3).

According to the data in Fig. 5, conductivity of the water from the sites varied between 0.25 and 1.4 dS.m^{-1} and may be classified, based on the Bauder *et al*., classification system, as excellent, good and permissible (Table 3). In August 2008, a higher value of 4.82 dS.m^{-1} was recorded for site P_3 and according to the classification scale (Table 3) this would be regarded as unsuitable for irrigation.

As indicator of the microbial activity for that month, the ACC of 2 130 cfu.mL^{-1} (site P_3, Table 3) was much lower than the 2.76 x 10^6 cfu.mL^{-1} for December 2008 at site P_1 which

had a conductivity of 0.29 $dS.m^{-1}$, and could, based on the conductivity value, be classified as good quality irrigation water. These trends show the poor association between water conductivity and microbial concentration. This suggests that conductivity is a poor indicator of the microbial quality in water.

Table 3 Suggested conductivity classification for irrigation water (Bauder *et al*., 2009)

Classes of water quality		Conductivity ($dS.m^{-1}$)
Class 1	Excellent	≤ 0.25
Class 2	Good	0.26 - 0.75
Class 3	Permissible	0.76 - 2.00
Class 4	Doubtful[2]	2.01 - 3.00
Class 5	Unsuitable[2]	≥ 3.01

[1]Leaching needed; [2]Good drainage needed and sensitive plants will have difficulty growing

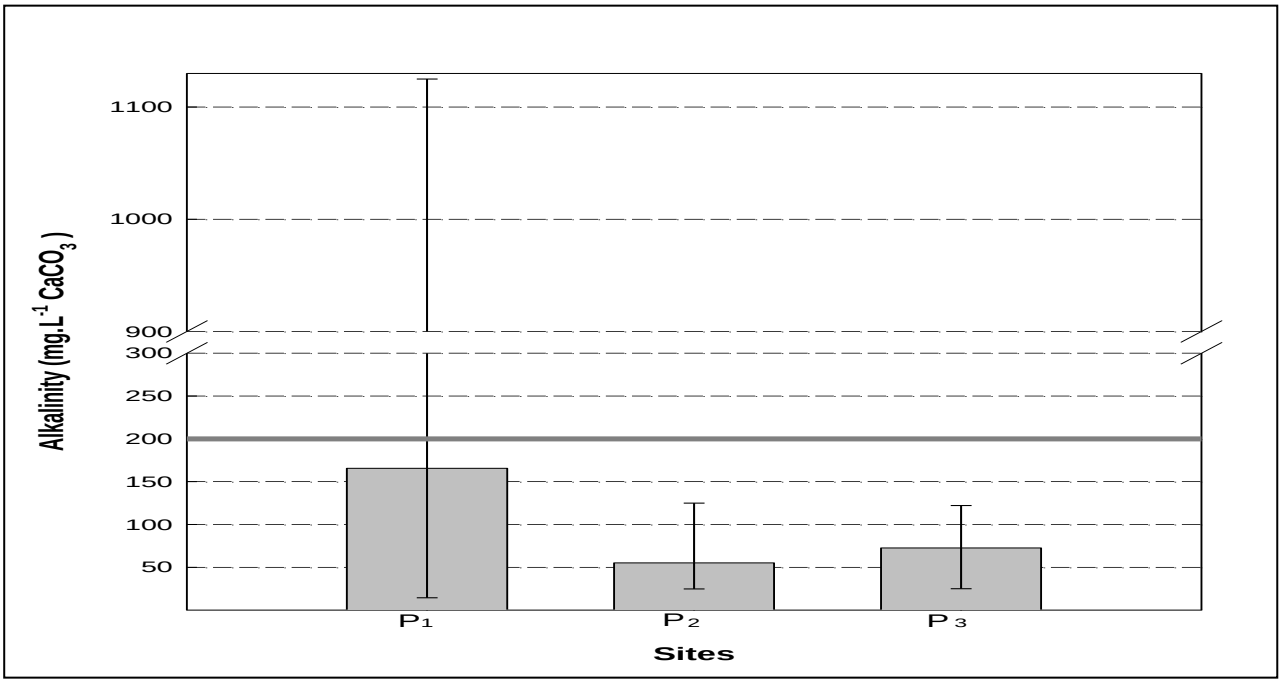

Figure 5 Water conductivity for sites P_1, P_2 and P_3 for the 13 month sampling period

Chemical Oxygen demand

Chemical Oxygen Demand (COD) is generally used as an indicator of water quality as it serves as an indirect indicator of the organic matter content of a water sample. The COD values of the three sites are shown in Fig. 6. Normally, COD concentrations between 50 – 150 $mg.L^{-1}$ would be regarded as satisfactory for treated wastewater whilst those >150

mg.L^{-1} are regarded as marginal and imply that corrective action should be taken to improve the water quality (Bahri, 2009). The COD concentrations in Fig. 6 were found to vary considerably from one sampling event to the next, ranging between 0 and 75 mg.L^{-1}. This variation could possibly lead to the incorrect assumption that the water quality might be microbiologically acceptable.

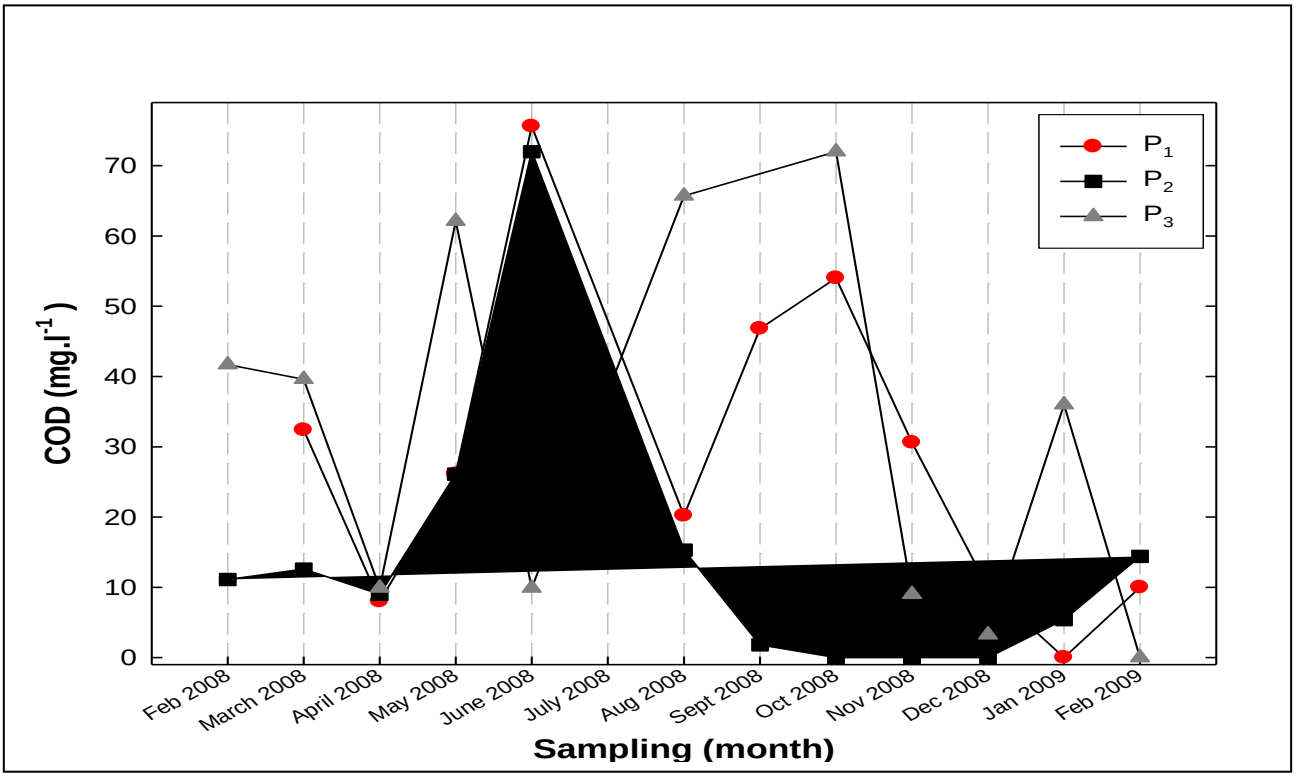

Figure 6 COD values of sites P_1, P_2 and P_3 for the 13 month sampling period

The continuous dilution factor associated with the flowing river water and rainfall is another important aspect that should be taken into consideration. Treated wastewater and polluted water is diluted when discharged into rivers thus, rivers with the same COD concentrations as that of adequately treated wastewater, could possibly indicate the discharge of insufficiently treated wastewater or polluted water into the rivers, further upstream. Therefore, the data presented in Fig. 6 should be classified as marginal, especially data from site P_1 and P_2 in June 2008 and site P_3 during May, August and October of 2008 (Fig. 6). These could possibly indicate the discharge of polluted water into these two rivers.

The COD values (Fig. 6) can also be compared to the ACC of the sites (P_1, P_2 and P_3) as summarised in Fig. 7. No visual trend was observed between the ACC and COD as COD values above 60 mg.L^{-1} were associated with low ACC levels ranging from 2 130 cfu.mL^{-1} (site P_3, August 2008) to 378 000 cfu.mL^{-1} (site P_1, June 2008). Similarly, COD levels

below 20 mg.L^{-1} were associated with high ACC values ranging up to 2.7 x 10^6 cfu.mL^{-1} (site P_1, December 2008) and 7.1 x 10^6 cfu.mL^{-1} (site P_2, Feb 2009). This suggests that high COD levels cannot always be linked to a higher level of microbial pollution. From the data obtained in this study it was concluded that COD should also be considered a poor indicator of the microbial quality of water.

Microbiological testing

Water samples from the three sites (site P_1, P_2 and P_3) in the Plankenburg and Eerste Rivers were subjected to microbial testing for the detection of faecal indicators and potential pathogens. A summary of the data obtained are presented in Tables 4, 5 and 6.

Indicator organisms - total and faecal coliforms

Total coliforms (TC) are Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production at 35°C in 24 to 48 h. They (TC) refer to the family *Enterobacteriaceae* with genera *Escherichia coli*, *Enterobacter*, *Klebsiella* and *Citrobacter* and are used as “indicators” of possible faecal pollution (Hurst *et al*., 2002). TC does, however, have limited use as indicators of faecal pollution as they are able to multiply in warm, tropical climates and present overgrowth on selective medium by non-faecal related colonies (Park *et al*., 2006). Although their presence cannot explicitly be linked to faecal pollution they are, however, still of sanitary significance as they exhibit a survival rate much lower than that of pathogenic organisms (Hurst *et al*., 2002).

Faecal coliforms (FC), also known as thermotolerant coliforms, is a subset of the TC group and regarded as more specific “indicators” of homeothermic faecal pollution (Park *et al*., 2006). This group conforms to all the criteria used to define TC plus the requirement that they grow and ferment lactose with gas and acid production at 44.5°C (Hurst *et al*., 2002). Crucial guidelines formulated by DWAF (2002), for water intended to be used for irrigational purposes require FC concentrations ≤4 000 cfu.100 mL^{-1}. This guideline associates a high health risk with irrigation water when it contains a FC concentration in excess of the allowed 4 000 cfu.100 mL^{-1}.

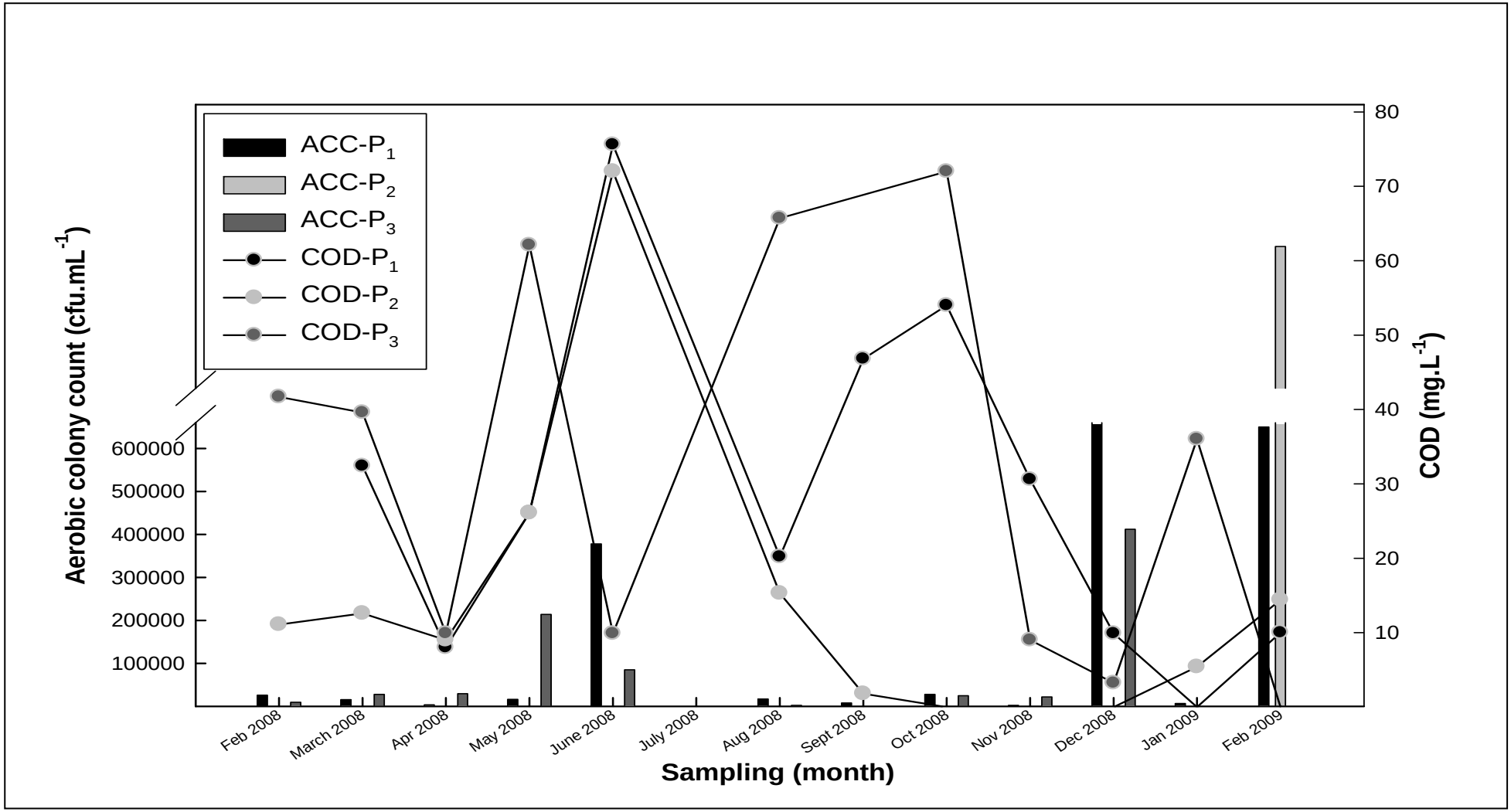

Figure 7 COD and ACC values of sites P_1, P_2 and P_3

Table 4 Results of the microbial testing conducted on water samples collected from site P_1

Sampling	ACC	Aerobic endospores	Anaerobic endospores	Total Coliforms	Faecal coliforms	*E. coli*	*Salmonella*	*Listeria*	*Staphylococcus*	Enterococci
	cfu.mL^{-1}			cfu. 100 mL^{-1}			present/absent		cfu.mL^{-1}	cfu.100 mL^{-1}
Feb 2008	25 900	ND	70	350 000	28 000	TG	ND	TG	390	65
March 2008	15 500	ND	ND	35 000	35 000	TG	TG	TG	ND	8
Apr 2008	3 600	160	0	490	490	TG	TG	TG	0	43
May 2008	16 100	3 600	ND	3 300	1 300	TG	TG	TG	ND	11
June 2008	378 000	370	560	33 000	13 000	TG	TG	ND	ND	152
July 2008	Excessive rainfall									
Aug 2008	17 000	1 200	ND	160 000	160 000	TG	TG	TG	10 000	131
Sept 2008	7700	460	270	22 000	2200	TG	TG	TG	ND	6 993
Oct 2008	27 800	130	231	49 000	33 000	TG	ND	TG	5 200	37
Nov 2008	2 230	3 300	ND	33 000	3300	TG	TG	ND	5 300	114
Dec 2008	2 760 000	ND	ND	790 000	490 000	TG	TG	TG	32 800	1 168
Jan 2009	6 700	16 000	20 000	790 000	790 000	TG	TG	TG	6 900	3 400
Feb 2009	650 000	150	ND	7 000 000	7 000 000	TG	TG	TG	94 000	1 540

TG - Typical growth; ND - None detected; ACC – Aerobic colony count

Table 5 Results of the microbial testing conducted on water samples collected from site P_2

Sampling	ACC	Aerobic endospores	Anaerobic endospores	Total Coliforms	Faecal coliforms	*E. coli*	*Salmonella*	*Listeria*	*Staphylococcus*	Enterococci
	cfu.mL^{-1}			cfu. 100 mL^{-1}			present/absent		cfu.mL^{-1}	cfu.100 mL^{-1}
Feb 2008	990	ND	ND	2 200	2 200	TG	TG	TG	ND	86
March 2008	ND	ND	ND	580	580	TG	TG	TG	720 00	47
Apr 2008	410	ND	ND	790	230	TG	TG	TG	ND	25
May 2008	920	ND	ND	17 000	3 300	TG	TG	ND	ND	9
June 2008	610	ND	ND	1 300	1 300	TG	TG	TG	3 400	38
July 2008	Excessive rainfall									
Aug 2008	ND	42	ND	700	490	TG	TG	ND	ND	2
Sept 2008	940	ND	ND	460	310	TG	TG	TG	ND	45
Oct 2008	320	90	ND	790	790	TG	ND	TG	ND	22
Nov 2008	ND	440	ND	3 300	3 300	TG	TG	ND	ND	172
Dec 2008	740	ND	ND	330	330	TG	TG	TG	ND	12
Jan 2009	740	ND	ND	35 000	ND	ND	TG	TG	ND	86
Feb 2009	7 100 000	ND	ND	7 900	790	TG	TG	ND	800	53

TG - Typical growth; ND - None detected; ACC – Aerobic colony count

Table 6 Results of the microbial testing conducted on water samples collected from site P_3

Sampling	ACC	Aerobic endospores	Anaerobic endospores	Total Coliforms	Faecal coliforms	*E. coli*	*Salmonella*	*Listeria*	*Staphylococcus*	Enterococci
	cfu.mL^{-1}			cfu. 100 mL^{-1}			present/absent		cfu.mL^{-1}	cfu.100 mL^{-1}
Feb 2008	9 400	ND	10	4 900	22 000	TG	TG	TG	3 100	7
March 2008	27 900	ND	ND	130 000	130 000	TG	TG	TG	ND	2 760
Apr 2008	29 600	ND	ND	49 000	2 200	TG	TG	TG	24 300	11
May 2008	214 000	ND	ND	33 000	33 000	TG	TG	TG	2 390	482
June 2008	85 000	360	ND	2 300000	64 000	TG	TG	TG	1.2 x 10^6	26
July 2008	Excessive rainfall									
Aug 2008	2 130	ND	ND	70 000	70 000	TG	TG	TG	ND	683
Sept 2008	Flooding*									
Oct 2008	24 800	340	230	130 000	49 000	TG	ND	TG	2 900	826
Nov 2008	21 700	ND	ND	23 000	23 000	TG	TG	TG	2 600	1 230
Dec 2008	412 000	410	100	130 000	130 000	TG	TG	TG	68 000	736
Jan 2009	1 560	ND	ND	310	310	TG	TG	TG	ND	72
Feb 2009	ND	ND	ND	790	790	TG	TG	ND	ND	35

TG - Typical growth; ND - None detected; ACC – Aerobic colony count; * Flooding prohibited access to this sampling site

The TC and FC concentrations determined for water samples obtained from the three sites (P_1, P_2 and P_3) for the sampling period of 13 months, are illustrated in Fig. 8. This data shows FC concentrations in excess of the ≤4 000 cfu.100 mL^{-1} DWAF guideline (2002) in 67% of the cases at site P_1, not once at site P_2 and again 67% at site P_3. The World Health Organisation (WHO, 1989) guideline is more stringent, only permitting up to 1 000 FC.100 mL^{-1} for water used during unrestricted irrigation of fresh fruit and vegetables. Similar standards required by the Canadian Council of Ministers of the Environment and the European Protection Agency both recommend FC concentrations ≤1 000 cfu.100 mL^{-1} for surface water, including river water used for the irrigation of fruit and vegetables (Steele & Odumeru, 2004). The data summarised in Fig. 8 shows a non-conformance rate to these guidelines of up to 85% of the cases at site P_1, 31% at site P_2 and 69% at site P_3.

TC and FC concentrations exceeding DWAF (2002), WHO (1989), EPA and Canadian regulations (Steele & Odumeru, 2004) could also suggest improper treatment of wastewater, possible pollution of rivers with untreated sewage effluent and therefore a high probability that potential pathogenic organisms will be present in these rivers.

Based on these results a high risk is associated with the transfer of potential pathogenic organisms from the water to produce. It could, therefore, be concluded that water from the Plankenburg and Eerste Rivers not only presents a high health risk but is also unfit to be used as irrigation source. Consequently, South Africa could risk losing much needed export licenses.

Escherichia coli – Indicator of faecal pollution

Escherichia coli, the common cause of infantile, travellers' and bloody diarrhoea as well as acute inflammation of the colonic mucosa (Beuchat, 1995; Jagals *et al*., 2006; Olaniran & Naicker, 2009) is a Gram-negative bacterium which commonly occurs in the intestines of warm-blooded animals and is therefore used as indicator of faecal pollution. The use of *E. coli* as health-related water-quality indicator is based on the assumption that *E. coli* is present in water when faecal material is present, and therefore indicates the presence of potential pathogens (Chandran & Hatha, 2005; Jagals *et al*., 2006; Wilkes *et al*., 2009).

The varying levels of *E. coli* presented in Fig. 9 indicate the presence of this organism at all three sites. With the exception of site P_2, *E. coli* was frequently detected at concentrations higher than 20 000 cfu.100 mL^{-1}, with the highest concentrations found at sites P_1 and P_3. The water quality from site P_1 was considered poor as it failed to comply to the ≤1 000 or the ≤4 000 cfu.100 mL^{-1} WHO (1989) and DWAF (2002) guidelines in 85

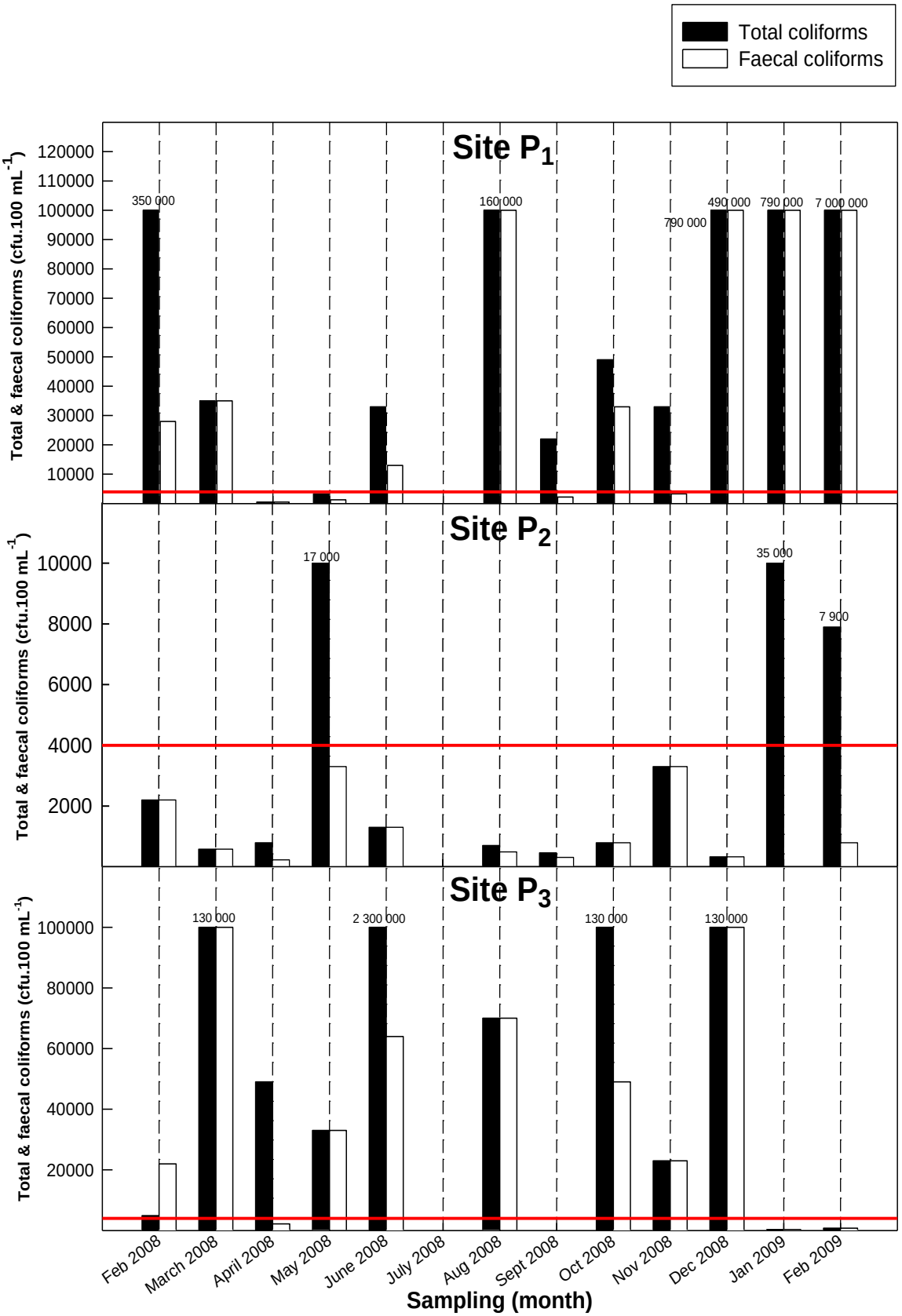

Figure 8 The total and faecal coliforms concentrations from sites P_1, P_2 and P_3. Reference line depicts DWAF's (2002) faecal coliform guideline of 4 000 cfu.100 mL^{-1} for irrigation water. Counts ranging above 100 000 cfu.100 mL^{-1} were omitted (but the values given) in order to show fluctuations in the lower ranges

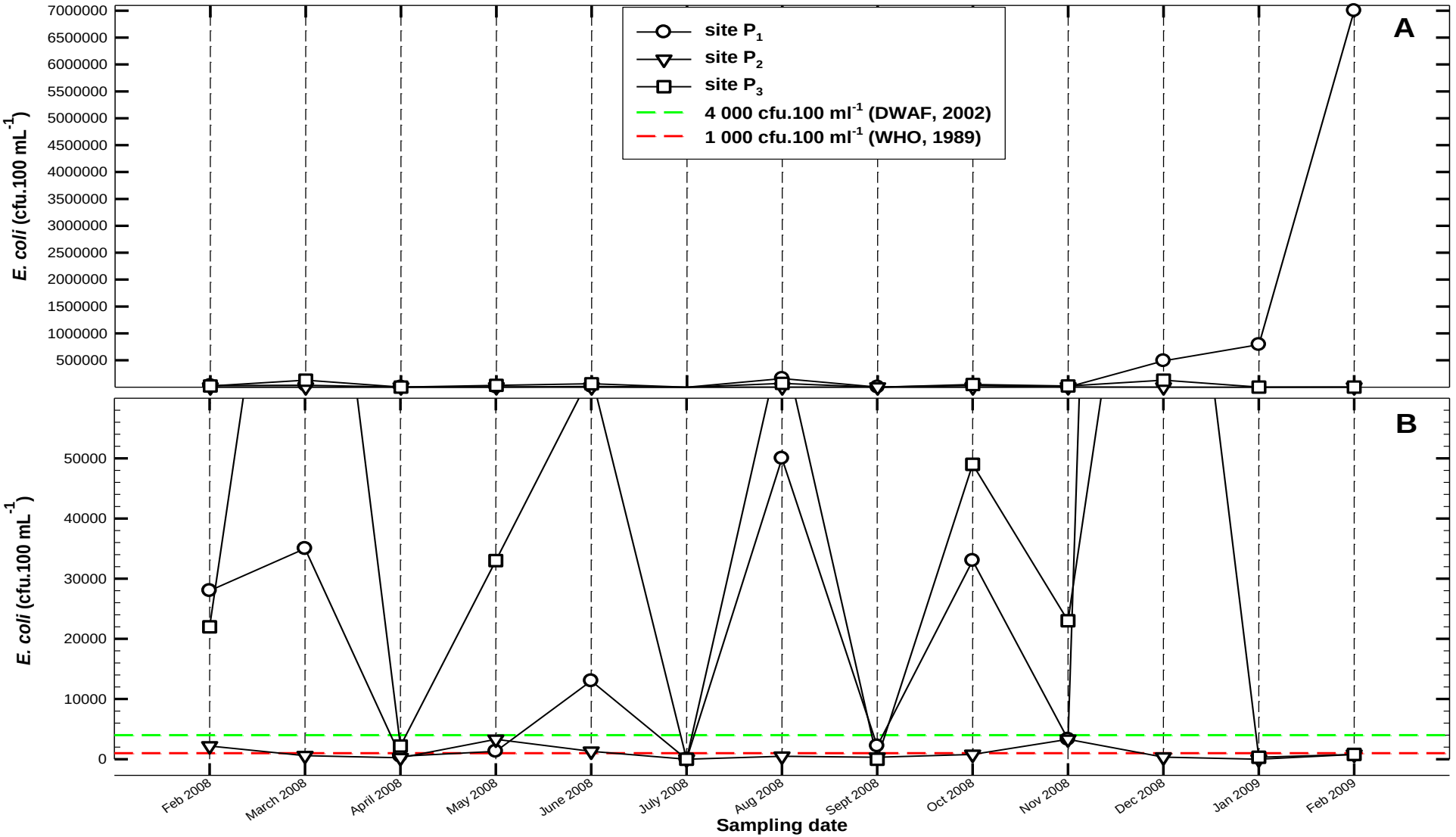

Figure 9 *E. coli* levels of the three sites (P_1, P_2 and P_3) for the sampling period of 13 months. Counts ranging above 50 000 cfu.100 mL^{-1} were excluded from Fig. 9B to show fluctuations in the lower ranges.

and 62% of the sampling sessions, respectively, and contained *E. coli* concentrations ranging up to 7.0 x 10^6 cfu.100 mL^{-1} (January 2009).

Water quality from site P_2 was better as only 8 and 38% of the samples did not comply with the respective WHO and DWAF guidelines (WHO, 1989; DWAF, 2002). Once again polluted water from the Plankenburg River influenced the water quality of site P_3. A total of 62 and 69% of the sampling sessions did not comply with either WHO (1989) or DWAF (2002) guidelines.

In 2009, Wilkes and co-workers studied relationships between indicator bacteria, pathogens and parasite oocysts/cysts, and concluded that the presence of pathogens could be associated with indicator densities greater than 100 cfu.100 mL^{-1}. The excessively high *E. coli* levels detected from Feb. 2008 to March 2009 therefore not only indicated faecal pollution, but also a high risk for the presence of pathogenic organisms.

It could therefore be concluded that water from the Plankenburg River, and to a lesser extent the Eerste River, is not suitable for irrigational purposes as a result of faecal pollution.

Potential pathogenic organisms

Previous microbial results (Figs. 8 and 9) confirmed the presence of faecal indicator organisms such as total and faecal coliforms and *E. coli* at all three sites (P_1, P_2 and P_3), thereby confirming faecal pollution in the Plankenburg and Eerste Rivers. According to recommended guidelines formulated by institutions and governmental agencies such as DWAF (2002) and WHO (1989), a faecal coliform concentration exceeding the recommended concentrations of 4 000 and 1 000 cfu.100 mL^{-1}, respectively, indicates a high risk of infection with pathogenic organisms present in such polluted water resources.

As a result, additional microbial tests for the detection of potential pathogenic organisms such as *Listeria*, *Salmonella* and *Staphylococcus* were conducted and the results presented in Table 7. Whilst some detection methods used in this study give results indicating the concentration (cfu.mL^{-1} sampling water) at which the specific organisms are present, other methods only indicate the presence or absence of a specific microbial type.

All of the organisms which had been tested for were detected at all three sites (Table 7), with the exception of the anaerobic endosporeformers which were not detected at site P_2. The overall potential pathogenic organism concentration was highest at sites P_1 and P_3. This once again suggests that water from site P_1 to be of the worst microbial quality of all three sites. High concentrations of aerobic endosporeformers (16 000 cfu.mL^{-1}), anaerobic endosporeformers (20 000 cfu.mL^{-1}), *Staphylococcus*

(94 000 cfu.mL^{-1}) and Enterococci (6 993 cfu.100 mL^{-1}) were detected at site P_1. Except for *Staphylococcus* concentrations that ranged up to 72 000 cfu.mL^{-1} and *Salmonella* which was detected in 92% of the sampling sessions, the overall microbial load at site P_2 remained much lower than that of site P_1 throughout the 13 month sampling period. At site P_3, however, high microbial loads were once again detected. Microbial concentrations at site P_3 were usually higher than found at site P_2 and lower than that of site P_1 with the exception of the 1.21 x 10^6 cfu.mL^{-1} *Staphylococcus* that was detected at site P_3 in June 2008.

Enterococci, another indicator of faecal pollution (Wilkes *et al*., 2009), was found to be present in all samples at concentrations from 2 to 6 993 cfu.100 mL^{-1} with the highest Enterococci concentration of 6 993 cfu.100 mL^{-1} found at site P_1 in September 2008.

Table 7 Organisms detected at sites P_1, P_2 and P_3 during the 13 month sampling period. Depending on the limitations of the specific identification methods, results are presented either in concentrations of cfu.mL^{-1}, cfu.100 mL^{-1} or the presence/absence of the organism (calculated as the percentage of sampling sessions during which the organism was detected)

Organisms	Site P_1	Site P_2	Site P_3
Aerobic endosporeformers (cfu.mL^{-1})	Max: 16 000 Min: 130	Max: 440 Min: 42	Max: 410 Min: 340
Anaerobic endosporeformers (cfu.mL^{-1})	Max: 20 000 Min: 70	ND	Max: 230 Min: 10
Salmonella (% presence)	83%	92%	91%
Staphylococcus (cfu.mL^{-1})	Max: 94 000 Min: 390	Max: 72 000 Min: 800	Max: 1 210 000 Min: 2390
Listeria (% presence)	83%	67%	92%
Enterococci (cfu.100 mL^{-1})	Max: 6 993 Min: 8	Max: 172 Min: 2	Max: 2 760 Min: 7

Min – Minimum microbial concentration detected for the 13 month sampling period; Max – Maximum microbial concentration detected for the 13 month sampling period; % - Percentage of sampling sessions during which the organism were found to be present; ND – None detected

Listeria and *Salmonella*, two well-known potential pathogenic organisms which have been reported as the cause of serious foodborne outbreaks in the past (Brackett, 1999; Goss & Richards, 2008), were also detected at all three sites. Occurrence rates of these organisms varied from 67 to 92% (*Salmonella*) and 83 to 92% (*Listeria*), respectively (Table 7).

Based on the results from this study it was therefore concluded that the water from these two rivers are polluted with faecal matter and as a result, contain high concentrations of potential pathogenic organisms. The microbial quality of water from these sites (P_1, P_2 and P_3) is therefore unacceptable and presents a health risk to both humans and animals through human contact or when consumed in adequate amounts (Jagals *et al*., 2006).

Comparison of the upstream (site P_0) and downstream (site P_1) water quality

An additional river sampling site (site P_0) 5 km upstream from site P_1 was identified towards the end of the sampling period to asses the impact of the Informal Settlement on the water quality of the Plankenburg River. Samples obtained from site P_0 during four sampling sessions were analysed using the same methods, and the data presented in Table 8.

Table 8 Microbial concentrations detected at sites P_1 and P_0 (n = 4). Depending on the limitations of the specific identification methods, results are presented either present or absent or in concentrations of cfu.mL^{-1} and cfu.100 mL^{-1}

Organisms	**Sites**	
	P_0	**P_1**
Total coliforms (cfu.100mL^{-1})*	390	7 900 000
Faecal coliforms (cfu.100mL^{-1})*	180	160 000
E. coli (cfu.100mL^{-1})	ND	TG
Salmonella (present/absent)	ND	TG
Listeria (present/absent)	ND	TG
Enterococci (cfu.100 mL^{-1})*	45	641
Staphylococcus aureus (cfu.mL^{-1})*	ND	7 583

ND – None detected; TG – Typical growth; * - average of 4 values

Microbial concentrations detected at site P_1 were very high in comparison to the lower concentrations obtained from site P_0 (Table 8). The maximum concentrations of total and faecal coliforms obtained from these sites differed by more than 20 and 10 fold, respectively, and whilst no *E. coli*, *Salmonella* or *Listeria* were detected at site P_0, they were found to be present downstream at site P_1.

The maximum concentration of Enterococci (641 cfu.100 mL^{-1}) detected at site P_1 could also be compared to the much lower load of 45 cfu.100 mL^{-1} detected upstream at site P_0. The trend of continuously detecting low microbial concentrations upstream (site P_0) and very high concentrations downstream (P_1) was, however, expected as these results indicated a pollution source downstream of site P_0 in the vicinity of site P_1, with the Informal Settlement as most likely cause of faecal pollution and confirmed by the high concentrations of faecal indicator organisms.

Microbial diversity in water samples from the different sites

Results obtained during the 13 month sampling period indicated that various hurdles such as enrichment steps, incubation temperatures and selective differential media which form part of the standard identification methods for the detection of specific organisms, sometimes failed to exclude unwanted organisms. As a result, many non-typical colonies were obtained on the final evaluation medium that did not resemble the typical colony characteristics recommended in the standard methods. Examples of such organisms included *Enterobacter aerogenes* and *Klebsiella* spp. that were frequently isolated from XLD agar, which is the selective medium used for the detection of *Salmonella* spp.

All colonies obtained from the various selective media, whether they resembled typical colony characteristics or not, were subjected to further testing to establish the identity of these organisms. Characteristics of purified isolates were determined by means of Gram-staining, catalase and oxidase tests and further characterization with the API test kits (Biomerieux) and the results presented in Table 9. Only API identification results with a ≥98% (classified as good, very good and excellent identifications) were regarded as positive identifications.

The data presented in Table 9 is a list of the types of microbes isolated and identified during the 13 month sampling period from all four sampling sites. The lower microbial load of site P_0 in comparison to the much more polluted site P_1 could again be seen with several potential pathogenic organisms detected at site P_1, being absent at site P_0. Fewer organisms isolated from site P_2 also indicated better water quality at this site in contrast to the poor water quality of site P_1. The poor water quality of site P_3, influenced by the poor water quality from the Plankenburg River (site P_1) was also represented by the many potential pathogenic organisms detected at this site.

Most importantly, however, were all the microbial genera and species highlighted in grey (Table 9). These organisms were isolated from sites P_1, P_2 and P_3 and not only included faecal indicator organisms such as faecal coliforms, *E. coli* and Enterococci, but also

potential pathogenic organisms such as *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus*. The presence of organisms such as *Klebsiella*, *Listeria*, *Salmonella* and *Staphylococcus* in river water are disturbing as most of them, if not all, have been implicated in both waterborne and foodborne outbreaks in the past (Beuchat, 1995; Heijnen & Medema, 2009; Wilkes *et al*., 2009). It can therefore be concluded that the water quality from these sites were of unacceptable microbiological quality and could potentially present a threat to the health of any person or animal that comes into contact with it.

Table 9 Microbial genera and species isolated from sites P_0, P_1, P_2 and P_3

Organisms	**Site P_0**	**Site P_1**	**Site P_2**	**Site P_3**
Aeromonas	-	X	-	X
Coliforms	X	X	X	X
Faecal coliforms	X	X	X	X
E. coli	-	X	X	X
Enterobacter	X	X	X	X
Enterobacter aerogenes	-	X	X	X
Enterobacter cloacae	-	X	-	X
Enterococci	X	X	X	X
Klebsiella	X	X	X	X
Klebsiella pneumoniae	-	X	X	X
Listeria innocua	-	X	X	X
Listeria grayi	-	X	X	X
Listeria welshimeri	-	X	-	-
Listeria monocytogenes	-	X	X	X
Salmonella enteritidis	-	X	X	X
Salmonella typhimurium	-	X		X
Serratia marcescens	-	X	X	X
Shigella	-	X		X
Staphylococcus aureus	-	X	X	X
Staphylococcus xylosus	-	X	-	-

X – presence of the organism at the specific site; - absence or not detected

CONCLUSIONS

Chemical analyses, including pH, alkalinity, COD and conductivity, showed no direct trend in relation to the corresponding ACC that were obtained during this study. Chemical

analyses data frequently indicated no risk in contrast to high ACC concentrations which indicated high microbial concentrations and therefore a potential health risk, or visa versa. The microbiological testing of the three sites indicated high microbial concentrations during summer (December, January and February) and lower concentrations during the months of June, July and August (winter), clearly indicating a seasonal impact probably as a result of lower average temperatures and increased rainfall. On average, after the rainy season, microbial counts started to increase usually in September.

High microbial loads detected over the 13 month sampling period as well as the increase of microbial load just as the rainy season had passed, indicated the continuous addition of pollutants to the rivers. This constantly added to the microbial load in the river and consequently prevented the rivers from being permanently cleansed during the winter months.

The presence of faecal indicator organisms such as total coliforms, faecal coliforms, *E. coli* and Enterococci were also detected at all three sampling sites. Faecal coliforms and *E. coli* concentrations frequently exceeded the recommended ≤1 000 cfu.100 mL^{-1} (WHO, 1989) and ≤4 000 cfu.100 mL^{-1} (DWAF, 2002) guidelines at sites P_1 and P_3, and to a lesser extent, site P_2. The high concentrations of these faecal indicator organisms indicates the increased likelihood of infection and an increased health risk is therefore associated with the water from these rivers. The potential health threat was confirmed when potential pathogenic organisms such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumoniae* ssp. *pneumoniae* and *Aerococcus viridans* were found to be present in the river water.

The data from this study indicated a faecal pollution problem in the Plankenburg, and to a lesser extent, in the Eerste River. This contaminated water is consequently a serious health hazard to all who come into contact with it. In addition, the violation of crucial guidelines established by DWAF (2002) and the WHO (1989) is also a cause of great concern as it could not only lead to serious health implications, but it also presents an increased risk of pathogen transfer from the water to fresh produce when used as source of irrigation. Consequently, the possible increase in foodborne outbreaks related to the consumption of fresh or minimally processed fruit and vegetables could cause damage to public trust, loss of export licenses, thousands of job losses and ultimately great financial losses, to not only the agricultural sector, but to the South African economy as well.

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CHAPTER 4

INVESTIGATING THE LINK BETWEEN THE MICROBIAL QUALITY OF IRRIGATION WATER AND FRESH PRODUCE

ABSTRACT

Foodborne outbreaks related to the consumption of fresh fruit and vegetables have increased internationally over the past few years. This study was, therefore, aimed at investigating the impact of poor quality irrigation water on the microbial quality of fresh produce. Standard microbial methods for the detection of well-known indicator organisms which included total and faecal coliforms and potential pathogens such as *E. coli*, *Salmonella*, *Staphylococcus*, *Listeria* and Enterococci were employed. Whilst the quality of the irrigation water was found to be acceptable with faecal coliform counts rarely detected and not once exceeding the DWAF's guideline (2002) of 4 000 cfu.100 mL^{-1}, results obtained from fresh produce indicated a different scenario. Total and faecal coliform concentrations ranging up to 1.3 x 10^6 and 7.9 x 10^3 cfu.100 mL^{-1}, respectively, and *E. coli* detected in 60% of the fresh produce samples suggested the possible transfer of organisms from polluted irrigation water onto fresh produce. Individual product characteristics played an important role regarding the extent of transfer as much lower microbial concentrations were detected on the smooth surface of grapes in contrast to the higher microbial concentrations present on the hairy surface of green beans. The accumulation of organisms on the surface of fresh produce as a result of multiple irrigations was also observed with the faecal coliform concentration increasing from 220 to 4.4 x 10^5 cfu.100mL^{-1} on the surface of green beans which had been irrigated with polluted irrigation water on a daily basis for 10 consecutive days. Finally, as part of an additional exploratory study, the microbial quality of produce at point of sale was determined. The presence of potentially harmful organisms such as *E. coli* and Enterococci were recovered from green beans purchased at 4 different outlets. Faecal coliform concentrations as high as 2.2 x 10^5 cfu.100 mL^{-1} were observed. These results indicated the possibility that the products may have been contaminated by faecal pollution. The detection of *Salmonella* and *Listeria* presented further evidence of the potential health hazard at point of sale. In conclusion, this study indicated that polluted irrigation water does present a health risk to the consumers when irrigating products such as fresh fruit and vegetables that are intended to be consumed raw or in a minimally processed state, even up to the point of sale.

INTRODUCTION

The continuous growth of the world population has simultaneously caused an increased demand for food products such as fresh fruit and vegetables (Abadias *et al*., 2008) and a decrease in the availability of fresh water resources, which has since lead to serious water constraints (Sadovski *et al*., 1978). As a result, the use of wastewater and poor quality water as irrigation sources has become an increasing reality leading to an international trend in the growing number of foodborne outbreaks related to the consumption of fresh produce (Abong'o *et al*., 2008).

Fruit and vegetables, especially those intended to be consumed raw or in a minimally processed state, present a considerably higher health risk when irrigated with polluted or improperly treated wastewater. This is due to the possible carryover of pathogenic organisms from the polluted irrigation source to the produce (Jackson *et al*., 2006; Abadias *et al*., 2008; Abong'o *et al*., 2008; Warner *et al*., 2008). Due to the inevitable dependency of agriculture on the availability of water, farmers have been forced to make do with irrigation water, not only of poor quality, but often water which has been polluted with untreated sewage.

Based on the desired safe reuse of wastewater for agriculture, in especially arid-zones, a heightened interest regarding the risks associated with the consumption of fresh fruit and vegetables and the impact of the quality of irrigation water on the safety of fresh produce has since sparked numerous research projects. In 1978, Sadovski *et al*. confirmed the contamination of vegetables with potentially pathogenic organisms when irrigated with sewage effluent. They also found that different irrigation methods influenced the extent of contamination. Results indicated that plastic sheet covers and drip irrigation methods were effective barriers against contamination of crops (Sadovski *et al*., 1978).

Other studies also indicated the prevalence and persistence of potential pathogenic organisms such as *E. coli* O157:H7 (Abong'o *et al*., 2008), *Salmonella* and *Listeria monocytogenes* (Brackett, 1999; Abadias *et al*., 2008; Goss & Richards, 2008) on the surface of fresh produce and in soil. According to the Centres for Disease Control (2005), pathogen/biofilm interaction on the surface of fresh produce accounts for the majority of bacterial life on produce and consequently the various outbreaks of foodborne disease after consuming fresh produce.

Outbreaks related to the consumption of contaminated produce such as cantaloupe, tomatoes, lettuce and alfalfa sprouts (Brackett, 1999) include a case where 10 000 people were infected with *E. coli* O157:H7 as a result of contaminated radish sprouts. Contaminated tomatoes have been implicated in numerous *Salmonella* outbreaks in the

USA (Steele & Odumeru, 2004; Stine *et al*., 2005). A large scale *E. coli* O157:H7 outbreak in the USA during September 2006 resulted in 200 cases (including three fatalities) of Haemolytic-uremic Syndrome (Abadias *et al*., 2008). Outbreaks such as these are not only potentially fatal, but could also result in an economic disaster. High risks associated with the consumption of fresh produce could damage public trust, cause consumers to become more hesitant to buy local produce and export licenses could be terminated (Cape Times, 2005), ultimately causing damage to the economic stability of South Africa.

The aim of this study was to investigate the carryover of potentially pathogenic organisms from the polluted Plankenburg and Eerste rivers to fresh produce. This will be done by comparing the microbial loads of the river, irrigation water and fresh produce using standard methods for the detection of specific indicator and index organisms. Furthermore, the impact of multiple irrigations will be determined to establish the cumulative microbial effect of using poor quality irrigation water. In addition to these aims an exploratory study investigating the microbial load of produce at point-of-sale will be conducted to identify possible health risks to consumers.

MATERIALS AND METHODS

Site selection

Two sampling sites, P_4 and P_5, were identified as sampling points where water was abstracted for the purpose of crop irrigation (Fig. 1).

Site P_4 (Fig. 1) is situated downstream of the Plankenburg and Eerste Rivers in an irrigation canal. This canal originates at the point where the Plankenburg and Eerste River merge (site P_3 as given in Chapter 3) and is a source of irrigation water to several farms in the surrounding area.

Site P_5 was a tap that forms part of the irrigation system on a near-by farm. Water is drawn from the canal which then flows into an irrigation dam from where the water is distributed across the farm by means of a series of pipe systems and taps.

Sites P_6, P_7, P_8 and sample P_9 are not identified in Fig. 1. Site P_6 refers to irrigation water obtained right at the point of irrigation (usually at the sprayer-heads) from a near-by farm situated at points P_4 and P_5 (Fig.1).

Sites P_7 and P_8 refers to green beans and grapes which had been irrigated with water from site P_6.

Sample P_9 is green beans which had been irrigated and analysed daily for 10 consecutive days, using irrigation water from site P_6.

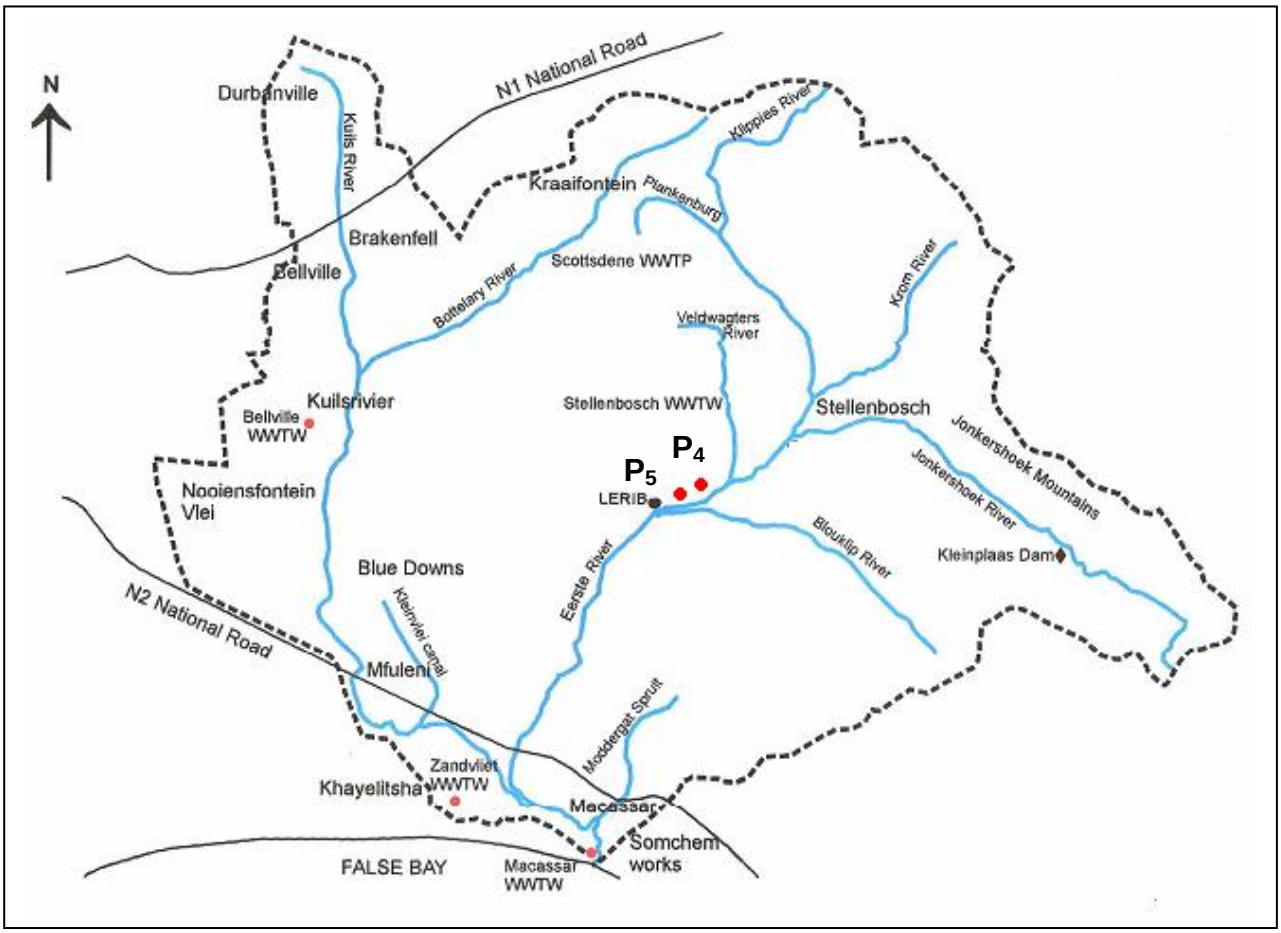

Figure 1 Location of sampling sites P_4 and P_5 (Ngwenya, 2006)

Water sample collection

Water samples (1 L) were taken once every four weeks according to sampling guidelines formulated by SANS 5667-6 (2006). Samples were obtained from the canal (P_4) by submerging a sealed, sterile Schott bottle as far as possible beneath the surface of the water and only then unscrewing the cap. The full bottle was then sealed whilst still submerged.

Water samples (1 L) from site P_5 were taken by opening the tap and allowing the water to run for 10 min. The sterile sampling bottle was opened whilst holding it underneath the running water and closing it as soon as the sample had been taken. Temperature readings were taken during each sampling session of both sites (site P_4 and P_5). Samples were placed on ice, transported to the laboratory and analysed within 60 min.

Transfer of organisms from polluted irrigation water to fresh produce

The transfer of potentially pathogenic organisms from the polluted rivers to the surface of fresh produce via irrigation was investigated by subjecting fresh produce samples (P_7 and P_8) and irrigation water (site P_6) used to irrigate these crops to microbial testing for the

detection of faecal indicators and potential pathogenic organisms. Fresh produce samples were obtained from a farm situated at points P_4 and P_5 (Fig. 1). This farm utilises water drawn from the Plankenburg and Eerste Rivers via the irrigation canal (site P_4) for irrigation by distributing it across the farm using the pipe system of which site P_5 forms a part.

Irrigation water sampling

Irrigation water samples were collected at the point of irrigation (site P_6) by switching the irrigation system on and allowing the water to run for 10 min before taking the sample. Samples (1 L) were taken by opening the sterile Schott bottle directly at the sprayer head and filling. It was then transported on ice and analysed within 60 min.

Fresh produce sampling

Sampling of fresh produce included green beans (P_7) and grapes (P_8) and was restricted to specific months due to seasonal availability. The availability of the grapes was from December to February, whilst green beans were available from middle February to the end of April. Samples consisting of 400 g were obtained on a weekly basis. Produce (beans and grapes) was selected randomly across a 100 m^2 plot in order to obtain a representative sample of that area. A 2 m wide zone on the perimeter of the plot was excluded to minimise the effect of contamination from domestic animals and humans.

Sterile latex gloves were worn during sampling and samples taken in sterile stomacher bags. Samples were transported on ice and analysed within 60 min.

Preparation of fresh produce samples

Ordinarily the first step of an experimental procedure would involve obtaining a homogenous sample. These fresh produce samples could, however, not be homogenised as this would cause damage to the cell structures which would lead to the leakage of cell sap. This could influence the number of organisms present in the sample due to the lowering of the pH. Analyses performed on such a sample would therefore not be representative of the actual contamination levels.

Fresh produce samples (grapes and green beans) were prepared for analyses by adding 400 mL of sterile physiological saline solution (PSS) to the 400 g sample in the sterile stomacher bag. The bag was sealed and gently shaken for 10 min to release the organisms attached to the surface of the produce into the PSS. The wash water was then

decanted aseptically into a sterile 500 mL Schott bottle to promote the effective handling of the sample during microbial testing.

Microbial methods

Standard methods (Table 1) for the detection and identification of the specific organisms were used during microbial testing.

Table 1 Standard methods used during microbial testing

Indicator and index organism	Standard methods reference	Typical growth
Aerobic colony count	**SABS 4833, 2007**	All colonies
Aerobic and anaerobic endosporeformers	**MFLP-44, 1998**	All colonies
Staphylococcus aureus and other species	**SANS-6888-1, 1999**	Black colonies surrounded by clear zone
Salmonella	**SABS-6579, 2004**	Black colonies
Listeria monocytogenes	**SABS 11290-1, 1996**	Olive green/grey colony with black centre and black discoloration of agar
Intestinal Enterococci	**SANS 7899-2, 2004**	Brownish pink colonies
Coliforms, faecal coliforms and *E. coli*	**MFHPB 19**, **2002**	Coliforms - tubes with gas Faecal coliforms - tubes with gas and fluoresced under UV light *E. coli* - dark colonies with metallic green sheen

Colonies obtained from the different selective media, whether typical (Table 1) or not, were purified, characterized and identified using API test kits (Biomerieux, South Africa). Organisms with API identification percentages ≥98% were regarded as positive identifications.

Impact of multiple irrigation sessions

The carryover and retention of potentially pathogenic organisms on the surface of fresh produce as a result of multiple irrigations was investigated by irrigating a plot (16 m^2) of green beans (sample P_9) on a daily basis for 10 consecutive days (9 irrigation sessions)

using irrigation water obtained from the Plankenburg River (site P_1). Each irrigation session consisted of 6 L of irrigation water. The faecal coliform concentration in the irrigation water was monitored to determine whether there was a link between the microbial consortium in the irrigation water and the extent to which carryover occurred. The faecal coliform load on the green beans was analysed 20 - 22 h after each irrigation session (prior to the next) to establish the build-up of faecal coliforms as a result of multiple irrigation sessions. The Most Probable Number method (Health Canada, 2002) was used for the testing of the faecal coliform concentration present both on the surface of the green beans as well as in the irrigation water.

A randomly selected sample of green beans (400 g) obtained from the 16 m^2 plot was analysed prior to the irrigation trial in order to obtain a base-line value of the microbial population that naturally occurs on the surface of fresh produce. Thereafter, the plot of beans was irrigated using a pressurised spray bottle (Mr Farmer, Stellenbosch) to simulate the over-head irrigation technique.

Irrigation water and green bean samples were transported on ice and the faecal coliform concentration tested within 60 min. This process was repeated for a total of 10 days and included 9 irrigation water and 10 green bean samples. Therefore, a total of 19 samples were tested during this trial.

Microbial quality of retail green beans at point-of-sale

As part of an additional exploratory study, green beans purchased at various shops, farm stalls and markets, both during and out-of-season, were subjected to microbial testing using the methods given in Table 1.

A sample (400 g) of each purchased product was placed aseptically in a sterile stomacher bag. Sterile PSS (400 mL) was added to the bag, sealed and gently shaken for 10 min. The wash water was then decanted into a sterile Schott bottle to promote effective handling of the sample. Microbial testing were conducted according to standard methods (Table 1).

RESULTS AND DISCUSSION

Water quality of sites P_4 and P_5

Water samples (1 L) obtained from the irrigation canal (site P_4) and irrigation system after the dam (site P_5), were subjected to microbial testing for the detection of faecal indicator

indicators and other potentially pathogenic organisms. The results are presented in Tables 2 and 3 and in Figs. 2 and 3.

Table 2 Results of microbial testing on water samples obtained from site P_4 (irrigation canal)

Date	Temp	pH	ACC*	Endosporeformers		Staphylococcus	Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
				Aerobic	Anaerobic							
	°C		cfu.Ll^{-1}				cfu.100 mL^{-1}				Present/absent	
March 2008	26.4	7.33	9 200	ND	ND	4 500	1 400	7 000	ND	16	TG	TG
April 2008	18.3	6.81	17 100	ND	ND	ND	7 000	7 000	TG	1	TG	TG
May 2008	21.9	4.49	1 340 000	ND	ND	ND	ND	ND	ND	2	TG	ND
June 2008	15.6	6.78	92 000	310	ND	ND	2	ND	ND	ND	TG	ND
July 2008	Excessive rainfall											
Aug 2008	11.1	6.28	1 490	320	ND	ND	11 000	11 000	TG	204	TG	ND
Sept 2008	15.5	7.19	1 800	310	240	ND	3 300	3 300	TG	18	TG	TG
Oct 2008	18.2	6.60	12 100	670	80	ND	2 200	2 200	TG	15	ND	TG
Nov 2008	24.4	8.91	4 070	11 600	ND	900	7 900	7 900	TG	> 300	TG	TG
Dec 2008	22.5	6.38	1 290	390	ND	ND	440 000	17 000	TG	54	TG	ND
Jan 2009	No water**											
Feb 2009	21.8	6.6	1 770	380	80	ND	17 000	1 700	TG	77	TG	TG

* Aerobic colony count; ** No water in irrigation canal; ND – None detected; TG – Typical growth

Table 3 Results of microbial testing on water samples collected at site P_5 (tap that forms part of the irrigation system)

Date	Temp	pH	ACC*	Endosporeformers		Staphylococcus	Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
				Aerobic	Anaerobic							
	°C		cfu.mL^{-1}				cfu.100 mL^{-1}				present/absent	
March 2008	22.7	6.35	3 700	ND	ND	ND	230	230	TG	ND	TG	TG
Apr 2008	20.3	6.39	ND	ND	ND	ND	230	79	ND	ND	TG	TG
May 2008	18.2	6.84	490	ND	ND	ND	2	2	ND	29	TG	ND
June 2008	14.1	6.67	106 000	ND	ND	ND	43	43	TG	ND	ND	ND
July 2008							No water					
Aug 2008	13.4	5.88	340	ND	ND	ND	ND	ND	ND		TG	ND
Sept 2008	14.2	6.45	13 500	ND	ND	ND	ND	ND	ND	5	TG	TG
Oct 2008	15.3	6.65	380	80	ND	ND	78	ND	ND	31	ND	TG
Nov 2008	19.4	6.49	ND	ND	ND	ND	ND	ND	ND	52	TG	ND
Dec 2008	22.3	6.45	2 430	ND	ND	ND	ND	ND	ND	7	TG	ND
Jan 2009	23.2	6.49	840	> 300	ND	600	4 600	ND	ND	4	TG	ND
Feb 2009	22.7	6.67	2 600	> 300	ND	ND	17 000	11 000	TG	2	TG	TG

* Aerobic colony count; ND – None detected; TG – Typical growth

Table 4 Results of microbial testing on water samples collected from site P_3 (data taken from Chapter 2, Table 6)

Date	ACC	Aerobic endospores	Anaerobic endospores	Total Coliforms	Faecal coliforms	*E. coli*	*Salmonella*	*Listeria*	*Staphylococcus*	Enterococci
	cfu.mL^{-1}			cfu. 100 mL^{-1}			present/absent		cfu.mL^{-1}	cfu.100 mL^{-1}
Feb 2008	9 400	ND	10	4 900	22 000	TG	TG	TG	3 100	7
March 2008	27 900	ND	ND	130 000	130 000	TG	TG	TG	ND	2 760
Apr 2008	29 600	ND	ND	49 000	2 200	TG	TG	TG	24 300	11
May 2008	214 000	ND	ND	33 000	33 000	TG	TG	TG	2 390	482
June 2008	85 000	360	ND	2 300000	64 000	TG	TG	TG	1.2 x 10^{6}	26
July 2008	Excessive rainfall									
Aug 2008	2 130	ND	ND	70 000	70 000	TG	TG	TG	ND	683
Sept 2008	Flooding*									
Oct 2008	24 800	340	230	130 000	49 000	TG	ND	TG	2 900	826
Nov 2008	21 700	ND	ND	23 000	23 000	TG	TG	TG	2 600	1 230
Dec 2008	412 000	410	100	130 000	130 000	TG	TG	TG	68 000	736
Jan 2009	1 560	ND	ND	310	310	TG	TG	TG	ND	72
Feb 2009	ND	ND	ND	790	790	TG	TG	ND	ND	35

TG - Typical growth; ND - None detected; ACC – Aerobic colony count; * Flooding prohibited access to this sampling site

pH

According to DWAF (1996a), irrigation water can be grouped into three classes based on pH. Class 1 = the most desirable: water with a pH <6.5. Class 2 is water with a pH between 6.5 and 8.4 and Class 3 is water with a pH >8.4. Water from site P_4 was mostly classified as Class 2 irrigation water (60%) whilst site P_5 was mostly (60%) classified as Class 1 irrigation water. No link could, however, be observed between pH and corresponding ACC when used as an indicator of the microbial load present. Results in Tables 3 and 4 indicated the association of high ACC such as 1 340 000 cfu.mL^{-1} (Table 3, May 2008) and 13 500 cfu.mL^{-1} (Table 4, Sept 2008) as well as lower ACC such as 1 290 cfu.mL^{-1} (Table 3, Dec 2008) and 340 cfu.mL^{-1} (Table 4, Aug 2008) with class 1 irrigation water (pH values <6.5). As a result, the pH of irrigation water is regarded as a poor indicator of irrigation water quality.

Aerobic colony count

Aerobic colony counts were determined for sites P_4 and P_5 and the results (Tables 2 and 3) compared to that of site P_3 (data extracted from chapter 3 and presented in Table 4), a site situated upstream of sites P_4 and P_5. Apart from two exceptions (1.34 x 10^6 and 9.2 x 10^4 cfu.mL^{-1} detected at site P_4 during May and June 2008) the ACC at sites P_4 and P_5 were lower than the ACC detected at site P_3 (Fig. 2). The ACC values of up to 2.14 x 10^5 and 4.12 x 10^5 cfu.mL^{-1} detected at site P_3 were usually followed by much lower (≤17 100 cfu.mL^{-1}) ACC just a few kilometres downstream at sites P_4 and P_5 (Fig. 2).

Following the first rain in early May of 2008, the ACC concentrations of 2.14 x 10^5 and 1.34 x 10^6 cfu.mL^{-1} were detected towards the end of May 2008 at site P_3 and P_4, respectively. The high ACC for these sites were possibly as a result of waste that had accumulated upstream on the river banks during summer being washed downstream by the rain.

The Stellenbosch area received an average of 260 mm (Wentzel, 2009) rainfall during July 2008. Most of this rainfall occurred over a short period of time resulting in strong water flows in the Plankenburg and Eerste Rivers. Sampling conditions were regarded as unsafe, causing all sampling sessions to be cancelled during this month and thus no data was generated during July.

Overall, the ACC results indicated a decrease in microbial loads from site P_3 to P_4 and P_5. Apart from one exception in September 2008 (Fig. 2), the ACC concentrations at site P_5 were much lower than concentrations detected at site P_4. The load lowering was probably

as a result of the dilution-effect caused by surface run-off and rainfall experienced in winter (Table 1 of Chapter 3) and increased UV exposure (Schultz-Fadem recht & Horn, 2008) during the summer months.

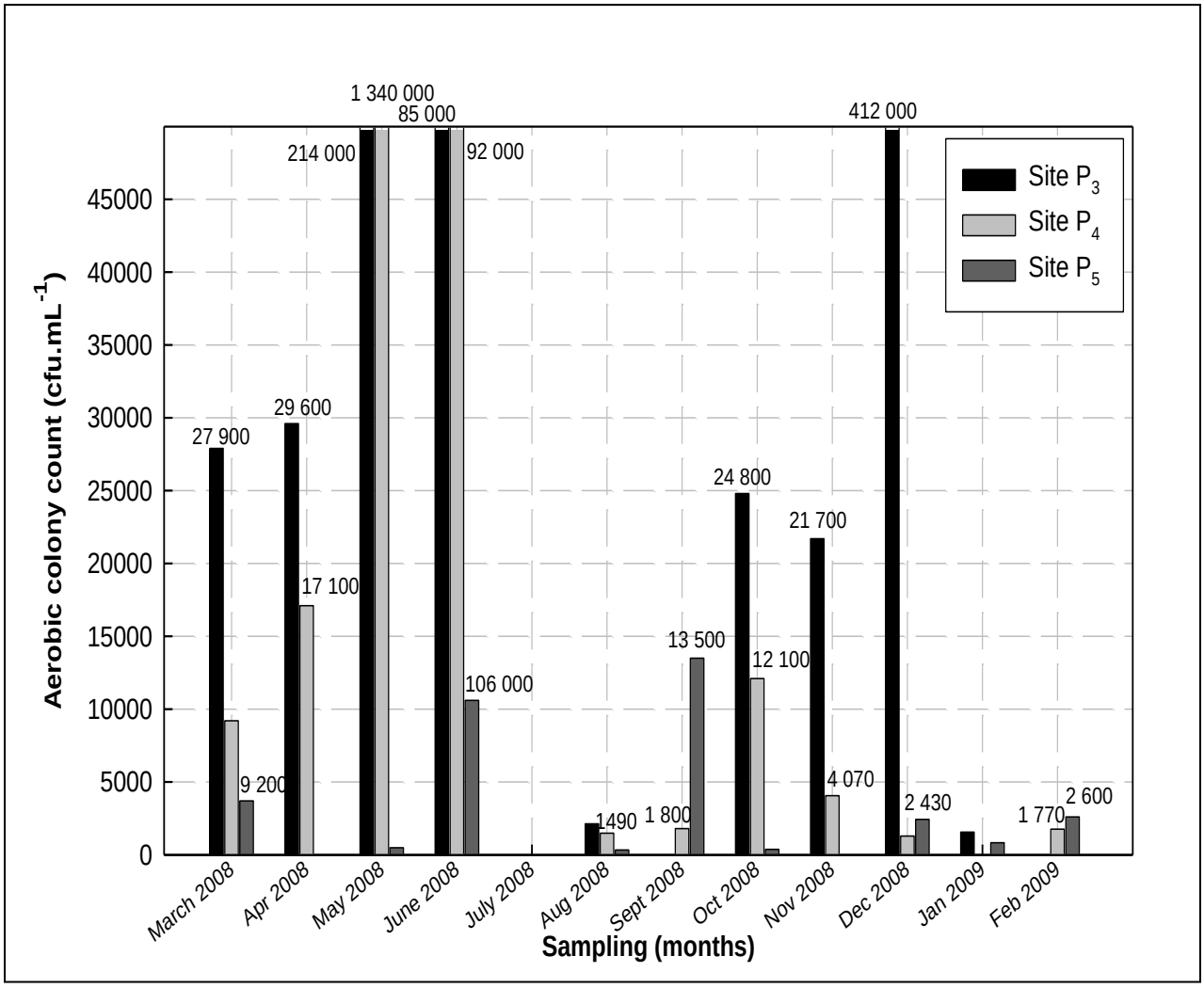

Figure 2 Aerobic colony counts (ACC) detected at sites P_3, P_4 and P_5 during the 12 month sampling period. Counts ranging above 50 000 cfu.mL^{-1} were omitted (but values given) to show fluctuations in the lower ranges

Faecal indicators – Faecal coliforms and E. coli

Faecal coliform concentrations at site P_4 (Table 3) exceeded the maximum recommended faecal coliform concentration of 4 000 cfu.100 mL^{-1} for microbially safe irrigation water (DWAF, 2002) on five occasions (March, April, August, November and December of 2008; Fig. 3) and did not comply to the much stricter <1 000 cfu.100 mL^{-1} guidelines of the WHO (1989) on eight sampling occasions (Fig. 3). *E. coli* concentrations detected at site P_4 (Fig. 3) exceeded DWAF (2002) and WHO (1989) guidelines on four (31%) and seven (54%) sampling occasions, respectively.

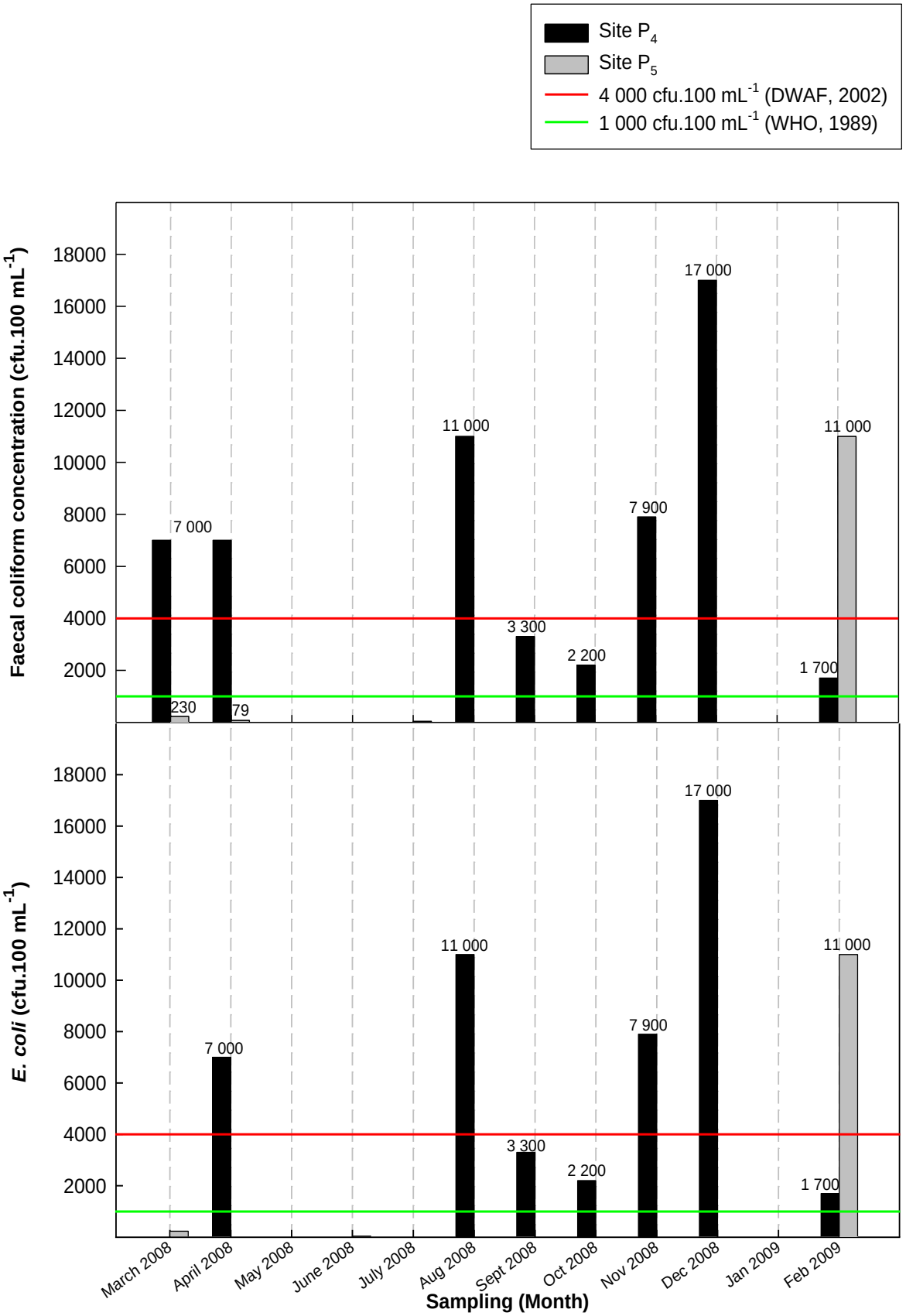

Figure 3 Faecal coliform and *E. coli* concentrations at sites P_4 and P_5 during the 12 month sampling period (Red line denotes DWAF guideline of 4 000 cfu.100 ml^{-1} (2002), green line represents the 1 000 cfu.100 mL^{-1} WHO guideline of 1989)

The faecal coliform and *E. coli* concentrations of site P_5 (Fig. 3) were consistently lower than concentrations detected at any of the other two sites (P_3 and P_4). Apart from one exception, during February 2009 when a faecal coliform concentration of 11 000 cfu.100 mL^{-1} was detected, total and faecal coliform concentrations at site P_5 never exceeding the recommended irrigation water guidelines of DWAF (2002) or the WHO (1989).

E. coli, a well-known faecal indicator, occurs in the intestines of humans and warm blooded animals; thereby indicating faecal pollution and the presence of potentially pathogenic organisms and highly infectious viruses. The data in Fig. 3 therefore shows that water from site P_4 and, to a lesser extent, site P_5 had been polluted with faecal contaminants. The possibility therefore exist that potentially harmful organisms might be present in these water resources thereby creating a potential health threat to any human or animal that consumes or comes into contact with this water.

Potential pathogenic organisms

The presence of faecal indicators showed faecal pollution of the water at sites P_4 and P_5 (Tables 2 & 3). Organisms such as *Salmonella* (90% incidence) and *Listeria* (60% incidence) were both detected frequently at site P_4 with Enterococci and *Staphylococcus* concentrations ranging from 1 to 204 cfu.100 mL^{-1} and 900 to 4 500 cfu.mL^{-1}, respectively (Table 2). Even though all of these organisms were detected at site P_5 (Table 3), results indicated a slightly lower frequency of occurrence for *Listeria* (45% incidence), a distinct drop in *Staphylococcus* counts which were only detected once at 600 cfu.mL^{-1} (Jan. 2009) and Enterococci concentrations ranging between 2 and 52 cfu.100 mL^{-1} (Table 4). The high frequency of occurrence of Enterococci (90 and 64% incidence) (Table 5), another indicator of faecal pollution, once again indicated faecal pollution at both sites (P_4 and P_5).

The presence of potentially harmful organisms such as *Salmonella*, *Listeria* and *Staphylococcus* confirmed that water from sites P_4 and P_5 could present a health risk to any human or animal that came into contact or consumed this water. With further identification (API web, Biomerieux) the following confirmations were made: *Salmonella* spp. were detected in 9 (90%); *Salmonella typhimurium* in 3 (30%); *Staphylococcus aureus*, *Listeria monocytogenes* and *Aeromonas* in 2 (20%); and *Klebsiella pneumoniae* in 3 (30%) of the 10 samples at site P_4.

Organisms isolated from water taken from site P_5 included: *Enterobacter* detected in 7 (64%); *Enterobacter aerogenes* in 2 (18%); *Listeria innocua* in 6 (55%); *Klebsiella* in 4 (36%); *Salmonella* spp. in 10 (90%); *Salmonella typhimurium* in 3 (27%) and *Aeromonas* and *Staphylococcus aureus* in 1 (9%) of the 11 samples.

Table 5 Potential pathogenic organisms and their respective frequencies of occurrence (% incidence) at sites P_4 and P_5

	Sampling sites	
Indicator organisms	**P_4**	**P_5**
Salmonella	90%	90%
Staphylococcus	20%	10%
Enterococci	90%	64%
E. coli	58%	25%
Listeria	60%	45%

% = indicates the frequency of occurrence calculated as a percentage of the sampling sessions during which a particular organism was detected

Even though the microbial concentrations decreased from sites P_3 to P_4 and P_5, the above confirmations (Tables 4 & 5) suggests that potential pathogenic organisms are able to survive in irrigation systems. The possibility therefore exists that potentially harmful organisms could be transferred to fresh produce during irrigation and ultimately result in foodborne illnesses if consumed in sufficient amounts.

Transfer of organisms from irrigation water (site P_6) to produce (P_7 and P_8)

Irrigation water quality

The carryover of potential pathogenic organisms from irrigation water (site P_6) to fresh produce (sites P_7 and P_8) was investigated by conducting microbial testing on irrigation water taken at the irrigation point and on the surface of green beans and grapes, irrigated with this water.

Microbial results indicated varying irrigation water quality at site P_6 (Table 6) with microbial concentrations lower than detection levels in 2 (18%) of the 11 samples. The absence of faecal indicator organisms in 5 samples suggest that no faecal contamination was present. The microbial results of the remaining 6 (55% of samples) irrigation water samples from site P_6 (especially samples 1 and 6) (Table 6) did, however, indicate a potential health risk associated with the use or consumption of this water due to the presence of faecal coliforms and *E. coli*. Even though the faecal coliform concentrations never exceeded the <4 000 cfu.100 mL^{-1} DWAF guideline (2002) it did, however, fail to

Table 6 Microbial counts found in irrigation water (site P_6) used to irrigate crops to determine the transfer of organisms to green beans and grapes

Sampling session	ACC	Endosporeformers		*Staphylococcus*	Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
		Aerobic	Anaerobic							
	cfu.mL^{-1}				cfu.100 mL^{-1}				Present/absent	
1	550 000	1 030	ND	11 100	1 300	1 300	TG	62	TG	TG
2	1 320	ND	ND	ND	4 300	23	ND	80	TG	TG
3	1 470	620	420	ND	3 300	110	ND	6	TG	TG
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND	34	ND	TG
6	19 800	ND	ND	750	79 000	2 300	TG	16	TG	ND
7	ND	ND	ND	ND	33	33	ND	ND	TG	ND
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND	30	ND	ND
10	2 800	ND	ND	ND	ND	ND	ND	22	ND	TG
11	7 800	ND	ND	ND	230	ND	ND	28	TG	ND

ND – None detected; TG – Typical growth; ACC – Aerobic colony count

comply with the <1 000 cfu.100 mL^{-1} WHO guideline (1989) in two instances (sampling session 1 and 6). Enterococci, another indicator of faecal pollution was, however, detected more often (73% of samples). Although Enterococci concentrations in the irrigation water only ranged up to a maximum of 80 cfu.100 mL^{-1}, the presence not only indicates faecal pollution of the water source, but also the ability of organisms to survive in irrigation water. Anaerobic and aerobic endosporeformers were detected in 2 of the 11 sampling sessions (Table 6).

DWAF water quality guidelines of 1996 stipulate that ACC >1 000 cfu.mL^{-1} indicates an increased risk of infectious disease transmission. The irrigation water samples had ACC ranging from ND (none detected) to 550 000 cfu.mL^{-1} with >1 000 cfu.mL^{-1} detected in 6 of the 11 cases (Table 6). The risk of transmission of infectious diseases via irrigation water was therefore increased.

Other potential pathogenic organisms such as *Salmonella*, *Listeria* and *Staphylococcus* were also able to survive in the irrigation system (Table 6), as they were detected in 6, 5 and 2 of the 11 irrigation water samples, respectively. Of these, *Salmonella typhimurium* and *Listeria grayi* were detected in 5 (45% of samples) cases and *Listeria monocytogenes* and *Staphylococcus aureus* detected twice (18% of samples). The presence of these organisms indicated the potential health hazard that the contaminated irrigation water could present, if used to irrigate produce intended to be consumed raw or in a minimally processed state (Brackett, 1999). Based on these results it could therefore be concluded that the irrigation water from site P_6 as found during this study was generally of unacceptable quality and that it could be the source of unwanted carry-over of potential pathogenic organisms from the irrigation water to fresh produce.

Microbial quality of fresh produce – Green beans (Phaseolus vulgaris)

Microbial analyses conducted on green beans (*Phaseolus vulgaris*) cultivated at site P_7 that had been irrigated with water from site P_6 showed microbial counts (Table 7) much higher than those found in the irrigation water (Table 6).

According to guidelines promulgated by the National Department of Health (DoH, 2006), food samples should not contain an ACC >1 000 cfu.g^{-1} of product. As indicated in Table 7 the ACC of the green beans ranged from 970 to 258 000 cfu.g^{-1} and exceeded the DoH's guidelines (2006) in 9 of the 10 sampling sessions. These guidelines (DoH, 2006) also stipulate that no *Salmonella* should be present in 25 g of product, total coliforms should not exceed 10 cfu.g^{-1} and no *E. coli* should be present in 1 g of product.

Table 7 Microbial counts of green beans (400 g) irrigated with irrigation water from site P_6 (Table 6)

Sampling session	*ACC	Endosporeformers		*Staphylococcus*	Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
		Aerobic	Anaerobic							
	cfu.mL^{-1}				cfu.100 mL^{-1}				present/absent	
1	211 000	ND	ND	2 800	ND	ND	ND	8	TG	TG
2	10 900	ND	ND	330	23	ND	ND	ND	TG	TG
3	7 100	ND	ND	ND	240	230	TG	5	TG	TG
4	258 000	160	330	ND	1 300 000	2 300	TG	>300	TG	TG
5	>300	570	ND	ND	33	17	TG	64	TG	TG
6	123 000	420	ND	900	33 000	ND	ND	>300	TG	TG
7	2 300	310	ND	ND	ND	ND	ND	30	TG	TG
8	970	520	460	ND	790	490	TG	>300	TG	TG
9	48 000	110	ND	4 500	7 900	7 900	TG	3 410	TG	TG
10	7 600	460	1 830	ND	1 100	1 100	TG	17	TG	ND

TNTC – To numerous to count; TG – Typical growth; ND – None detected; *ACC – Aerobic colony count

Salmonella was, however, detected in all the green bean samples. Total coliform concentrations exceeded the DoH (2006) guidelines on 3 occasions (sample 4, 6 and 9; Table 7) with the total coliform concentration of sample 4 exceeding the guideline by more than 4 300 fold (1.3×10^6 cfu.100 mL^{-1}). *E. coli* was detected in 6 and Enterococci, detected in 9 of the samples (Table 7), respectively.

The presence of faecal indicator organisms (faecal coliforms, *E. coli* and Enterococci) indicates that the green beans had been irrigated with water contaminated with faecal matter. Potentially harmful organisms originating from faecal matter in the polluted irrigation water could therefore also have been transferred to the produce which could lead to serious health risk if these products are consumed raw or minimally processed. The resultant health risk that faecal contamination of food products present was confirmed (API web, Biomerieux) when potential pathogens such as *Salmonella typhimurium* and *Klebsiella* spp. were detected in 4 (40%), *Listeria innocua* in 6 (60%), *Staphylococcus aureus* in 4 (40%), *Listeria monocytogenes* in 2 (20%) and *Aerococcus viridans*, *Klebsiella pneumoniae* and *Listeria grayi* detected in 1 (10%) of the 10 samples, respectively. The identification of isolates was confirmed using the API Web data base (Biomerieux). Isolates which obtained identification percentages ≥ 98% were regarded as positive identifications.

Based on the results presented in Table 7 it could be concluded that the microbial quality of the green beans was unacceptable and could present serious health risks if consumed raw or without any processing step.

Microbial quality of fresh produce – Grapes (Vitis vinifera)

The frequency of the microbial testing of grapes (site P_8) was limited as a result of seasonal availability. Therefore, only four samples of grapes were tested. The specific cultivar tested during this study was *Vitis vinifera*, or otherwise known as green table grapes and was also irrigated with water from sites P_4 and P_5 (Fig. 1). Grapes were irrigated weekly by running the irrigation system at maximum capacity for six hours per irrigation session.

Results obtained during the microbial testing of the grapes (site P_8) indicated much lower microbial concentrations (Table 8) than those recovered from the surface of the green beans (site P_7). The green beans and grapes had both been irrigated by means of the over-head irrigation technique using water from the same source and microbial quality. The lower microbial concentrations isolated from the grapes were therefore ascribed to the smoother surface of grapes in comparison to the hairy surface of green beans which are

Table 8 Microbial counts on table grapes (400 g) irrigated over-head with irrigation water from site P_6

Sampling session	Endosporeformers				Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
	ACC*	Aerobic	Anaerobic	*Staphylococcus*						
	cfu.mL^{-1}				cfu.100 mL^{-1}				present/absent	
1	5 000	ND	ND	ND	490	ND	ND	ND	TG	TG
2	410 000	190	ND	3 200	21 000	490	ND	116	TG	TG
3	2 250	ND	ND	ND	ND	ND	ND	21	TG	ND
4	7 600	460	1 830	ND	2 300	2 300	TG	17	TG	ND

ND – None detected; TG – Typical growth; * Aerobic colony count

thought to promote water retention and consequently also the adherence of micro-organisms.

The identification of organisms isolated from the grapes indicated the presence of faecal indicators as well as potential pathogenic organisms. *E. coli* was detected in 1 of the 4 grape samples (25%), faecal coliforms in 2 (50%) and total coliforms recovered from 3 (75%) of the 4 samples with concentrations peaking at 21 000 cfu.100 mL^{-1} (Table 8). The ACC also exceeded guidelines (DoH, 2006) that require < 1 000 $cfu.g^{-1}$, with counts ranging up to 4.1 x 10^5 $cfu.mL^{-1}$ (Table 8).

The isolation, characterization and identification of isolates using the API Web data base (Biomerieux) revealed the presence of potential pathogens on the grape samples. Isolates that obtained an identification percentage ≤ 98% were regarded as positive identifications. These results showed the presence of potential pathogens such as: *Enterobacter* spp. (50% of the samples); *Enterobacter aerogenes* (25%); *Salmonella typhimurium* (75%); *Aeromonas* (25%) and *Listeria innocua* (25%) on the surface of the grape samples. The presence of non-pathogenic organisms such as *L. innocua*, although not harmful, indicated the use of polluted irrigation water.

The microbial quality of the grapes was thus unacceptable. The presence of faecal indicators such as faecal coliforms, *E. coli* and Enterococci suggest the possibility that these samples (P_8) could have been contaminated with faecal pollution. Also, potential pathogens such as *Salmonella typhimurium* confirmed a possible health risk associated with the consumption of these grapes.

Establishing the link between irrigation water quality and the safety of fresh produce

The microbial genera and species isolated from river site P_3 (the point where the Plankenburg and Eerste Rivers merge), the irrigation point P_6 and fresh produce (P_7 and P_8) are presented in Table 9 to examine the link between the quality of the irrigation water and the safety of fresh produce.

All colonies obtained during the microbial testing of these samples were selected and purified on Nutrient agar. Thereafter, they were subjected to further testing that included Gram-stain, inspection of morphological and cultural characteristics, catalase and oxidase tests and identification by means of API test kits (Biomerieux). Isolates that obtained identification percentages ≥ 98% were regarded as positive identifications and presented in Table 9.

Bacillus species, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Serratia marcescens* and *Shigella* were detected in 2 of the 3 sites. This does, however, not necessarily imply

that a specific organism was not present at a specific site, but rather that the organism might have been present at concentrations below the detection level or it could have been absent in the samples taken during this study.

Table 9 Microbial genera and species isolated from sites P_3, P_6, P_7 (green beans) and P_8 (grapes)

Organisms*	Sites		
	P_3	P_6	P_7 & P_8
Aerobic sporeformers	X	X	X
Anaerobic sporeformers	X	X	X
Aerococcus viridans	X	X	X
Bacillus spp.	X		X
Total coliforms	X	X	X
Faecal coliforms	X	X	X
E. coli	X	X	X
Enterobacter	X	X	X
Enterobacter aerogenes	X	X	X
Enterobacter cloacae	X		
Enterococci	X	X	X
Klebsiella	X	X	X
Klebsiella pneumoniae	X		X
Listeria innocua	X	X	X
Listeria grayi	X	X	X
Listeria monocytogenes	X	X	X
Salmonella enteritidis	X		
Salmonella typhimurium	X		X
Serratia marcescens	X	X	
Shigella spp.	X	X	
Staphylococcus aureus	X	X	X
Staphylococcus lentus	X		

X – Organism detected at the specific site

* Organisms high-lighted are those which had been recovered from all three locations

A total of 64% of the organisms listed in Table 9 were isolated from all 3 sites with an additional 14% isolated from 2 of the 3 sites. These results suggest similar microbial

populations in the rivers, irrigation water and on the produce and therefore, the likelihood that the microbial population isolated from the surface of the green beans and grapes could be influenced by the microbial population of the irrigation water and consequently, the microbial quality of the rivers. These results therefore also suggest the transfer of potential pathogens from the irrigation water to produce.

Impact of multiple irrigations on the microbial quality of fresh produce

The frequency of irrigation sessions could also play an important role in influencing the microbial quality of fresh produce as it creates the opportunity for organisms to be transferred to the surface of fresh produce repeatedly. If true, this could also suggest that the microbial quality of irrigation water does not necessarily have to exceed the recommended guidelines in order to present a possible health risk to anyone who consumes these fruit and vegetables raw or minimally processed.

It can be seen from the data in Fig. 4 that the faecal coliform concentration present in the irrigation water (P_6) exceeded the ≤ 4 000 cfu.100 mL^{-1} faecal coliform DWAF (2002) guideline for all 9 irrigation sessions. These counts ranged between 4 600 and 790 000 cfu.100 mL^{-1} and therefore also exceeded the ≤ 1 000 cfu.100 mL^{-1} WHO guideline (1989). According to these guidelines a high health risk is associated with irrigation water of which the faecal coliform concentration exceeds these stipulated requirements. The microbial quality was therefore concluded to be of unacceptable quality.

The microbial concentration recovered from the surface of green beans (P_9) increased from irrigation session 0, when the microbial base-line value on the beans was determined as zero detected, to irrigation session 6 with a maximum faecal coliform concentration of 9.2×10^4 cfu.100 mL^{-1}. On day 7 (irrigation session 7) a decrease in faecal coliform concentrations on the green beans was observed. This was possibly as a result of rainfall the night before which could have “rinsed” some of the microbial load off the surface of the beans. The microbial concentration of the irrigation water was also lower at irrigation session 7 (17 000 cfu.100 mL^{-1}) than the microbial concentration detected for water used during irrigation session 6 (44 000 cfu.100 mL^{-1}). This decreased microbial concentration could have been as a result of the dilution effect caused by rainfall. The microbial load then once again increased following the next irrigation session from a concentration of 17 000 to 35 000 cfu.100 mL^{-1}.

On the final day of analyses (irrigation session 9) microbial concentrations on green beans decreased from the previously detected 35 000 cfu.100 mL^{-1} to 22 000 cfu.100 mL^{-1} whilst still remaining much higher than the recommended guidelines of the DoH (2006). The

drop in faecal coliform concentrations from irrigation session 8 to 9 could have been as a result of the limited amount of beans available for sampling. The previous 8 days of sampling depleted most of the beans in the plot, leaving only the small beans hanging right by the stem of the stalk. This resulted in a sample of beans which had been sheltered from most of the irrigation water throughout the 9 day trial, thereby possibly resulting in lower microbial concentrations.

Based on these results it could be concluded that multiple irrigations sessions contribute to the build-up of organisms on the surface of fresh produce and thus promote the carryover of potential pathogenic organisms to fresh produce.

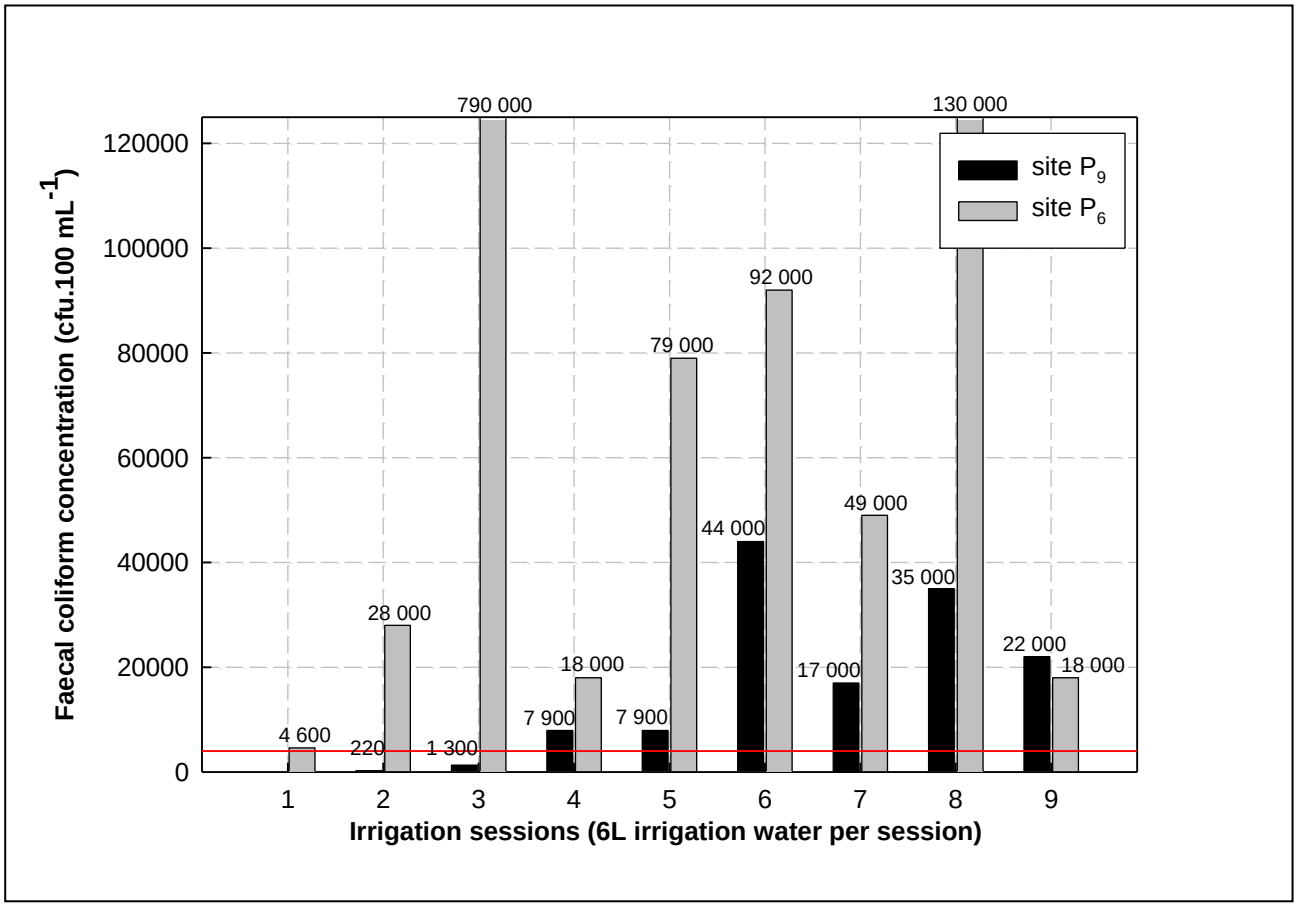

Figure 4 Accumulation of organisms on the surface of green beans due to multiple irrigations (reference line indicates DWAF's 4 000 cfu.100 mL^{-1} faecal coliform guideline for irrigation water (2002)

Microbial quality of fresh produce at point of retail

Green beans purchased at various outlets in and around Stellenbosch were subjected to microbial testing as part of an exploratory study to establish the microbial quality of fresh produce at the point of sale. The origin of these products was unknown and therefore

information regarding the quality of the irrigation water used to irrigate these specific products was unavailable. It is therefore important to note that the microbial quality of these samples cannot be linked to the pollution levels in the Plankenburg and Eerste Rivers. The microbial load on these bean samples was analysed to determine if organisms isolated from green beans and grapes in previous trials also occurred on commercially available products and if, in fact, potential pathogens are able to survive post-harvest processes up to point-of-sale to consequently present a potential health threat to consumers.

Beans were purchased from two supermarkets, a farm stall and one fruit and vegetable market on three occasions and tested. The microbial results of these products are presented in Table 10 and Fig. 5.

The presence of specific indicator and potential pathogenic organisms were detected (Table 10) and the total coliform, faecal coliform and Enterococci concentrations presented in Fig. 5. The microbial concentrations recovered from the surface of the beans were, in general, much higher than those of the beans presented in Table 7. ACC counts ranged up to 1.13 x 10^6 cfu.mL^{-1}, aerobic and anaerobic endosporeformers were detected in 100 and 50% of the samples, respectively, and *Staphylococcus aureus* concentrations as high as 39 600 cfu.mL^{-1}, were detected (Table 10).

According to guidelines established by the Department of Health (DoH, 2006), raw fruit and vegetables should not contain any *Salmonella* or *Listeria* per 25 g and 1 g of product, respectively. The total coliform concentration should not exceed 200 cfu.g^{-1} and no *E. coli* should be present per g of product. All the products analysed in this trial (Table 10) contained *Salmonella*, in fact, four samples tested positive for *Salmonella typhimurium*. *Listeria* spp. that included *L. innocua* and *L. grayi* were detected in 83% of the samples. Total coliform concentrations exceeded the DoH's guidelines (2006) in four cases, ranging up to 7.9 x 10^5 cfu.100 mL^{-1} and *E. coli* were present in 50% of the samples (Table 10).

High concentrations of faecal coliforms and Enterococci, two indicators of faecal pollution, were also detected at all four outlets. Faecal coliform concentrations as high as 140 000 (farm stall) and 220 000 cfu.100 mL^{-1} (Grocery store 2) and Enterococci concentrations ranging up to 2 592 (Grocery store 1) and 2 880 cfu.100 mL^{-1} (Fruit & vegetable market) (Fig. 5) were detected. These results not only indicated faecal contamination of these products, but also the possible presence of potential pathogenic organisms. Based on these results it could be concluded that all of these retail outlets sold samples of unacceptable microbial quality. Results not only suggest possible faecal contamination of products, but the presence of organisms such as *Salmonella*, *Listeria* and *Staphylococcus*

Table 10 Microbial counts found on green bean samples (400 g) purchased at four retail outlets on three sampling occasions

Site	ACC	Endosporeformers Aerobic	Endosporeformers Anaerobic	Staphylococcus	Total coliforms	Faecal coliforms	*E. coli*	Enterococci	*Salmonella*	*Listeria*
	cfu.mL^{-1}				cfu.100 mL^{-1}				present/absent	
Sample session 1										
Farm stall	271 000	730	690	39 600	14 000	180	TG	82	TG	TG
F & V Market	580 000	3 300	ND	4 600	1 300	ND	ND	31	TG	TG
Grocery 1	225 000	2 800	320	35 000	49 000	14 000	TG	96	TG	TG
Grocery 2	30 500	590	1 190	ND	2 200	170	ND	1 120	TG	TG
Sample session 2										
Farm stall	241 000	4 600	ND	11 600	790 000	140 000	TG	124	TG	TG
F & V Market	2 420	1 810	1 560	5 100	9 500	9 500	ND	2 880	TG	ND
Grocery 1	37 200	440	ND	ND	49 000	2 200	ND	2 592	TG	TG
Grocery 2	4 640	340	ND	ND	4 900	ND	ND	182	TG	ND
Sample session 3										
F & V Market	820 000	330	350	ND	2 300	390	TG	327	TG	TG
Farm stall	123 000	240	ND	5 800	7 900	ND	ND	> 300	TG	TG
Grocery 1	7 900	> 300	ND	60	23	23	TG	91	TG	TG
Grocery 2	1.13 x10^{6}	160	270	6 890	230 000	220 000	TG	583	TG	TG

ND – None detected; TG – Typical growth; *ACC – Aerobic colony count

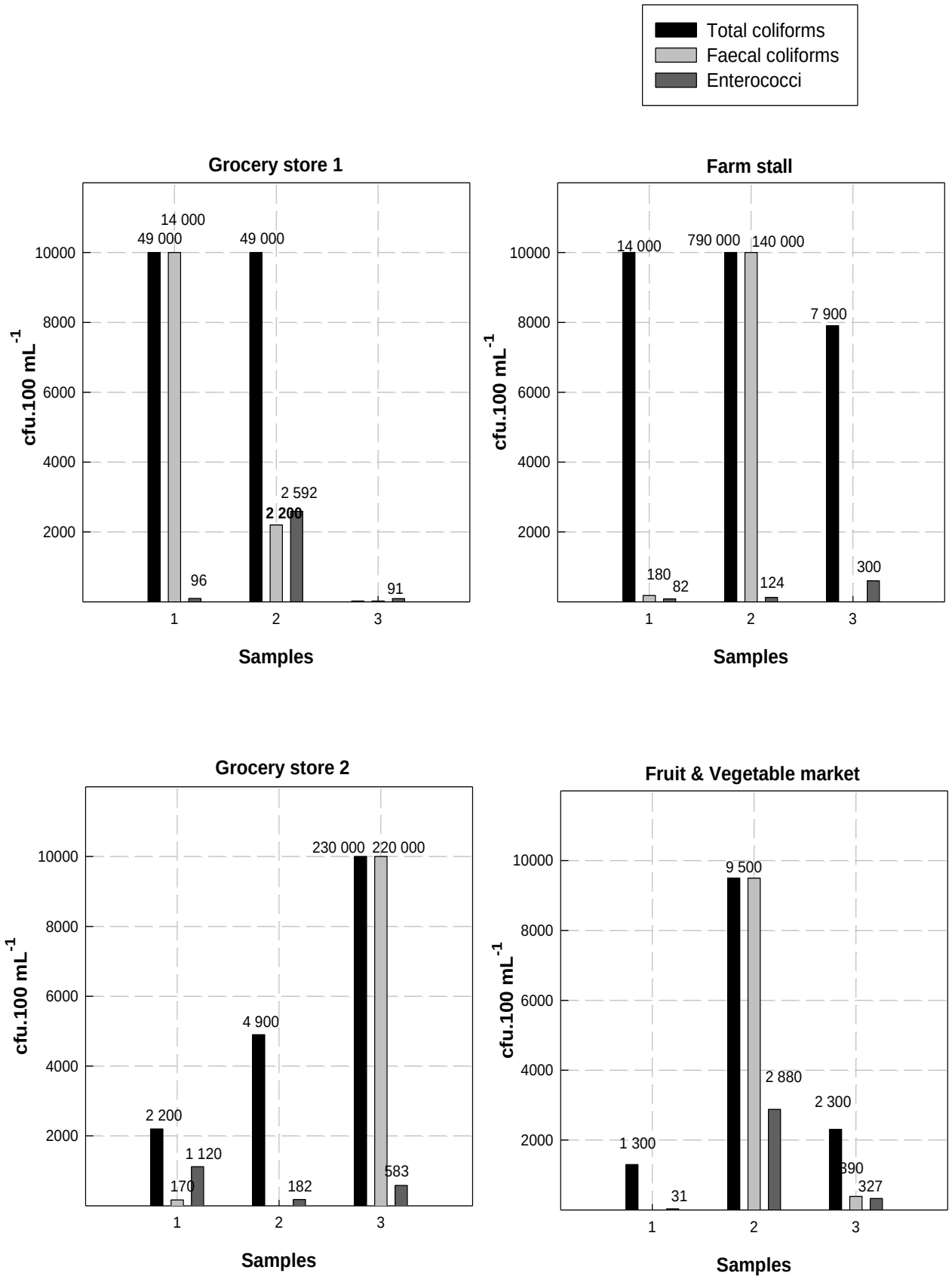

Figure 5 Total coliform, faecal coliform and Enterococci concentrations of green beans (300 g) purchased at various locations in and around Stellenbosch. Counts ranging above 10 000 cfu.100 mL^{-1} were omitted to show fluctuations in the lower ranges.

confirmed the potential health threat presented by these products if consumed in adequate amounts.

CONCLUSIONS

The detection of faecal indicators such as faecal coliforms, *E. coli* and Enterococci in water from sites P_4 and P_5, irrigation water, site P_6, and fresh produce samples, sites P_7 and P_8, clearly suggest faecal pollution and, consequently an increased health risk is associated with these water resources and products. The potential health threat was confirmed by the detection of potential pathogens in sites P_4, P_5, P_6, P_7 and P_8 which included *Staphylococcus*, *Salmonella*, *Klebsiella* and *Listeria*.

Although the faecal coliform concentration at site P_4 exceeded the DWAF guideline for irrigation water (2002) on five occasions, faecal coliform concentrations at this site (site P_4), apart from one exception, never came close to the excessively high counts obtained at site P_3 (130 000 cfu.100 mL^{-1}). Microbial concentrations at sampling site P_5 were even lower than the concentrations at site P_4. A steady decline in microbial concentrations from the rivers (site P_3) to the irrigations systems was therefore observed. This drop in microbial load was found consistently over the 13 month sampling period, most likely due to the increased UV exposure, die off and dilution caused by surface runoff and rainfall.

Microbial testing of irrigation water (site P_6) indicated fluctuating microbial quality. Microbial results varied between some samples with very low microbial growth concentrations and others that contained faecal coliform concentrations that exceeded WHO guidelines (1989) for irrigation water with counts of 1 300 and 2 300 cfu.100 mL^{-1}, respectively. The microbial quality of irrigation therefore varied between some samples being of acceptable quality for irrigational purposes and others associated with a high health risk if used to irrigate crops intended to be consumed raw.

Microbial analyses performed on grapes and green beans (sites P_7 and P_8) irrigated with water from site P_6 indicated a difference in microbial quality of the grapes (P_8) and green beans (P_7). The microbial load on the surface of green beans was much higher than those retrieved from the surface of the grapes. Differences in microbial loads were most probably as a result of the unique characteristics of the fruit and vegetable in combination with multiple irrigation sessions.

Nonetheless, the microbial genera and species recovered from both the green beans (site P_7) and grapes (site P_8) resembled that of the irrigation water (site P_6) and rivers (site P3) with 61% of the organisms isolated from the rivers, the irrigation water as

well as produce (Table 9). These results therefore indicated a possible transfer of potential pathogenic organisms from the irrigation water to the fresh produce.

The negative impact of multiple irrigation sessions was indicated by the build-up of faecal coliforms on the surface of the green beans from initially “none detected” to a maximum concentration of 44 000 cfu.100 mL^{-1} (Fig. 4). It could also be concluded that even though the microbial load of irrigation water complied with the different guidelines (WHO, 1989; DWAF, 2002), the presence of potential pathogenic organisms could still present a threat to the health of humans due to the build-up of organisms on produce as a result of multiple irrigations. Potential pathogenic organisms transferred from polluted rivers to fresh produce via irrigation water and the build-up of organisms, as a result of multiple irrigations, could therefore lead to increased associated health risks as a result of using poor quality irrigation water.

As part of an exploratory study green beans purchased at four retail outlets in Stellenbosch were subjected to microbial testing to determine if any of the above mentioned risks exists at the point of sale. Although the organisms obtained from these products could not be linked to the pollution problem in the Plankenburg and Eerste Rivers it could, however, be concluded that organisms are able to survive unfavourable conditions and hurdles (low storage temperatures and washing) presented during packaging and storage of the product.

Data from this study therefore indicate that the water from site P_4 (irrigation canal) and to a lesser extent site P_5, had been polluted with faecal contaminants. These contaminants were transferred from the irrigation water (P_6) to the fresh produce (sites P_7 and P_8) when results indicated that similar microbial genera and species had been isolated from the rivers (sites P_1, P_2 and P_3 presented in chapter 4 of this thesis), irrigation water (site P_6) as well as produce (sites P_7 and P_8). Multiple-irrigations also contributed to the pollution problem as it caused the build-up of organisms on the surface of fresh produce. According to guidelines (WHO, 1989; DWAF, 2002) a high health risk is associated with water which has been contaminated with faecal matter as the probability is high that potentially pathogenic organisms could be present. The detection of potential pathogens in the rivers, irrigation water, freshly harvested produce as well as produce purchased at various retail outlets confirmed the potential health threat presented to consumers. This is especially alarming as many fruit and vegetables are often consumed raw or in a minimally processed state. Washing of fruit and vegetables has also been proven to be insufficient in removing all organisms and consumers are therefore facing a high risk of being infected with potential pathogenic organisms.

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CHAPTER 5

INCORPORATING API 20E AND MULTI-ANTIBIOTIC RESISTANCE PROFILING TO LINK THE CARRY-OVER OF COLIFORMS FROM IRRIGATION WATER TO FRESH PRODUCE

ABSTRACT

The transfer of faecal coliforms from polluted irrigation water to fresh produce was explored by incorporating phenotypic and genotypic profiling as source trackers. Antibiotic resistance profiles were determined for 105 *E. coli* isolates (92 environmental strains obtained from irrigation water and fresh produce and 13 reference strains). Similarity clustering was done using numerical classification systems to group isolates. Variation was found for both genotypic and phenotypic characteristics of the 105 isolates. Based on the phenotypic API 20E data, the 105 isolates were grouped into 15 clusters of which 53 isolates (51%) were successfully classified as *E. coli*. The Multi-Antibiotic Resistance (MAR) genotypic data also indicated variation with the isolates grouped into 27 different clusters. The majority of isolates showed resistance to vancomycin and were either inhibited or sensitive to ofloxacin. Most importantly, however, was the fact that 34% of the *E. coli* isolates, consisting of isolates obtained from irrigation water and fresh produce, were classified together based on both genotypic and phenotypic characteristics. These results indicated similar genotypic and phenotypic characteristics and therefore showed that these isolates were the same species. It could therefore be concluded that the isolates obtained from the irrigation water and those isolated from the surface of the green beans originated from the same source of pollution, thereby confirming the transfer of organisms from polluted irrigation water to fresh produce.

INTRODUCTION

Faecal contamination of water resources is a serious problem which many countries are facing (Lu *et al*., 2004). South Africa's water resources are often severely polluted with microbial contaminants originating from humans, animals and other environmental practices (Olaniran *et al*., 2009). Waters contaminated with human faecal matter are generally regarded as a greater risk as they are more likely to contain human-specific enteric pathogens such as *E. coli*, *Salmonella enterica* serovar *Typhi*, *Shigella* spp. and the hepatitis A virus (Scott *et al*., 2002). As a result, it is estimated that more than 3 million

people, mostly children, die each year from water-related diseases (Wilkes *et al*., 2009). It is therefore critical that a clear understanding of the various contamination sources of faecal pollution be achieved for the successful risk assessment and development of corrective management techniques (Scott *et al*., 2002; Lu *et al*., 2004; Field & Samadpour, 2007; Carroll *et al*., 2009).

Microbial Source Tracking (MST) is based on identifying the source of faecal pollution by identifying source-specific features by means of microbiological, genotypic, phenotypic or chemical methods (Scott *et al*., 2002; Graves *et al*., 2006; Yan & Sadowsky, 2007). These methods are used to group organisms according to their origin or host, based on subtle differences within groups. Members or sub-groups within a species become more adapted to a particular host or environment, therefore, over time, organisms within a particular host or environment should possess a similar or identical genetic or phenotypic fingerprint (Scott *et al*., 2002).

Several MST techniques have been evaluated in recent years (Carroll *et al*., 2009) and although this field of study is still under development, consensus is yet to be reached regarding the effectiveness of using microbial source tracking tools. Whilst some researchers regard MST purely as a useful supplementary tool (Stapleton *et al*., 2007), others advocate the feasibility of MST techniques to predict the source of faecal contamination in surface waters (Carroll *et al*., 2009; Parajuli *et al*., 2009).

In 2009, Carrol and colleagues studied the antibiotic resistance profiles of 1 005 *E. coli* isolates. Discriminant Analysis and Partial Least Square (PLS) was used to differentiate between the different Antibiotic Resistance Profiles (ARP's) and consequently identify the contamination source. Results indicated that the majority of faecal contamination could be ascribed to non-human sources. It was also established that the percentage of human faecal isolates increased closer to urban areas. From this and using PLS regression, they were able to develop predictive models which indicated a high correlation between human source isolates and surrounding urban areas. A potential source of human faecal contamination was therefore identified. Although classified as a useful tool, researchers also stated the lack of quantitative source apportionment data that MST provided (Carrol *et al*., 2009).

The aim of this study was to link the suspected carry-over of faecal organisms present in the water from the Plankenburg River to fresh produce irrigated with this water. Faecal indicators will be isolated from the irrigation water and from the surface of green beans that will be irrigated with this water. Genotypic and phenotypic characteristics of isolated

strains will be characterised using API 20E kits and Multi-Antibiotic Resistance (MAR) profiles and the data compared using numeric methods. This study will be conducted on the basis that a high numerical similarity indicates isolates that originate from the same source. *E. coli* was chosen as faecal indicator of choice during this study as this organism have been widely accepted as an indicator of faecal contamination and is therefore also an indicator of the possible presence of other enteric pathogens.

MATERIALS AND METHODS

Isolation of faecal coliforms from irrigation water and irrigated green beans

Plot design and sampling procedure

A 16 m^2 plot, as part of a larger field of green beans (*Phaseolus vulgaris*), was irrigated daily for nine consecutive days using irrigation water collected from the Plankenburg River (site P_1)(Fig. 1). The beans were irrigated using 6 L irrigation water per session, simulating the over-head irrigation technique used by most commercial farmers.

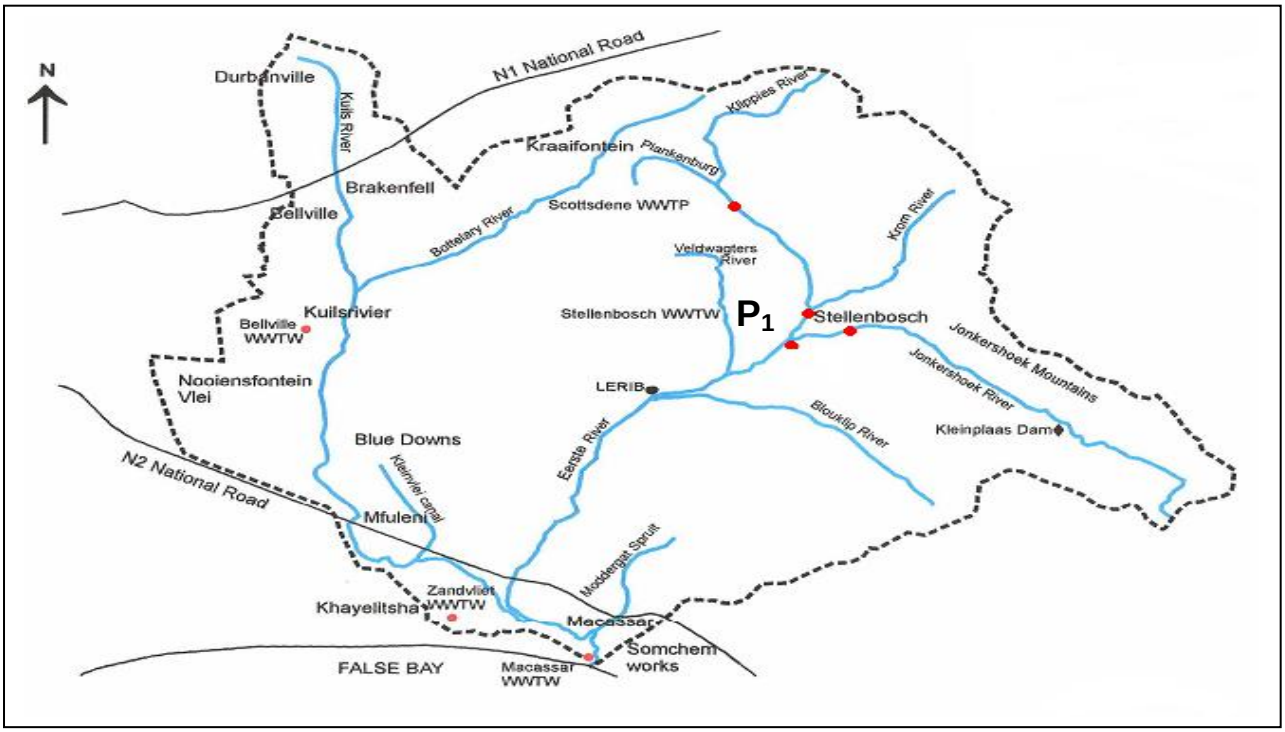


Figure 1 Sampling site in the Plankenburg River that was used as source of irrigation water (map as given by Ngwenya, 2006)

Samples of green beans (400 g) were analysed prior to the first irrigation session to establish a base-line value of the faecal indicators that are present on the surface of the

beans. Thereafter, two sample sets of both green beans (400 g) and irrigation water (1 L) were analysed daily for nine consecutive days using the Multiple Tube Fermentation technique - MFHPB 19 (Health Canada, 2002) for the detection of coliforms, faecal coliforms and *E. coli*. The presence of *E. coli* was confirmed as dark purple colonies with a metallic green sheen on Eosin Methylene Blue (L-EMB) agar (Oxoid) in the final step of the MTF method. Five metallic green colonies from the L-EMB agar were randomly selected per sample and purified. The purity of all isolates was confirmed by means of Gram-staining and microscopic characterisation. Further confirmation of purity was done by transferring the isolates to Nutrient agar (Merck) and then plating them out on three selective media, MacConkey agar (Oxoid), Chromogenic *E. coli* agar (Oxoid) and Brilliance *E. coli*/ coliform agar (Oxoid), to monitor individual colony pigments and morphological traits. This process was repeated for nine days and included a total of nine green bean and nine irrigation water samples.

Irrigation water sampling

An irrigation water sample containing 7 L of water was collected from site P_1 (Fig. 1) prior to each irrigation session. The sample was collected in a sterile Schott bottle according to guidelines of the South African Bureau of Standards (SABS, 1984). After mixing well, 6 L of the sample was used to irrigate the plot of green beans and the remaining 1 L used for microbial analyses in the laboratory. The microbial load of the 1 L sample was analysed within 60 min after arrival at the laboratory.

Green bean sampling

Green bean samples consisting of 400 g each were obtained daily, prior to each irrigation session, by randomly selecting beans across the 16 m^2 plot in order to obtain a representative sample of that area. Sterile latex gloves were worn during sampling and samples taken in sterile stomacher bags. Samples were transported on ice and analysed within 60 min upon arrival at the laboratory.

Preparation of green bean samples

Green bean samples were prepared for analyses by adding 400 mL of sterile physiological saline solution (PSS) to the 400 g sample in the sterile stomacher bag. The bag was sealed and gently shaken for 5 min to release the organisms attached to the surface of the

beans into the PSS. The wash water was then decanted aseptically into a sterile 500 mL Schott bottle to promote the effective handling of the sample during microbial testing.

Multiple Tube Fermentation (MTF) method

The MTF method (Health Canada, 2002) as illustrated in Fig. 2, was used to determine the coliform, faecal coliform and *E. coli* concentrations present on the surface of the green beans as well as in the irrigation water. The presence of *E. coli* was confirmed by streaking out all positive EC tubes (tubes with gas and fluorescing under UV light) onto Eosin Methylene Blue (L-EMB) agar (Oxoid) and enumerating typical *E. coli* colonies as those that produce dark purple or black colonies with a distinct metallic sheen on the surface of the L-EMB agar (Merck Manual, 2005).

Colony pigmentation and morphological characteristics

Five metallic green colonies were selected from each green bean and irrigation water sample and transferred to three selective media: Chromogenic *E. coli* agar (Oxoid), Chromogenic agar (Oxoid) and Brilliance *E. coli* agar (Oxoid).

Purified isolates were also subjected to Gram-staining, catalase and oxidase tests to assess the morphological characteristics of each isolate.

Storage

Pure isolates were sub-cultured in Nutrient (NA) broth (Merck) and incubated at 35°C for 24 h. An 80% sterile glycerol solution was prepared in sealable plastic tubes in units of 500 µL per tube. An aliquot (500 µL) of NA broth (containing the sub-cultured isolates) were transferred into the clearly marked tubes and stored at -80˚C for further use.

Reference and marker strains

An additional set of strains (M:1 to M:13) from clinical sources and kindly supplied by Dr J.M. Barnes (Department of Community Health, University of Stellenbosch) were also included as medically identified *E. coli* markers. An ATCC *E. coli* strain, strain 58 (ATCC 11775) was characterised in duplicate and the data added for quality control purposes.

Numerical analysis

For numerical clustering the characteristics of 105 strains composed of 54 isolates from the irrigation water, 38 from the irrigated beans, 13 medical reference strains and 1 ATCC

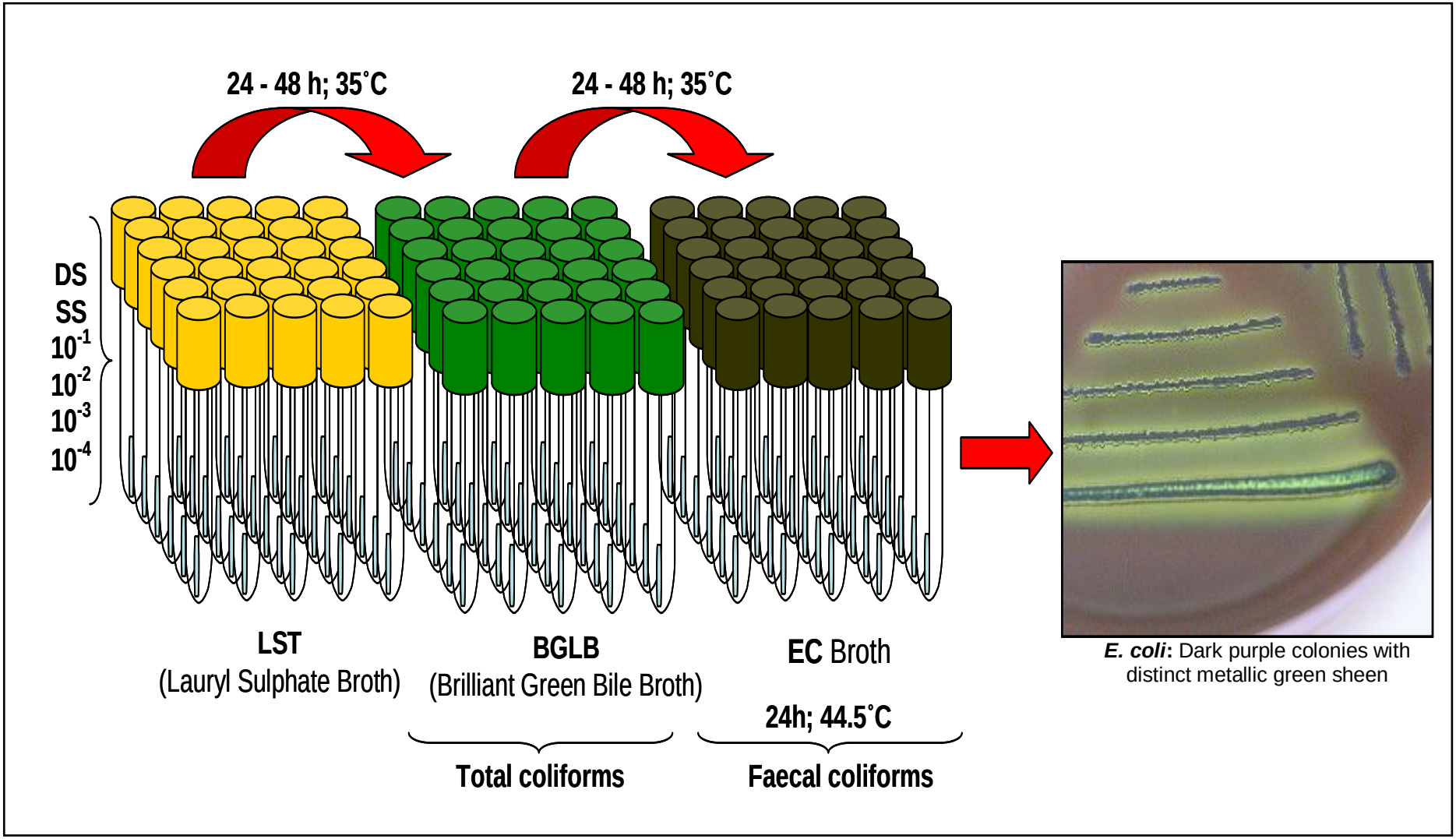


Figure 2 The Multiple Tube Fermentation technique (Health Canada, 2002)

reference strain, were included. Twenty-seven characters were included in the data set and analysed using the Jaccard (SJ) and Sokal & Michener (SM) coefficients and the unsorted similarity matrix was rearranged into groups by average linkage cluster analysis (Lockhart & Liston, 1970). Tests that gave uniform results for all the strains were excluded from the numerical analyses. Dendrogram distances were calculated based on the phenotypic characteristics as calculation concept.

Source tracking techniques

API 20E

The 92 Gram negative isolates, 13 medical reference strains and one ATCC reference strain were characterised using API 20E kits (Biomerieux). The API 20E was used to generate phenotypic profiles for each isolate based on their ability to metabolise the different substrates (Biomerieux Manual). Isolates were then classified into various groups based on their phenotypic characteristics, with a high degree of similarity indicated by isolates that were grouped together or closer to each other.

Multi-Antibiotic Resistance (MAR) profiling

MAR was used to differentiate *E. coli* from different sources by isolating and culturing the target organism and then replica plating the isolates on media containing the different antibiotics at varying concentrations (Scott *et al*., 2002). Organisms were then scored according to their resistance to the antibiotics resulting in Antibiotic Resistance Profiles (ARP).

Mueller-Hinton agar (Oxoid) was sterilised at 121°C for 15 min, allowed to cool, poured into petridishes (approximately 20 mL per dish) and allowed to set. Spread plates were prepared on Mueller-Hinton agar by transferring 0.1 mL suspension of each isolate to a petridish and spreading the organism across the surface of the agar using a sterile spreader.

Twenty-three different antibiotic discs (Davies Diagnostics), each containing a specific type of antibiotic at a specific concentration (Table 1), were dispensed onto the surface of the clearly marked petridishes using a Multi-precision disc dispenser (Davies Diagnostics). Six antibiotic discs were positioned per plate and all plates incubated at 35˚C for 24 h.

Table 1 Antibiotics used in this study (Davies Diagnostics)

Type of antibiotic	Antibiotic class	Concentration
Cefotaxime	Cephalosporin	3 and 5 µg
Vancomycin		5 and 30 µg
Trimethoprim		2.5 µg
Tetracycline	Tetracyclines	10 and 25 µg
Gentamicin	Aminoglycosides	10 µg
Cefuroxime	Cephalosporin	30 µg
Cotrimoxazole		25 µg
Ciprofloxacin	Fluoroquinolones	1 and 5 µg
Amoxycillin	β-lactam	25 µg
Amikacin	Aminoglycosides	30 µg
Cephazolin		30 µg
Ofloxacin	Aminoglycoside	5 µg
Chloramphenicol	Phenicols	10 and 30 µg
Neomycin	Aminoglycosides	30 µg
Ceftriaxone	Cephalosporin	30 µg
Ampicillin	β-lactam	2 and 25 µg
Erythromycin	Macrolides	15 µg

After incubation, multi-antibiotic resistance profiles were compiled for each isolate by measuring the diameters of the inhibition zones (mm) caused by the various antibiotics and recording the data. This trial was performed in duplicate, average diameter readings were calculated and used during numerical clustering.

RESULTS AND DISCUSSION

Growth characteristics

A total of 92 *E. coli* isolates were obtained from the irrigation water from site P_1 in the Plankenburg River and the green beans which had been irrigated using water from site P_1 by the random selection of well-isolated dark purple colonies with metallic green sheen from the L-EMB agar. The 13 confirmed medical *E. coli* strains (supplied by J.M. Barnes, Department of Community Health, University of Stellenbosch) and one ATCC reference strain were characterised with the 92 environmental strains giving 105 faecal strains that were used during this study.

Table 2 Colony pigmentation of the 104 faecal isolates on four indicator media*

Isolate	Source	L-EMB*	McConkey*	Chromogenic *E. coli**	Brilliance *E. coli**
01:01	Irrigation water	Metallic green	Blood red	Purple, slight pink halo	White
01:02	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	dark purple
01:03	Irrigation water	Metallic green	Blood red & slight discoloration of agar	Purple with prominent pink halo	dark purple
01:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	dark purple
01:05	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
03:01	Beans	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Dark purple
03:02	Beans	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Dark purple
03:03	Beans	Metallic green	Blood red & slight discoloration of agar	Pink colony	Dark purple
03:04	Beans	Metallic green	Blood red colony & slight discoloration of agar	Pink colony	Dark purple with grey shine
03:05	Beans	Metallic green	Blood red & slight discoloration of agar	Pink colony	Dark purple
04:01	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
04:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
04:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
04:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
04:05	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
05:01	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
05:02	Irrigation water	Metallic green	Blood red	Purple with sllight pink halo	Dark purple
05:03	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
05:04	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
05:05	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
06:01	Beans	Metallic green	Blood red	Purple with slight pink halo	White
06:02	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple
06:03	Beans	Metallic green	Blood red	Purple with slight pink halo	White
06:04	Beans	Metallic green	Blood red	Purple with slight pink	Dark purple
06:05	Beans	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
07:01	Irrigation water	Metallic green	Blood red with slight discoloration of agar	Purple with prominent pink halo	Dark purple
07:02	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple

07:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Purple
07:04	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	White
07:05	Irrigation water	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Light pink
08:01	Beans	Metallic green	Red/Brown with discoloration of media	Purple with prominent pink halo	Dark purple
08:02	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
08:03	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple
08:04	Beans	Metallic green	Blood red	Light purple/pink	Dark purple
08:05	Beans	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
09:01	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
09:02	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Light pink, dark purple & white
09:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
09:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	White
09:05	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
10:01	Beans	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
10:02	Beans	Metallic green	Blood red	pink colony	Dark purple
10:03	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
10:04	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple
10:05	Beans	Metallic green	Blood red	Purple with prominent pink halo	Dark purple with grey sheen
11:01	Irrigation water	Metallic green	Blood red	Purple colony with slight pink halo	Dark purple
11:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
11:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
11:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
11:05	Irrigation water	Metallic green	Blood red	Purple with pink halo	White
12:01	Beans	Metallic green	Blood red	Light purple/pink	Dark purple
12:02	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple
12:03	Beans	Metallic green	Blood red	Light purple/pink	Dark purple
12:04	Beans	Metallic green	Blood red	Light purple/pink	Dark purple
12:05	Beans	Metallic green	Blood red	Light purple/pink	Dark purple
13:01	Irrigation water	Metallic green	Blood red	Light purple/pink	Dark purple
13:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple

13:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
13:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
13:05	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
14:01	Beans	Metallic green	Brown/orange colony with discoloration of agar	Purple with prominent pink halo	White
14:02	Beans	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Dark purple
14:03	Beans	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Dark purple
14:04	Beans	Metallic green	Brown/orange colony with discoloration of agar	Pink colony	Dark purple
15:01	Irrigation water	Metallic green	Blood red with slight discoloration of agar	Purple with slight pink halo	Dark purple, light pink & white
15:02	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
15:03	Irrigation water	Metallic green	Blood red with slight discoloration of agar	Purple with slight pink halo	Dark purple
15:04	Irrigation water	Metallic green	Blood red	Purple with pink halo	Dark purple
15:05	Irrigation water	Metallic green	Blood red with slight discoloration of agar	Purple with slight pink halo	Dark purple
16:01	Beans	Metallic green	Blood red	Purple with slight pink halo	Dark purple
16:02	Beans	Metallic green	Blood red with slight discoloration of agar	Purple with slight pink halo	White
16:03	Beans	Metallic green	Blood red	Purple with slight pink halo	Light pink
16:04	Beans	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
16:05	Beans	Metallic green	Blood red	Purple with slight pink halo	White
17:01	Irrigation water	Metallic green	Blood red	Purple with prominent pink halo	Dark purple
17:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
17:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
17:04	Irrigation water	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
18:01	Beans	Metallic green	Blood red	Pink colony	Dark purple
18:02	Beans	Metallic green	Blood red/Pink	Purple with prominent pink halo	Dark purple & white
18:03	Beans	Metallic green	Blood red	Purple with prominent pink halo	Purple
18:04	Beans	Metallic green	Pink colony	Pink colony	Dark purple
20:01	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
20:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple

20:03	Irrigation water	Metallic green	Light pink colony with discoloration of agar	Pink colony	Dark purple & white
20:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
20:05	Irrigation water	Metallic green	Light pink colony with discoloration of agar	Pink colony	Dark purple
21:01	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
21:02	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
21:03	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
21:04	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple & white
21:05	Irrigation water	Metallic green	Blood red	Purple with slight pink halo	Dark purple
M:1	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:2	Human	Metallic green	Blood red	Dark blue	Dark purple
M:3	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:4	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:5	Human	Metallic green	Light pink mucoid colony with discoloration of agar	Dark purple with slight pink halo	Dark purple & white
M:6	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:7	Human	Metallic green	Creamy/pink mucoid colony with discoloration of agar	Dark purple with slight pink halo	Dark purple
M:8	Human	Metallic green	Light pink mucoid colony with discoloration of agar	White colony	White
M:9	Human	Metallic green	Light pink mucoid colony with discoloration of agar	White colony	White
M:10	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:11	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:12	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple
M:13	Human	Metallic green	Blood red	Dark purple with slight pink halo	Dark purple

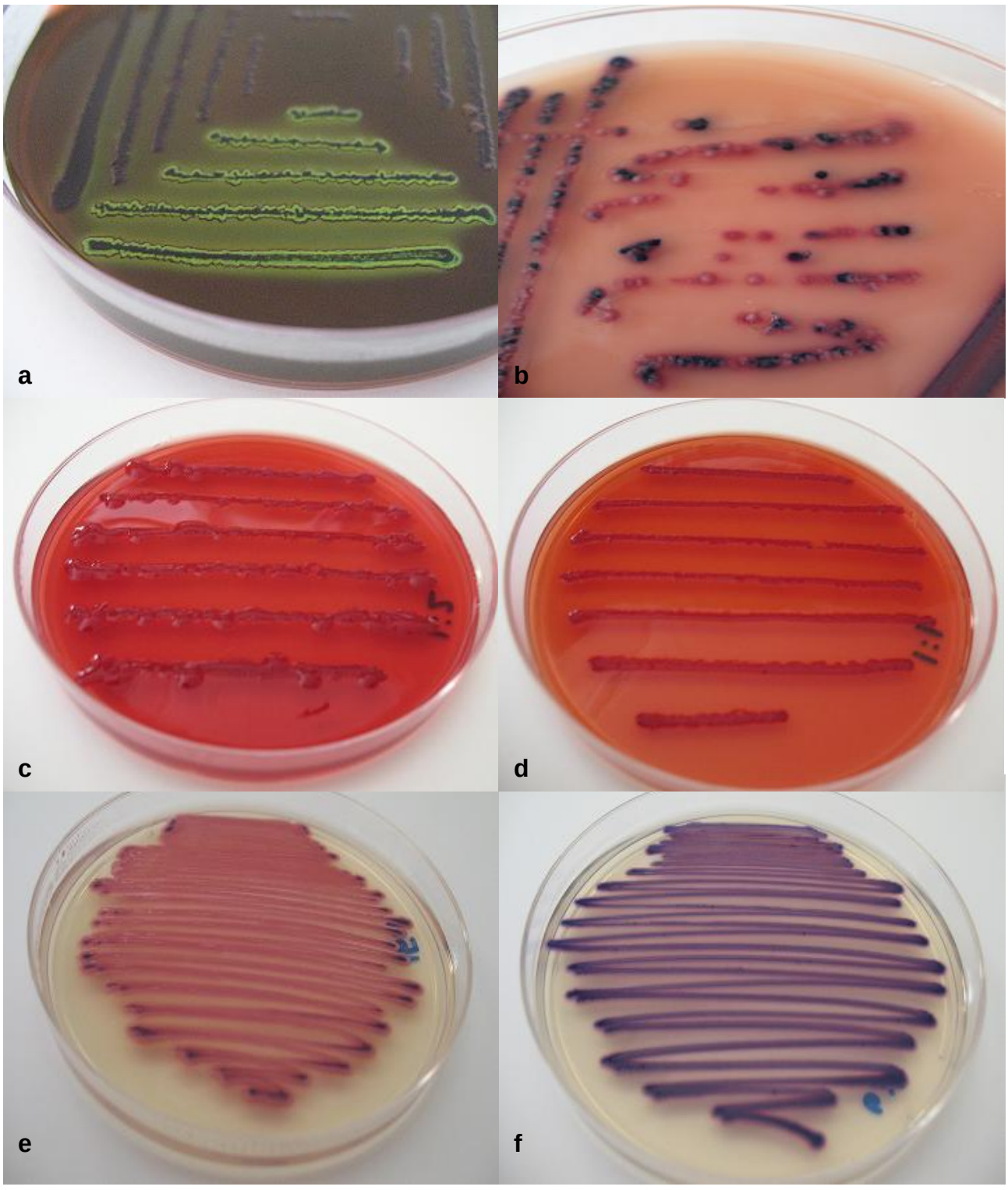


Figure 3 Different growth characteristics obtained on three differential media: (a) Dark purple colonies with a metallic green sheen represented the typical growth of *E. coli* on L-EMB agar. Dark red colonies with no discoloration of agar (c) and dark red colonies with precipitation zones (d) on MacConkey agar both representing typical growth of *E. coli* whilst light pink (b & e) and dark purple colonies (b & f) on *E. coli* chromogenic agar were identified as coliforms and *E. coli*, respectively.

The *E. coli* isolates were cultured on three indicator media: MacConkey agar (Oxoid), Chromogenic *E. coli* agar (Oxoid) and Brilliance *E. coli*/coliform agar (Oxoid) to compare the colony characteristics of the reference strains to that of the 92 environmental strains. Colony pigments obtained are presented in Tabel 2.

Typical colony characteristics of *E. coli* isolates on these media varied between reddish pink and dark purple colonies (Table 2). Although the majority of isolates exhibited the typical growth characteristics of *E. coli*, some strains did however, show unexpected colony characteristics.

Colonies on the MacConkey agar showed red colonies with (Fig. 3d) and without (Fig. 3c) discoloration of agar, pink colonies with and without discoloration of agar as well as a few brown/orange colonies (Table 2). All of these colony pigments did, however, fall within the specifications of the typical growth characteristics of *E. coli* as established by the manufacturer (Table 3) (Oxoid) and should therefore represent *E. coli*.

E. coli strains are known to possess both the β–glucuronidase and β–galactosidase enzymes which enable them to cleave chromogens in the *E. coli* chromogenic and Brilliance *E. coli*/coliform media resulting in the formation of dark purple colonies (Oxoid) (Fig. 3b & f). Microbial growth detected on both of these indicator media resulted in the formation of dark purple colonies, light pink colonies (Table 2) as well as white/colourless colonies (Table 2). According to the specifications of the manufacturer (Oxoid), pink colonies (Fig. 3b & e) indicate the presence of coliforms and white/colourless (Fig. 3b) or dark blue colonies indictae the presence of organisms that cannot be classified as either *E. coli* or coliforms (Table 3). At first, it was thought that some of these isolates might not be *E. coli* strains but rather contaminants which were able to pass through the hurdles presented by the MTF method. Very important, however, was that the 13 medical reference strains (M:1 to M:13, Addendum A) also displayed different growth characteristics on the MacConkey, Brilliance *E. coli*/Coliform and Chromogenic *E. coli* agar (Table 2).

Closer inspection of the growth characteristics of the medical reference strains (M:1 to M:13) showed that four strains (M:2; M:5; M:8 & M:9) produced colony characteristics different to the typical characteristics of *E. coli* as given by the manufacturer (Oxoid). Blood red colonies with no discoloration of agar and pink colonies that discoloured the agar were both recorded for these 13 strains on the MacConkey agar. Growth characteristics on Chromogenic *E. coli* agar consisted of mostly purple colonies, but also included dark blue and white colonies whilst the appearance of colonies on the Brilliance *E. coli*/coliform agar varied between dark purple and white colonies. All 13 medical strains

had been confirmed as *E. coli* by means of PCR (Dr J.M. Barnes, 2008) and it could therefore be concluded that traditional tools such as these confirmation media were unable to successfully confirm these isolates as *E. coli*. This could possibly be ascribed to variances that existed between different isolates of the same species, a phenomenon known to occur in nature as organisms are required to constantly adapt to a particular host or environment in order to survive (Scott *et al*., 2002).

Table 3 Identification media and corresponding typical colony characteristics of *E. coli* isolates

Growth Medium	**Typical growth characteristics of *E. coli***	**Growth characteristics recorded**
MacConkey Agar (Oxoid)	Pink to red colonies, with or without precipitation zone (Oxoid)	Blood red colonies with precipitation zones Blood red colonies without precipitation zones Brown/orange colony with precipitation zone Pink colonies Light pink colony with precipitation zone
E. coli Chromogenic agar (Oxoid)	Dark purple colonies – *E. coli* Pink colonies – coliforms Dark blue colonies – Organisms other than *E. coli* or coliforms (Oxoid)	Dark purple colony Pink colony Dark blue colony
Brilliance *E. coli*/coliform agar (Oxoid)	Dark purple colonies – *E. coli* Pink colonies – coliforms (Oxoid)	Dark purple colonies White colonies Light Pink colonies

Identification and clustering of isolates

The API 20E kit was used to characterise each of the 92 environmental and 13 medical reference strains using the API web based data base (Table 4). Identification percentages ≥98% were regarded as a positive identification. According to the API 20E database 53 of the 105 isolates were identified as *E. coli*, 4 as *Enterobacter*, 2 *Chromobacterium*, 3 as *Citrobacter*, 3 as *Raoultella*, 7 as Pseudomonas, 29 isolates showed a low identification discrimination between *Klebsiella pneumoniae* and *E. coli,* and the remaining 5 could not be identified using the API 20E. Phenotypic characteristics (Table 4) of each isolate were then used to determine similarities and grouping clusters as presented in the dendrogram in Fig. 3.

Table 4 Phenotypic characteristics and API classification of faecal isolates

Isolate	Source	Gram +/-	Morphology	Catalase +/-	Oxidase +/-	Identification*
01:01	Irrigation water	-	Rods	+	-	*Pseudomonas*
01:02	Irrigation water	-	Rods	+	-	*Klebsiella pneumoniae*
01:03	Irrigation water	-	Rods	+	-	*E. coli*
01:04	Irrigation water	-	Rods	+	-	*E. coli*
01:05	Irrigation water	-	Rods	+	-	*E. coli*
03:01	Beans	-	Rods	+	-	*E. coli*
03:02	Beans	-	Rods	+	-	*E. coli*
03:03	Beans	-	Rods	+	-	*Klebsiella pneumoniae*
03:04	Beans	-	Rods	+	-	*Klebsiella pneumoniae*
03:05	Beans	-	Rods	+	-	*Citrobacter*
04:01	Irrigation water	-	Rods	+	-	*Citrobacter*
04:02	Irrigation water	-	Rods	+	-	*Klebsiella*
04:03	Irrigation water	-	Rods	+	-	*E. coli*
04:04	Irrigation water	-	Rods	+	-	*E. coli*
04:05	Irrigation water	-	Rods	+	-	*E. coli*
05:01	Irrigation water	-	Rods	+	-	*Klebsiella*
05:02	Irrigation water	-	Rods	+	-	*Klebsiella*
05:03	Irrigation water	-	Rods	+	-	*E.coli*
05:04	Irrigation water	-	Rods	+	-	*Klebsiella*
05:05	Irrigation water	-	Rods	+	-	*E. coli*
06:01	Beans	-	Rods	+	-	*Unacceptable profile*
06:02	Beans	-	Rods	+	-	*E. coli*
06:03	Beans	-	Rods	+	-	*Pseudomonas*
06:04	Beans	-	Rods	+	-	*Klebsiella*
06:05	Beans	-	Rods	+	-	*Unacceptable profile*
07:01	Irrigation water	-	Rods	+	-	*E. coli*
07:02	Irrigation water	-	Rods	+	-	*Klebsiella*
07:03	Irrigation water	-	Rods	+	-	*Klebsiella*
07:04	Irrigation water	-	Rods	+	-	*Chromobacterium*
07:05	Irrigation water	-	Rods	+	-	*Enterobacter*
08:01	Beans	-	Rods	+	-	*Klebsiella*
08:02	Beans	-	Rods	+	-	*Klebsiella*
08:03	Beans	-	Rods	+	-	*E. coli*
08:04	Beans	-	Rods	+	-	*Klebsiella*
08:05	Beans	-	Rods	+	-	*E. coli*
09:01	Irrigation water	-	Rods	+	-	*Raoultella ornithinolytica*
09:02	Irrigation water	-	Rods	+	-	*E. coli*
09:03	Irrigation water	-	Rods	+	-	*E. coli*
09:04	Irrigation water	-	Rods	+	-	*Pseudomonas*
09:05	Irrigation water	-	Rods	+	-	*Klebsiella oxytoca*
10:01	Beans	-	Rods	+	-	*Citrobacter*
10:02	Beans	-	Rods	+	-	*Klebsiella*
10:03	Beans	-	Rods	+	-	*Klebsiella*
10:04	Beans	-	Rods	+	-	*Klebsiella*
10:05	Beans	-	Rods	+	-	*Klebsiella*
11:01	Irrigation water	-	Rods	+	-	*Raoultella*
11:02	Irrigation water	-	Rods	+	-	*Klebsiella*
11:03	Irrigation water	-	Rods	+	-	*E. coli*
11:04	Irrigation water	-	Rods	+	-	*E. coli*
11:05	Irrigation water	-	Rods	+	-	*Pseudomonas*
12:01	Beans	-	Rods	+	-	*E. coli*

12:02	Beans	-	Rods	+	-	*E. coli*
12:03	Beans	-	Rods	+	-	*E. coli*
12:04	Beans	-	Rods	+	-	*E. coli*
12:05	Beans	-	Rods	+	-	*E. coli*
13:01	Irrigation water	-	Rods	+	-	*Klebsiella*
13:02	Irrigation water	-	Rods	+	-	*E. coli*
13:03	Irrigation water	-	Rods	+	-	*E. coli*
13:04	Irrigation water	-	Rods	+	-	*Klebsiella*
13:05	Irrigation water	-	Rods	+	-	*Klebsiella*
14:01	Beans	-	Rods	+	-	*Pseudomonas*
14:02	Beans	-	Rods	+	-	*E. coli*
14:03	Beans	-	Rods	+	-	*E. coli*
14:04	Beans	-	Rods	+	-	*E. coli*
15:01	Irrigation water	-	Rods	+	-	*Pseudomonas*
15:02	Irrigation water	-	Rods	+	-	*Raoultella ornithinolytica*
15:03	Irrigation water	-	Rods	+	-	*E. coli*
15:04	Irrigation water	-	Rods	+	-	*E. coli*
15:05	Irrigation water	-	Rods	+	-	*E. coli*
16:01	Beans	-	Rods	+	-	*E. coli*
16:02	Beans	-	Rods	+	-	*Pseudomonas*
16:03	Beans	-	Rods	+	-	*Enterobacter*
16:04	Beans	-	Rods	+	-	*E. coli*
16:05	Beans	-	Rods	+	-	*Unacceptable profile*
17:01	Irrigation water	-	Rods	+	-	*E. coli*
17:02	Irrigation water	-	Rods	+	-	*E. coli*
17:03	Irrigation water	-	Rods	+	-	*Klebsiella*
17:04	Irrigation water	-	Rods	+	-	*E. coli*
18:01	Beans	-	Rods	+	-	*Klebsiella*
18:02	Beans	-	Rods	+	-	*Enterobacter*
18:03	Beans	-	Rods	+	-	*Klebsiella*
18:04	Beans	-	Rods	+	-	*Klebsiella*
20:01	Irrigation water	-	Rods	+	-	*E. coli*
20:02	Irrigation water	-	Rods	+	-	*E. coli*
20:03	Irrigation water	-	Rods	+	-	*E. coli*
20:04	Irrigation water	-	Rods	+	-	*E. coli*
20:05	Irrigation water	-	Rods	+	-	*E. coli*
21:01	Irrigation water	-	Rods	+	-	*Klebsiella pneumoniae*
21:02	Irrigation water	-	Rods	+	-	*Klebsiella pneumoniae*
21:03	Irrigation water	-	Rods	+	-	*Klebsiella pneumoniae*
21:04	Irrigation water	-	Rods	+	-	*E. coli*
21:05	Irrigation water	-	Rods	+	-	*E. coli*
M:1	Reference	-	Rods	+	-	*E. coli*
M:2	Reference	-	Rods	+	-	*E. coli*
M:3	Reference	-	Rods	+	-	*E. coli*
M:4	Reference	-	Rods	+	-	*E. coli*
M:5	Reference	-	Rods	+	-	*Chromobacterium*
M:6	Reference	-	Rods	+	-	*E. coli*
M:7	Reference	-	Rods	+	-	*E. coli*
M:8	Reference	-	Rods	+	-	*Unacceptable profile*
M:9	Reference	-	Rods	+	-	*Unacceptable profile*
M:10	Reference	-	Rods	+	-	*E. coli*
M:11	Reference	-	Rods	+	-	*E. coli*
M:12	Reference	-	Rods	+	-	*E. coli*
M:13	Reference	-	Rods	+	-	*E. coli*

*Identification percentages ≥98% were regarded as a positive identification

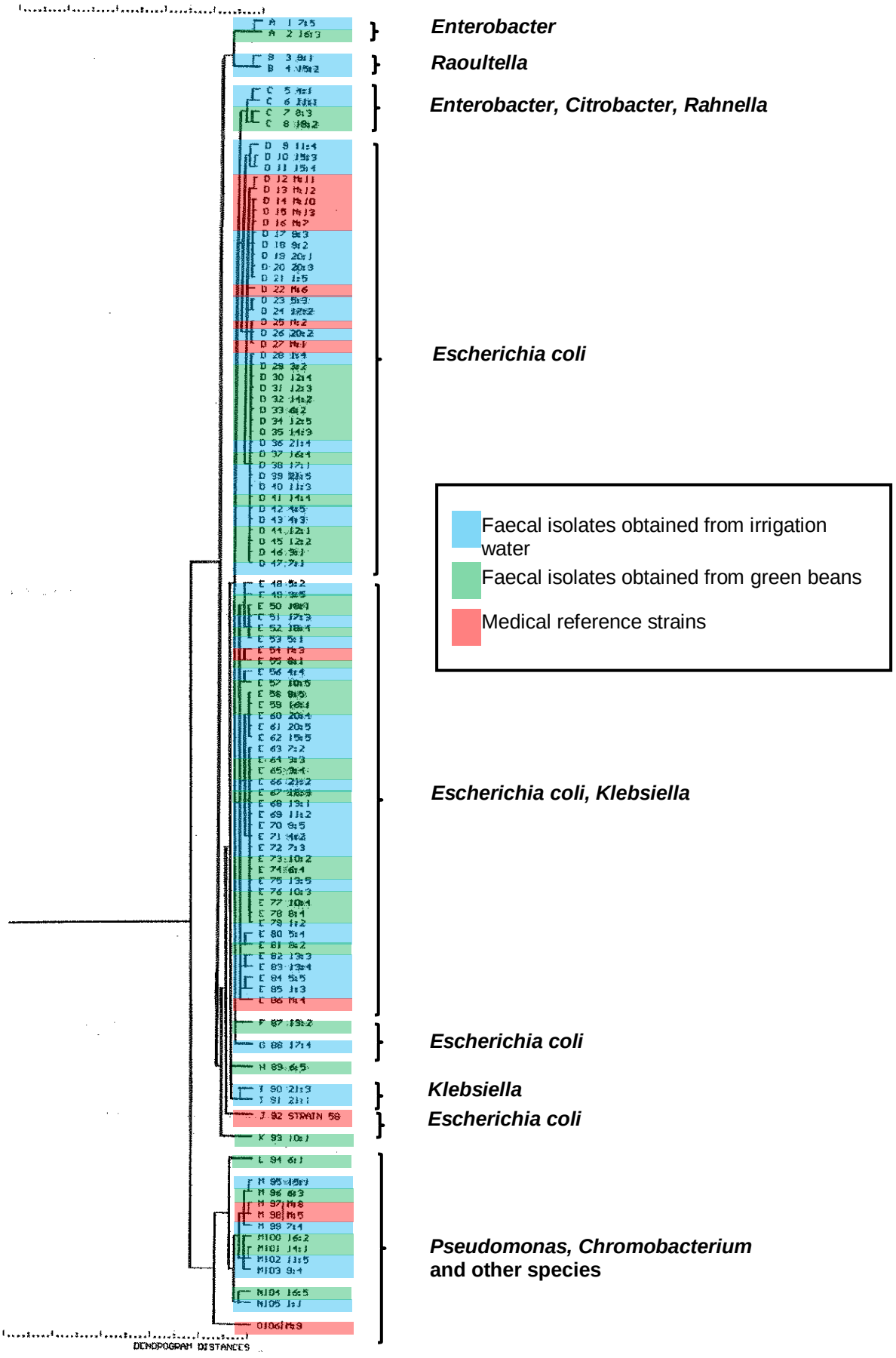


Figure 3 Dendrogram grouping of isolates based on API characterisation data

The dendrogram (Fig. 3) illustrates the spatial distribution of the isolates based on the degree of similarity and were grouped into 15 clusters (clusters A to O) of which the three main groups (Groups D, E and M) collectively contained 82% of the isolates.

In total, 39 of the 53 isolates which had been positively identified as *E. coli* were clustered in group D. Group E consisted of 39 isolates, of which 11 were positively identified as *E. coli* and the remaining 28 isolates were identified as *Klebsiella* with a low discrimination between *Klebsiella* and *E. coli*.

Very important also, was the identification and spatial distribution of the 13 medical strains (M:1 to M:13) as indicated in red in Fig.3. According to the API 20E database, 10 of these strains were identified as *E. coli* (strains grouped in groups D & E), 2 strains could not be identified and 1 was identified as *Chromobacterium*. The 13 medical reference strains were distributed across the entire length of the dendrogram, thereby indicating the variation within the group. The identification and clustering of these 13 isolates not only indicated a high degree of variance between isolates of the *E. coli* species, but also indicated similar phenotypic characteristics between some of the medical strains and the remaining 92 isolates which had been grouped as *E. coli*, *Klebsiella*, *Pseudomonas*, *Chromobacterium*, *Raoultella, Citrobacter* and other species.

Isolates originating from a specific environment often acclimatise or adapt to environmental conditions in order to survive and should therefore tend to share similar characteristics (Scott *et al*., 2002). Organisms grouped together based on similar phenotypic characteristics can therefore potentially have originated from the same source. Strains isolated from green beans (highlighted in green, Fig. 3) and irrigation water (highlighted in blue, Fig. 3) that were grouped together can thus be considered to be of the specific species in both these sources and therefore be taken, based on the API data, as an indication of direct transfer of isolates from irrigation water to fresh produce during the irrigation.

It could therefore be concluded that in spite of the obvious short-comings of using the API identification system to identify the 92 environmental strains as *E. coli*, this exercise still indicated that not all isolates, even if they are from the same species, share the exact set of characteristics. In addition, environmental strains are often exposed to extreme conditions that force them to adapt or acclimatise in order to survive, adaptations which could in turn make the organisms unrecognisable to the API identification system.

Very important, however, was that some of the phenotypic profiles of the 92 environmental *E. coli* strains resembled those of the 13 medical strains. Green bean and irrigation water

isolates that had been grouped together also indicated the possible transfer of organisms from irrigation water to fresh produce via irrigation.

Multi-antibiotic resistance (MAR) profiling

MAR profiling is based on the resistance or sensitivity of faecal isolates to different antibiotics to generate resistance profiles for every individual isolate (Carroll *et al*., 2009). For this study the resistance that an isolate displayed to a specific antibiotic was determined by grouping them based on the radius of each inhibition zone as either resistant (0 – 3 mm), intermediate (4 – 8 mm) or sensitive (≥ 9 mm). Inhibition profiles of the antibiotics are presented in Table 5.

The resistance of a specific isolate to the 23 antibiotics was combined to create a resistance profile. Isolates were then grouped in clusters based on the similarity of their MAR profiles using the SJ coefficient clustering similarities as presented in Fig. 4. The 105 faecal isolates were grouped into 27 clusters based on their multi-antibiotic resistance profiles (Addendum A), with isolates within each group exhibiting similar MAR traits. The five main clusters, clusters O, P, Q, X and Y, all contained between 5 and 34 isolates and collectively comprised 72% of the isolates. Isolates grouped in cluster O (5% of the isolates) were all resistant to Vancomycin and sensitive to Ofloxacin, Ceftriazone, Ciprofloxacin, Cefuroxime and Cefotaxime. Isolates grouped in cluster P (7% of isolates) were also resistant to Vancomycin, but were inhibited by Ampicillin, Ofloxacin, Ceftriaxone, Cefuroxime and Cefotaxime. The largest cluster (cluster Q) which comprised 33% of the total group, was resistant to Vancomycin, and inhibited by Ofloxacin, Ceftriaxone, Ciprofloxacin and Cefotaxime. The majority of isolates in this cluster also indicated high sensitivity towards Cefuroxime and Chloramphenicol. Clusters X (16% of isolates) and Y (11% of isolates), indicated resistance towards Vancomycin, Ampicillin, and Amoxicillin. However, cluster X was resistant to Tetracycline and Cotrimoxazole and cluster Y resistant to Trimethoprim. The majority of cluster X were inhibited by Ofloxacin, Ceftriaxone, Ciprofloxacin and Cefotaxime whilst cluster Y were only sensitive to Ceftriaxone and Ciprofloxacin.

The MAR profiles of the 13 medical strains varied and were distributed across the entire length of the dendrogram as illustrated in Fig. 4. The medical strains were grouped into 8 of the clusters (clusters G, I, K, M, P, S, X and Y) according to their unique MAR profiles. As in the case of the phenotypic classification, different MAR profiles of the 13 medical reference strains indicated the genotypic variance that exists between isolates from the same species.

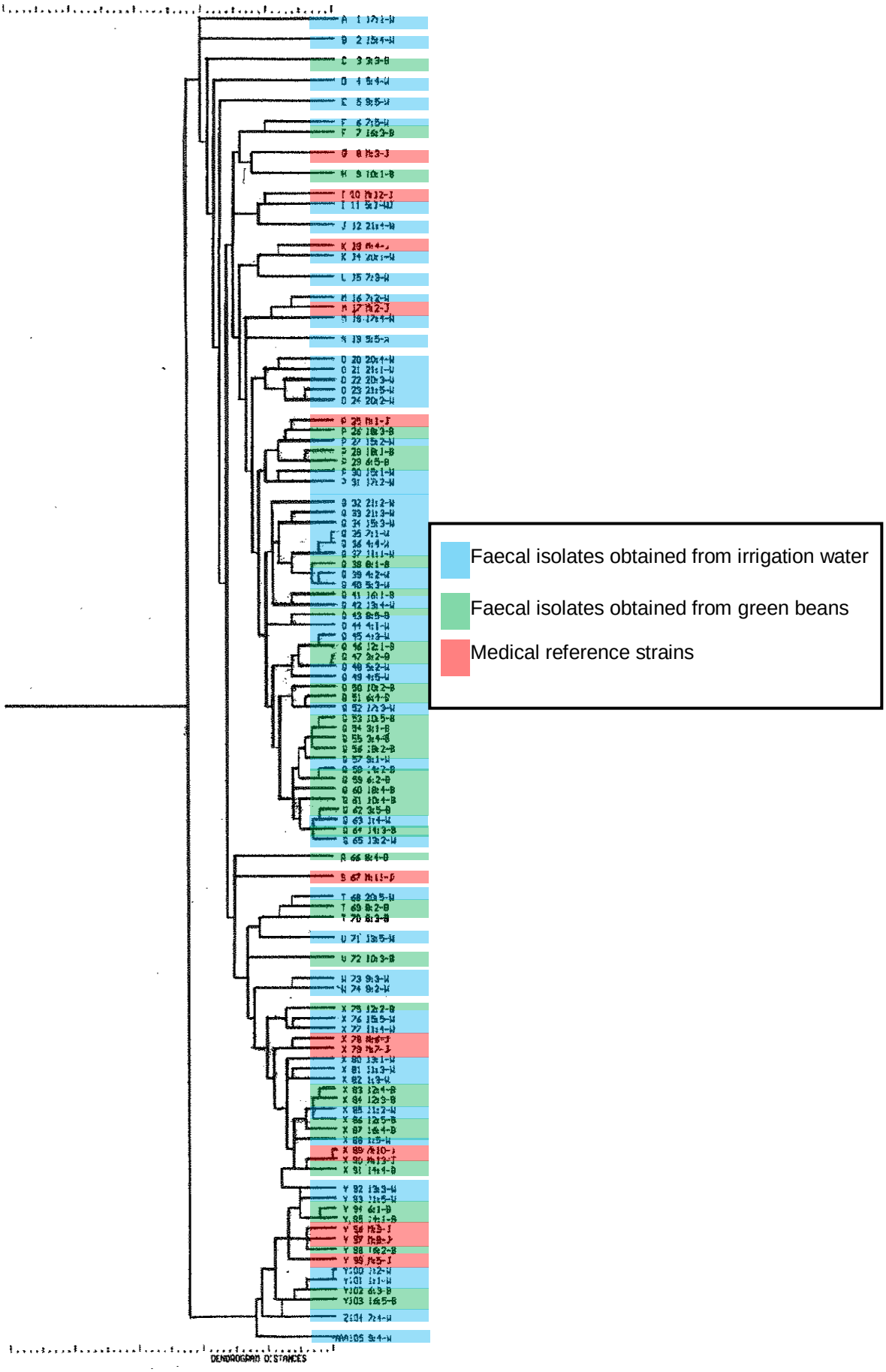


Figure 4 Dendrogram clustering of isolates based on their MAR profiles

Table 5 Inhibition profiles of antibiotics used in this study for the 105 faecal isolates

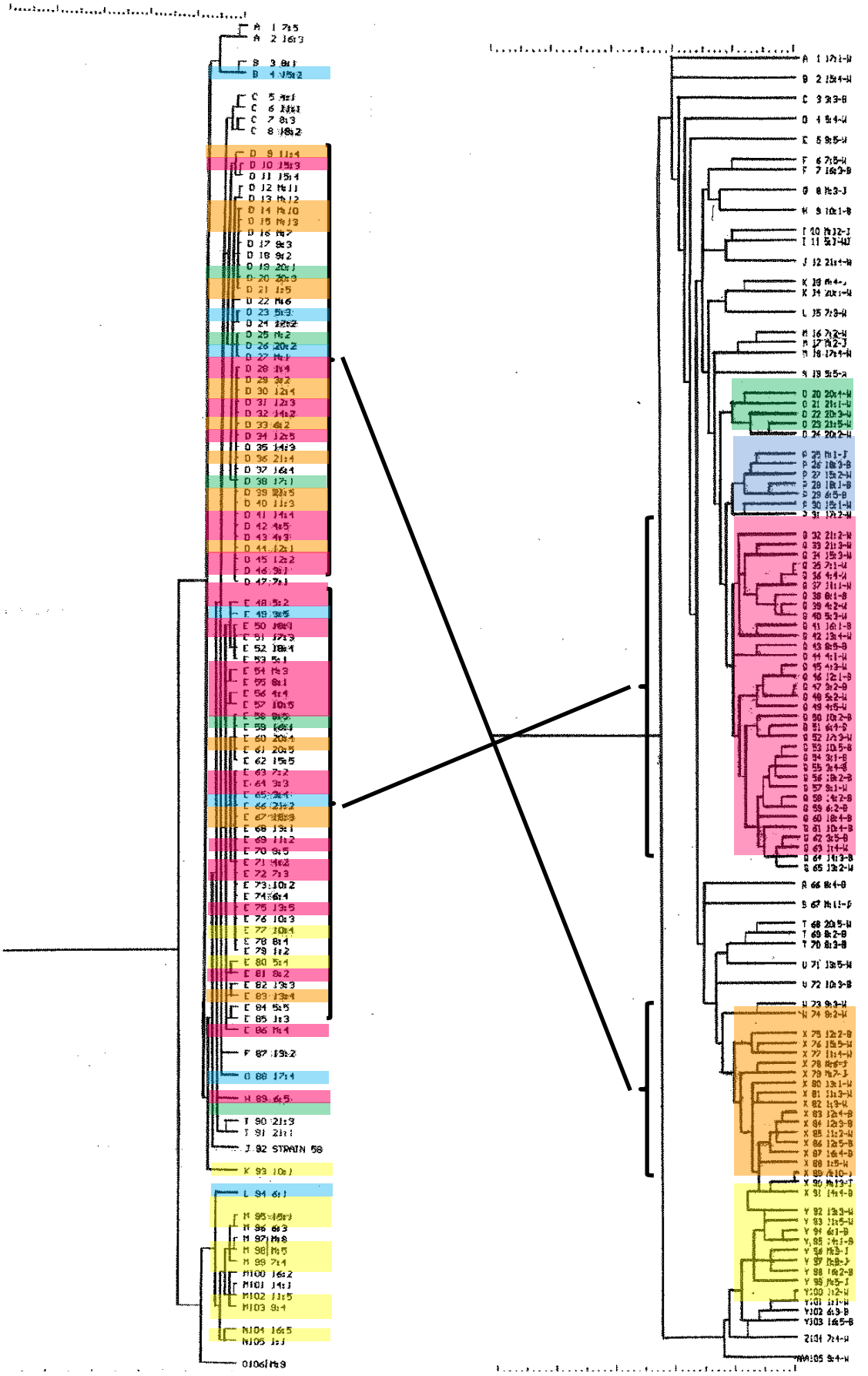
Type of antibiotic	Concentration µg	Resistant (0–3 mm)	Intermediate (4-8 mm)	Sensitive (≥ 9 mm)
Cefotaxime	5	22	10	73
Vancomycin	5	104	1	0
Vancomycin	30	103	1	1
Trimethoprim	2.5	103	1	1
Trimethoprim	5	42	50	13
Tetracycline	10	47	58	0
Tetracycline	25	45	60	0
Gentamicin	10	5	89	11
Cefuroxime	30	15	37	53
Cotrimoxazole	25	47	37	21
Ciprofloxacin	1	18	3	84
Ciproloxacin	5	3	3	99
Amoxycillin	25	46	52	7
Amikacin	30	17	69	19
Cephazolin	30	28	55	22
Ofloxacin	5	5	16	84
Chloramphenicol	10	14	85	6
Chloramphenicol	30	16	57	32
Neomycin	30	5	95	5
Ceftriaxone	30	7	6	92
Ampicillin	2	88	17	0
Ampicillin	25	38	20	47
Erythromycin	15	87	18	0

Isolates were grouped together based on similar resistance profiles and therefore indicated similar genotypic characteristics. These genotypic abilities could have been acquired through the repeated exposure to specific antibiotics. Similar MAR profiles therefore suggest exposure to the same environmental conditions and consequently, the conclusion that isolates which had been grouped together most probably originated from the same environment and therefore from the same species.

These results therefore suggest that irrigation water and green bean isolates which clustered together were exposed to similar antibiotics, therefore possibly originating from the same environment. This grouping consequently confirms the possible transfer of harmful faecal organisms from irrigation water to fresh produce via the irrigation system.

Comparison of the API 20E and MAR clustering data

Both the API 20E characteristics and MAR profiling were used to link organisms present in irrigation water to those isolated from the surface of green beans irrigated with this water. In other words, the 19 biochemical tests of the API 20E together with the 23 different antibiotic resistance zones all served to create unique profiles of each isolate and these



Dendrogram A: Clustering based on API data

Dendrogram B: Clustering based on MAR data

Figure 5 Comparison of the isolates' clustering based on both API and MAR data

could therefore be grouped based on information obtained from both the API 20E and MAR data (Fig. 5).

Isolates grouped together based on the combined data should therefore share some similar phenotypic and genotypic characteristics which could in turn suggest that these isolates originate from the same source of pollution. This information could then be used to positively confirm the transfer of potentially pathogenic organisms from polluted irrigation water to fresh produce.

If one considers the three main clusters (clusters Q, X and Y) presented in the MAR dendrogram (Fig. 4) and retrace the grouping of these isolates to the API data (Fig. 3) it becomes evident that a significant portion of these isolates were grouped together based on both genotypic and phenotypic characteristics. Almost half (47%) of cluster Q (Fig. 4) were grouped together in cluster E (Fig. 3), 76% of cluster X (Fig. 4) were grouped together in cluster D (Fig. 3) and 50% of cluster Y (Fig. 4) was grouped together in cluster M of the API dendrogram (Fig. 3). In total, 34% of the 105 isolates were grouped together based on both their genotypic and phenotypic characteristics.

Equally important, was that approximately a third (26%) of the isolates classified together based on both genotypic and phenotypic characteristics were isolated from irrigation water (site P_1), 54% obtained from green beans irrigated with water from site P_1, whilst the remaining 20% consisted of reference strains. These results therefore suggest that strains isolated from both the irrigation water and green beans originate from the same source of pollution. Potential pathogens present in the rivers because of faecal pollution could therefore be transferred to fresh produce when water from this river was used as irrigation source.

CONCLUSION

Faecal isolates obtained from irrigation water and the surface of green beans irrigated with this water were combined with 13 medical strains to form a library of faecal isolates which were subjected to microbial testing during this trial. Morphological, phenotypic and genotypic characteristics were identified for each strain by means of differential agar, API 20E and MAR profiling.

The 92 environmental strains used in this trial were obtained by using the Multiple Tube Fermentation technique (Health Canada, 2002). All 105 faecal strains (92 environmental and 13 medical strains) exhibited typical growth characteristics of *E. coli* on the L-EMB agar. Strains were identified as non-sporing, Gram-negative bacilli, oxidase-negative, catalase-positive and IMVIC ++--. These morphological characteristics resembled that of

typical *E. coli* colonies. The 105 faecal strains, did however, present varying colony characteristics on three differential media, MacConkey, *E. coli* chromogenic and Brilliance *E. coli*/coliform agar. Growth characteristics of both the environmental and medical strains ranged from colonies that exhibited the typical colony characteristics of *E. coli* to others which, based on these characteristics, would not be classified as coliforms.

Very important, however, was that the colony characteristics of the 13 confirmed *E. coli* medical strains did not conform to one specific combination of characteristics either, but ranged across the entire spectrum of possibilities. These varying colony characteristics indicated the variance that exists between different isolates within the faecal coliforms.

Classification based on phenotypic characteristics as determined by the API 20E tests also presented unexpected results. Firstly, the API classification system was unable to identify all 13 medical strains to the species level of *E. coli*. Secondly, the API results showed some similarities between the 92 environmental strains, 50% were classified as *E. coli*, 3% as *Enterobacter*, 7% as *Pseudomonas*, 28% indicated a low discrimination between *E. coli* and *Klebsiella* wilst the remaining 10% were identified as either *Raoultella*, *Citrobacter*, *Shigella* or *Chromobacterium*. These results indicated a large phenotypic variation between isolates which could possibly be because of adaptations brought about by changing environmental conditions.

Results of the MAR profiles also indicated variation between isolates of the same species based on the different responses to the 23 different antibiotics. The majority of isolates showed resistance to vancomycin, a glycopeptide antibiotic commonly used as reserve antibiotic for the treatment of infections caused by Gram-positive bacteria, and inhibition or sensitivity to ofloxacin, an antibiotic only used to treat serious bacterial infections such as chronic bronchitis, pneumonia and urinary tract infections (Peters *et al*., 2003). The MAR responses of the 13 medical strains once again showed a large variance. The reference strains were distributed across the entire length of the dendrogram, thereby reflecting the variance detected in the 92 environmental strains.

Isolates were clustered together based on similar phenotypic and genotypic characteristics, respectively. Strains from beans and irrigation water which were clustered together could have possibly originated from the same source and therefore thus indicates the transfer of potential pathogens from irrigation water to fresh produce.

Clustering based on similar MAR profiles indicated adaptations possibly caused by frequent exposure to the specific antibiotics or environmental conditions. Strains isolated

from green beans and irrigation water were once again clustered together, suggesting that they might have originated from the same environment and same strain, thereby contributing to the possibility that they might have been transferred from polluted irrigation water to fresh produce during irrigation.

Finally, just over a third of the 105 isolates were classified together based on both genotypic and phenotypic characteristics. These isolates included strains originating from irrigation water as well as fresh produce. Similar genotypic and phenotypic traits therefore suggest that isolates obtained from the green beans are closely related to strains from irrigation water. It was therefore concluded that the faecal strains on the surface of green beans were present due to the transfer of faecal isolates from the contaminated irrigation water to the fresh produce. Therefore, these results also indicate the transfer of potential pathogens from polluted irrigation water to the surface of fresh produce. The consumption of raw or minimally processed fruit and vegetables could therefore cause serious food infections when contaminated with polluted irrigation water.

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Appendix A

To chapter 5

To simplify the discussion of the results, the data illustrated in Figs. 3 - 5 have been included in this Appendix.

Appendix A:

Tabel 1 Antibiotics used to generate Multi-Antibiotic resistance profiles

Code	Antibiotic	Concentration (µg)
VA_{30}	Vancomycin	30
E	Erythromycin	15
AP_{25}	Ampicillin	25
OFX	Ofloxacin	15
CRO	Ceftriaxone	30
NE	Neomycin	30
AP_{2}	Ampicillin	2
C_{10}	Chloramphenicol	10
CIP_{1}	Ciprofloxacin	1
T_{25}	Tetracycline	25
A	Amoxycillin	25
CXM	Cefuroxime	30
T_{10}	Tetracycline	10
GM	Gentamicin	10
CZ	Cephazolin	30
AK	Amikacin	30
CIP_{5}	Ciprofloxacin	5
CTX	Cefotaxime	5
TM	Trimethoprim	2.5
VA_{5}	Vancomycin	5
TS	Cotrimoxazole	25
C_{3}	Chloramphenicol	30

Tabel 2 Multi-antibiotic resistance profiles of the 105 faecal isolates. Values indicate the radius of each inhibition zone which was classified as either resistant (0 – 3 mm), intermediate (4 – 8 mm) or sensitive (≥ 9 mm).

Code	Source	VA_{30}	E	AP_{25}	OFX	CRO	NE	AP_2	C_{10}	CIP_1	T_{25}	A	CXM	T_{10}	GM	CZ	AK	CIP_5	CTX	TM	VA_5	TS	C_3
01:01	Irrigation water	0	7	0	10	11	6	0	2	11	3	0	0	2	5	0	7	13	0	0	0	3	2
01:02	Irrigation water	0	2	11	12	13	5	3	5	12	6	7	10	4	7	8	3	12	12	6	0	10	7
01:03	Irrigation water	0	4	0	13	4	8	0	1	12	0	0	9	0	7	5	8	15	10	0	0	0	11
01:04	Irrigation water	0	2	10	11	12	6	0	5	12	8	7	8	5	7	7	9	15	10	6	0	5	8
01:05	Irrigation water	0	3	0	12	13	6	0	5	14	0	0	7	0	6	8	0	16	10	0	0	0	10
03:01	Beans	0	2	8	10	14	8	0	5	12	6	6	6	5	6	8	2	14	10	5	0	8	7
03:02	Beans	0	2	8	11	13	7	0	5	13	6	6	8	3	4	6	7	15	2	6	0	7	8
03:03	Beans	2	4	1	9	5	7	3	5	6	5	6	8	5	1	7	8	11	0	9	0	10	9
03:04	Beans	1	4	9	10	14	5	2	5	11	7	8	7	5	6	9	6	13	11	8	0	8	10
03:05	Beans	0	3	11	12	13	7	3	7	14	6	7	9	4	6	9	8	14	10	6	0	8	8
04:01	Irrigation water	0	2	0	11	13	7	0	5	11	6	0	10	5	7	8	0	13	10	7	0	7	7
04:02	Irrigation water	4	3	8	12	15	6	4	7	10	0	8	9	0	8	9	0	15	12	7	0	9	7
04:03	Irrigation water	0	2	6	10	13	7	0	5	11	6	4	7	4	6	5	6	15	8	9	0	9	9
04:04	Irrigation water	0	2	8	10	13	8	2	4	11	1	7	7	0	6	8	7	14	10	7	0	6	7
04:05	Irrigation water	0	3	9	11	15	7	0	7	12	8	0	10	4	7	9	7	13	0	6	0	8	8
05:01	Irrigation water	0	2	7	11	13	7	2	7	1	7	6	8	4	6	0	2	6	10	7	0	7	7
05:02	Irrigation water	0	2	8	11	13	7	2	6	13	7	6	7	4	0	7	6	14	0	6	0	7	8
05:03	Irrigation water	0	4	10	10	14	5	2	8	11	0	8	9	0	6	7	0	15	11	6	0	6	8
05:04	Irrigation water	0	8	11	10	8	5	2	5	12	8	5	10	5	9	8	8	2	12	10	0	9	9
05:05	Irrigation water	0	6	10	10	13	5	3	6	13	8	9	9	2	10	8	7	16	12	9	0	10	10
06:01	Beans	2	5	0	5	7	3	0	5	14	3	0	0	1	5	0	6	14	1	0	0	0	6
06:02	Beans	0	4	9	12	16	8	3	6	12	7	7	10	5	7	7	9	13	10	5	0	10	9
06:03	Beans	0	6	0	6	11	9	0	6	12	4	0	0	2	5	0	8	11	0	0	0	3	1
06:04	Beans	0	3	10	14	14	7	3	6	14	7	6	8	6	9	8	9	14	7	8	0	11	10
06:05	Beans	0	0	10	11	13	7	4	7	1	7	8	9	4	9	8	0	13	9	8	0	8	7
07:01	Irrigation water	0	0	8	11	12	7	1	2	12	0	7	8	0	6	8	8	14	12	6	0	8	7
07:02	Irrigation water	0	2	11	14	14	7	4	11	14	7	1	9	5	7	10	8	15	11	7	0	1	10
07:03	Irrigation water	0	3	9	11	13	6	4	7	13	5	0	8	3	8	8	0	15	13	11	0	10	10
07:04	Irrigation water	0	0	0	5	0	7	0	9	0	5	0	0	0	5	0	7	14	0	0	0	2	6
07:05	Irrigation water	0	0	2	11	12	7	0	4	12	6	0	8	4	7	0	6	14	8	8	0	10	11
08:01	Beans	0	2	9	13	14	7	4	7	13	0	9	9	0	7	9	0	17	10	0	0	6	8
08:02	Beans	0	2	10	11	13	6	4	7	12	0	9	6	0	6	9	7	15	10	0	0	8	10
08:03	Beans	0	2	0	0	14	5	0	8	13	0	7	9	0	1	8	7	16	10	0	3	6	4

08:04	Beans	0	2	7	13	15	6	5	8	13	0	6	10	0	8	10	0	16	12	0	0	0	9
08:05	Beans	0	3	9	11	14	7	0	7	13	7	1	8	6	4	8	0	15	10	6	0	8	6
09:01	Irrigation water	2	4	6	10	15	7	2	8	13	6	7	8	5	8	8	7	15	0	6	0	9	9
09:02	Irrigation water	0	0	0	5	14	7	0	4	6	0	0	8	4	6	7	8	7	11	0	0	2	9
09:03	Irrigation water	0	3	0	6	14	8	0	5	6	0	0	0	0	7	9	10	9	10	0	0	0	7
09:04	Irrigation water	20	8	0	4	9	3	0	2	0	3	0	0	1	5	0	7	12	0	0	0	0	7
09:05	Irrigation water	0	8	7	10	14	6	2	7	0	0	0	8	5	6	9	9	12	8	7	7	0	6
10:01	Beans	0	1	6	8	14	6	0	2	12	5	4	7	4	7	9	8	14	7	4	0	9	7
10:02	Beans	1	2	8	10	14	6	1	9	13	7	5	8	5	8	0	7	15	6	8	0	9	10
10:03	Beans	0	2	10	11	7	5	3	0	1	0	8	10	0	7	8	7	15	11	0	0	7	9
10:04	Beans	0	3	10	12	14	7	1	6	13	6	6	9	5	6	8	7	15	11	11	0	11	9
10:05	Beans	0	3	9	10	13	6	1	5	12	6	6	8	5	6	9	8	13	10	6	0	9	6
11:01	Irrigation water	0	2	10	12	15	5	3	3	12	0	7	9	4	6	8	7	15	10	5	0	5	7
11:02	Irrigation water	0	0	10	11	13	5	3	7	12	0	0	7	0	6	8	7	12	10	0	0	0	8
11:03	Irrigation water	0	2	0	11	14	5	0	4	11	0	0	8	4	6	7	7	12	11	10	0	4	8
11:04	Irrigation water	0	2	0	11	14	8	0	5	0	0	0	7	5	8	6	6	15	10	0	0	0	9
11:05	Irrigation water	0	0	0	7	9	3	0	7	12	3	0	2	2	5	0	8	15	0	0	0	4	4
12:01	Beans	0	2	8	11	15	7	0	6	13	6	6	8	4	8	7	8	15	9	7	0	7	9
12:02	Beans	0	2	0	12	15	7	0	7	1	0	0	8	0	6	6	10	15	9	0	0	0	8
12:03	Beans	0	2	0	12	14	5	0	6	13	0	0	10	0	6	7	9	16	11	0	0	0	8
12:04	Beans	0	2	0	12	14	7	0	6	13	0	0	8	0	6	8	9	12	10	0	0	0	7
12:05	Beans	0	2	0	12	3	8	0	6	13	0	0	8	0	6	8	7	15	10	0	0	0	8
13:01	Irrigation water	0	2	0	13	15	9	0	7	12	7	0	9	4	6	2	0	15	11	0	0	0	9
13:02	Irrigation water	0	0	10	12	14	7	4	7	13	8	7	8	5	6	7	7	15	10	4	0	8	8
13:03	Irrigation water	0	0	0	6	10	4	0	0	11	2	0	0	0	5	0	8	13	0	0	0	3	9
13:04	Irrigation water	0	2	10	13	15	8	5	7	13	6	8	8	4	6	10	8	16	13	5	0	7	6
13:05	Irrigation water	0	4	11	12	15	8	3	1	12	0	7	8	0	9	0	9	14	10	0	0	0	3
14:01	Beans	0	6	0	5	9	3	0	4	13	3	0	0	2	6	0	7	12	1	0	0	2	5
14:02	Beans	0	3	9	12	15	6	3	8	13	8	8	11	5	9	7	8	13	11	7	0	7	8
14:03	Beans	0	2	10	12	15	8	0	7	13	7	7	9	6	7	8	10	12	11	6	0	7	8
14:04	Beans	0	0	0	12	15	7	0	5	14	0	0	9	0	7	0	8	13	0	0	0	0	8
15:01	Irrigation water	0	3	10	12	16	6	4	8	13	8	9	10	6	7	0	0	13	9	8	0	8	6
15:02	Irrigation water	0	0	11	13	15	6	5	8	13	7	8	10	4	7	8	7	14	8	7	0	8	8
15:03	Irrigation water	0	2	8	10	14	6	3	7	2	0	7	9	5	8	8	7	13	8	7	0	7	7
15:04	Irrigation water	0	4	10	5	15	7	5	10	12	8	1	10	5	7	0	0	14	12	9	0	11	8
15:05	Irrigation water	0	0	0	8	15	7	0	7	3	0	0	10	6	9	6	10	12	10	0	0	0	8
16:01	Beans	0	3	11	11	16	7	5	7	13	0	9	9	0	7	9	7	15	10	5	0	3	6

16:02	Beans	0	0	0	7	0	4	0	4	13	3	0	0	0	6	0	7	13	0	0	0	0	10
16:03	Beans	0	1	3	10	12	6	0	7	0	8	0	2	4	5	0	6	11	8	7	0	9	8
16:04	Beans	0	2	0	12	15	11	0	7	14	0	0	9	0	8	5	8	15	10	0	0	0	6
16:05	Beans	0	6	0	9	11	8	0	0	3	4	0	0	0	6	0	8	14	2	0	0	2	8
17:01	Irrigation water	0	2	8	0	0	0	2	10	13	8	7	10	5	6	7	8	15	9	8	0	0	9
17:02	Irrigation water	0	2	11	11	15	7	2	6	2	7	6	10	6	7	0	9	14	10	8	0	0	9
17:03	Irrigation water	0	2	10	11	14	6	2	7	15	7	7	9	5	8	9	8	14	1	6	0	7	10
17:04	Irrigation water	0	3	10	11	14	8	4	10	10	8	9	9	6	7	8	8	14	10	0	0	0	9
18:01	Beans	0	3	10	11	17	7	2	8	1	6	8	10	4	8	8	3	13	12	7	0	7	6
18:02	Beans	0	2	8	11	12	6	3	8	10	7	7	9	5	7	0	8	14	12	5	0	8	7
18:03	Beans	0	2	10	11	15	8	3	7	0	8	8	10	5	8	0	9	13	12	6	0	6	5
18:04	Beans	0	2	9	11	16	4	2	7	16	8	8	9	5	9	9	10	16	12	7	0	8	6
20:01	Irrigation water	0	0	9	0	15	7	3	7	13	8	0	9	4	7	7	8	16	10	5	0	4	6
20:02	Irrigation water	0	2	6	11	13	5	4	6	13	8	8	9	4	6	8	7	13	9	9	0	0	0
20:03	Irrigation water	0	0	0	12	8	6	0	8	14	8	7	8	5	7	8	8	15	9	5	0	0	0
20:04	Irrigation water	0	2	7	13	13	5	2	7	15	7	6	10	4	8	8	8	15	9	10	0	0	5
20:05	Irrigation water	0	0	10	1	11	5	2	6	14	8	7	9	4	9	9	8	14	10	1	0	5	8
21:01	Irrigation water	0	2	10	13	14	7	4	5	13	0	7	11	0	6	8	8	16	10	10	0	0	0
21:02	Irrigation water	0	2	12	14	15	7	2	5	13	0	3	8	0	8	0	8	16	13	7	0	10	9
21:03	Irrigation water	0	4	0	13	15	8	2	5	1	0	9	9	0	7	9	9	16	11	8	0	9	9
21:04	Irrigation water	0	2	9	14	13	9	2	7	1	6	7	9	4	8	0	9	3	0	0	0	9	0
21:05	Irrigation water	0	2	10	11	17	7	3	7	14	6	8	9	4	8	8	7	15	8	9	0	10	0
M:1	Human	0	2	10	11	14	7	4	6	13	8	7	10	0	6	7	7	1	10	8	0	0	8
M:2	Human	0	2	10	11	17	6	3	5	10	7	0	8	5	8	10	7	14	0	7	0	0	9
M:3	Human	0	2	9	12	13	8	3	7	15	7	1	9	5	6	10	9	14	9	11	0	6	0
M:4	Human	0	0	9	0	14	8	4	9	15	0	0	10	4	7	9	0	16	10	8	0	0	0
M:5	Human	0	0	0	5	2	5	0	0	14	4	0	0	2	0	0	7	15	1	0	0	0	6
M:6	Human	0	3	0	13	17	7	0	0	15	0	0	9	0	10	7	9	16	9	0	0	0	0
M:7	Human	0	1	0	13	17	8	0	5	13	0	0	11	0	8	6	7	16	9	0	0	0	1
M:8	Human	0	0	0	7	0	3	0	0	12	5	0	0	2	6	0	8	12	0	0	0	0	0
M:9	Human	0	0	0	6	1	4	0	0	12	3	0	0	2	10	0	7	13	0	0	0	0	3
M:10	Human	0	1	0	12	12	8	2	5	16	0	0	8	0	7	0	7	17	11	0	0	0	0
M:11	Human	0	2	10	13	15	5	2	5	15	0	7	8	2	7	10	7	14	11	0	0	0	6
M:12	Human	0	0	8	14	14	7	2	8	3	8	4	9	4	7	9	6	8	11	0	0	0	8
M:13	Human	0	0	0	14	14	8	0	7	15	0	0	9	0	7	0	7	15	12	0	0	0	0

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Western Cape agriculture is a multi-billion rand industry contributing an estimated 21% share to South Africa's total gross farming revenue (WESGRO, 2010). The wellbeing of the local agricultural sector has recently become increasingly vulnerable due to deteriorating environmental conditions which negatively impact the quality of agricultural products. The growing demand for fresh water brought on by increased urbanisation, a rapidly growing population and climate change has resulted in water shortages. These shortages have forced farmers to become more dependent on surface water as primary source for irrigation. These water resources are often severely polluted and can lead to health risks as a result of the transfer of potential pathogens from the polluted irrigation water to the surface of fresh produce.

The aim of this study was firstly to evaluate the microbial quality of two sources of irrigation water, the Plankenburg and Eerste Rivers, by determining the base-line levels of faecal indicators as well as the presence of other potential pathogenic organisms. In the second phase of the study the impact of the polluted rivers on the safety of fresh produce was determined by assessing the microbial quality of fresh produce irrigated with this water and then comparing the microbial load and types to that in the irrigation water and the raw river water. Lastly, the link between the microbial quality of the irrigation water and that of fresh produce was investigated to confirm the transfer of microbes from the polluted irrigation water to produce. This aspect was achieved by implementing API 20E and Multi-Antibiotic Resistance profiling as source linking techniques.

The baseline study of the Plankenburg and Eerste Rivers was continued for 12 months and showed that both rivers continuously had high faecal contamination levels that exceeded DWAF and WHO faecal coliform guidelines of 1 000 MPN per 100 mL water for irrigation of fresh produce intended to be consumed raw (DWAF, 1996; WHO, 1989). The microbial results indicated especially high concentrations of faecal indicator organisms in the Plankenburg, and to a lesser extent, the Eerste River. Faecal coliforms and *E. coli* concentrations exceeded the ≤ 4 000 cfu.100 mL^{-1} guideline (DWAF, 2002) on 67% of the sampling occasions and were detected at concentrations as high as 7 x 10^6 cfu.100 mL^{-1}. Faecal contaminants from these rivers indicated faecal pollution probably from the up-river informal settlement. The presence of these indicators not only indicated unsanitary processes, but also the possible presence of potential pathogenic organisms. Several potential pathogens such as *Klebsiella pneumoniae*, *Listeria monocytogenes, Staphylococcus* and *Salmonella* were isolated from all three sites. Based on these results the microbial quality of these rivers was found to be of unacceptable standard and it could be concluded that a high health risk was associated with water from these two rivers, especially the Plankenburg River, when used as irrigation sources.

The aim of the second phase of the study was to assess the impact of the polluted rivers on the safety of fresh produce and comparing the microbial load and types from the water and the

fresh produce. The overall microbial groups found in the irrigation water from both rivers and those from the selected fresh produce that had been irrigated with the river water were found to be similar. These included faecal coliforms, *E. coli*, and members of the genera *Enterococcus*, *Salmonella*, *Staphylococcus, Shigella, Bacillus, Aerococcus, Enterobacter, Klebsiella* and *Listeria*. The presence of the same genera and even the same species present in the irrigation water and the produce indicate that carry-over had taken place. However, based on the data obtained this can only be taken as "presumptive carry-over" and must be confirmed using more specific methods to prove without a doubt that carry-over does take place.

One important aspect that was observed during the study was that the unique surface characteristics of produce were an element which could impact the microbial quality and attachment and thus impact carry-over to produce. It was clear that the hairy surface of green beans promoted the retention of irrigation water and consequently promoted the build-up of organisms as illustrated by the increase of faecal coliform concentration from an initial count of about 200 to 4.4 x 10^5 cfu.100mL^{-1} after multiple irrigations. Polluted irrigation water resources, the transfer of organisms from irrigation water to fresh produce and the build-up caused by multiple irrigations consequently presents a serious health threat to consumers.

In the third phase of the study the transfer of organisms from the polluted river water to fresh produce (green beans) was done via per-hand irrigation of bean plots with the river water. After the enumeration steps to monitor microbial levels, colonies from both the irrigation water and from the irrigated beans were isolated and unique phenotypic (API) and genotypic (MAR) profiles were generated for each of the 105 isolates. To link the transfer from water to produce the isolate profiles were grouped based on their degree of similarity using numerical clustering systems. In spite of the large degree of character variation between the different isolates, 34% were grouped together in several related sub-clusters based on both the MAR and API characteristics. Based on the groupings it was concluded that these isolates were related and originated from the same pollution source. It was thus concluded that from the data generated in this study that the presence of faecal contaminants, and specifically *E.coli*, on the surface of fresh produce were as a result of carry-over from polluted rivers to the fresh produce via irrigation.

Consequently, the impact of polluted irrigation water and the resultant faecal contamination of produce could be widespread with health impacts and financial implications at both local and international levels. It would then only be a matter of time before locally produced produce is associated with a high health risk placing export agreements in danger of cancellations. The inevitable discontinued support of the local market would contribute to the lower demand for locally produced fruit and vegetables which could ultimately lead to the collapse of the local agricultural sector leaving thousands of people without a source of income. The loss of income due to cancelled export agreements would also result in a lower GDP resulting in devastating implications on South Africa's economy.

RECOMMENDATIONS FOR FURTHER RESEARCH

Due to the serious implications of the data generated in this study, it would be beneficial to continue the microbial monitoring of these water resources for an extended period and to increase the number of sampling sites and sampling frequencies to gain an even better understanding of the pollution crises that exist within these river systems.

The incorporation of more sophisticated molecular techniques like doing PCR confirmations of the identity of the isolates should be included for the verification of indicator organisms and the more positive linking of faecal contaminants in irrigation water to those on the surface of irrigated fresh produce. The number of these faecal isolates should also be increased to provide more substantial linking data.

Some short-comings or limitations in terms of relying solely on traditional microbial methods were identified during this study when organisms were able to pass through the different selection hurdles presented by traditional microbial methods whilst in other cases these microbial methods were unable to accurately identify reference strains. The addition of molecular techniques will therefore also contribute towards presenting more accurate data as it will be less likely to produce false negative or false positive identifications.

In addition to linking, molecular techniques can also be incorporated as source tracking techniques to facilitate the identification of the specific sources of pollution. This information can then be used to develop corrective action plans specific to each source of pollution to eliminate the root of the pollution problem rather than merely treating the symptoms.

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