

**IMPACT OF SELECTED ENVIRONMENTAL FACTORS ON
E.COLI GROWTH IN RIVER WATER AND AN INVESTIGATION
OF CARRY-OVER TO FRESH PRODUCE DURING IRRIGATION**

by

Anneri van Blommestein

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Supervisor : Professor T.J. Britz
Co-Supervisor : Dr G.O. Sigge
Co-Supervisor : Dr C. Lamprecht

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ABSTRACT

The consumption of fresh produce has increased over the past few decades, but it has also resulted in an increase in foodborne outbreaks. Once fresh produce gets contaminated, microbes can survive or even multiply on the produce. There is, however, very little information available on what impact environmental conditions will have on the survival and growth of potential pathogens. Thus the purpose of the first phase of the study was to determine the impact of different environmental factors: carbon levels; temperature; incubation time; and initial microbial load on the growth of *E.coli* and other "indigenous" microbes present in the Plankenburg river water.

Water from the Plankenburg River was incubated at 10° to 35°C and the *E.coli* and aerobic (ACC) loads determined at 0, 6, 12 and 24 h. The impact of different COD:N:P ratios on growth were also evaluated by altering the water values. It was found that in non-sterile river water the *E.coli* levels increased with increase in incubation temperature. Minimal growth took place between 10° and 20°C and the largest growth increases at 35°C. The data showed that *E.coli* die-off was rapid when the nutrient levels were low. It was concluded that the carbon (COD) level is a major growth limiting factor in river water. The impact of adjusted carbon levels on the growth of *E.coli* present was then evaluated. The *E.coli* was found to die-off faster at the higher temperatures when the carbon levels were low (COD = <10 mg.L⁻¹), possibly due to nutrient limitations. At higher carbon levels (COD = >100 mg.L⁻¹), the growth profiles of *E.coli* showed major growth increases with no die-off during the 24 h period, probably because of the availability of sufficient nutrient levels.

Even though standard methods can be used to identify if irrigation water is faecally contaminated it is important to confirm that organisms on fresh produce are from the faecally polluted irrigation water. The purpose of the second study was to determine the effect of daily irrigation on carry-over, the effect of "once-off" irrigation on the survival of *E.coli* on the produce, identifying types of *E.coli* in the irrigation water and those on irrigated fresh produce, and then linking the *E.coli* types.

In the first trial, green beans planted in a tunnel and irrigated on a daily basis (14 days) with Plankenburg water and a "pure wild-type" *E.coli* at a concentration of 10⁶ cfu.mL⁻¹ were evaluated. The results showed that *E.coli* is carried over from irrigation water to the irrigated green beans, especially when the *E.coli* levels in the river water were high.

After the enumeration steps, colonies from both the irrigation water and from the irrigated beans were selected (67 isolates from the green beans and 72 from the irrigation water). Unique phenotypic (API) profiles were generated for each isolate. The first indication of linking was confirmed based on the degree of phenotypic similarity using numerical clustering systems. The results showed that these isolates were related and originated from the same pollution source.

The use of PCR further confirmed (*uidA* positive) that all the phenotypically identified strains were *E.coli*. With multiplex PCR, further linking confirmation was shown when strains

tested positive for the *mdh* gene. The presence of EPEC strains in the irrigation water was also revealed. Thirteen *E.coli* isolates, which showed positive carry-over links between the irrigation water and the green beans, were further analysed using triplex PCR. However, no direct phylogenetic link to the three main phenotypic *E.coli* clusters was found. However the triplex PCR could be of value in identifying the source of faecal pollution.

For further linking confirmation, DNA sequencing was done on selected phenotypically clustered strains. The *oriC*-locus sequencing was unsuccessful in distinguishing between the different *E.coli* strains while the *dnaJ* sequences showed clear differences and similarities between *E.coli* strains and some *E.cloacae* and *K.pneumoniae* strains respectively from the river water and from the beans. The probability of faecal coliforms being carried-over from irrigation water to fresh produce was shown. This showed the importance of a "multi-method" approach to confirm carry-over.

UITTREKSEL

Tydens die afgelope paar dekades het die verbruik van vars groente en vrugte vermeerder, maar dit het ook gepaard gegaan met 'n toename in voedselverwante uitbrekings. Indien vars groente of vrugte gekontamineer word, kan die mikrobies daarop oorleef en ook vermeerder. Daar is min informasie beskikbaar oor die impak van omgewingskondisies op die oorlewing en groei van potensiële patogene. Dus was die doel van die eerste fase van die studie om die impak van verskillende omgewingsfaktore te bepaal: koolstofvlakke; temperatuur; inkubasietyd; en aanvanklike mikrobiële lading op die groei van *E.coli* en ander "inheemse" mikrobies wat teenwoordig is in die Plankenburg Rivier water.

Water van die Plankenburg Rivier was geïnkubeer by 10° tot 35°C en die *E.coli* en aërobe kolonie tellings (AKT) was bepaal by 0, 6, 12 en 24 h. Die impak van verskeie CSB:S:F verhoudings op groei, was ook geëvalueer deur die waarders van die rivierwater te verander. Dit was gevind dat in die nie-steriele rivierwater, die *E.coli* vlakke vermeerder het soos die inkubasië temperatuur vermeerder het. Minimale groei het plaasgevind by 10° en 20°C en die meeste groei by 35°C. Die data het gewys dat die *E.coli* vinnig afgesterwe het as die nutriënt vlakke laag was. Die gevolgtrekking was dat die koolstofvlakke (CSB) die hoof faktor is wat die groei beperk in rivierwater. Die impak van die aangepaste koolstofvlakke op die groei van *E.coli* teenwoordig was ook geëvalueer. Daar was gevind dat die *E.coli* vinniger afsterf by die hoër temperature as die koolstofvlakke laag is (CSB = <10 mg.L⁻¹), omdat die nutriente moontlik beperk is. Tydens die hoë koolstofvlakke (CSB = >100 mg.L⁻¹), het die *E.coli* groeiprofiële baie groei getoon met geen afsterwe tydens die 24 h periode nie, omrede dat daar moontlik genoeg nutriente beskikbaar was.

Al kan standaard metodes gebruik word om fekale kontaminasie in besproeiingswater te identifiseer, is dit belangrik om te kan bevestig of die organismes op vars groente of vrugte van fekale gekontamineerde besproeiingswater is. Die doel van die tweede fase van die studie was om die effek van daaglikse besproeiing op oordrag te bepaal, effek van eenkeer se besproeiing op die oorlewing van *E.coli* op groenboontjies, identifisering van *E.coli* tipes in die besproeiingswater en op die groenboontjies, asook die koppeling van *E.coli* tipes.

In die eerste proef was groenboontjies geplant in 'n tunnel en daaglik besproei (14 dae) met Plankenburg water en 'n "suiwer wilde-tipe" *E.coli* met 'n konsentrasie van 10⁶ cfu.mL⁻¹. Die resultate het getoon dat *E.coli* oorgedra was van die besproeiingswater na die besproeide groenboontjies, veral toe die *E.coli* vlakke in die rivierwater hoog was.

Na al die isoleringsstappe, is kolonies van die besproeiingswater en van die besproeide groenboontjies geselekteer (67 isolate van die groenboontjies en 72 van die besproeiingswater). Unieke fenotipiese (API) profiele was gegenereer vir elke isolaat. Die eerste indikasie van koppeling was bewys deur die graad van soortgelyke fenotipiese profiele deur numeriese groepering. Die resultate het gewys die isolate was verwant en oorspronklik van dieselfde bron van kontaminasie.

Die gebruik van PKR het verder bewys (*uidA* positief) dat al die fenotipiese geïdentifiseerde isolate *E.coli* was. Verdere koppeling was geïdentifiseer met die multiplex PKR deurdat die isolate positief getoets het vir die *mdh* geen. Die teenwoordigheid van die EPEC isolate in die besproeiingswater was ook gevind. Dertien *E.coli* isolate, wat positiewe oordrag verbinding getoon het tussen die besproeiingswater en die groenboontjies, was verder geanaliseer deur triplex PKR. Geen direkte fenotipiese verbinding met die drie hoof fenotipiese *E.coli* groepe was egter gevind nie. Die triplex PKR kan wel van waarde wees om die bron van die fekale besoedeling te identifiseer.

Vir verdere koppeling bevestiging, was DNA volgorde gedoen op isolate van geselekteerde fenotipiese groepe. Die *oriC*-locus volgorde was onsuksesvol om te onderskei tussen die verskillende *E.coli* tipes, terwyl die *dnaJ* volgordes duidelike verskille en ooreenstemmings getoon het tussen die *E.coli* asook party *E.cloacae* en *K.pneumoniae* tipes, onderskeidelik van die rivierwater en boontjies. Die waarskynlikheid van oordrag van fekale kolvorme vanaf besproeiingswater na vars goedere was getoon. Dit wys die belangrikheid van 'n "multi-metode" benadering om oordrag te bewys.

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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

Over the last few decades the consumption of fresh produce has increased, but it has also resulted in an increase of food carried outbreaks (Mandrell, 2009). The major fresh produce commodity leading to the most disease outbreaks is leafy greens (Buchanan, 2006). It was reported that from 1986 - 1995 the consumption of leafy greens increased with 17.2% followed by an increase of 59% in outbreaks. From 1996 - 2005, leafy green consumption increased by 5% followed by an increase of 38.6% in outbreaks (Herman *et al.*, 2008). The microbial content of fresh produce could be of a health risk for the consumers, since contaminated fresh produce can lead to infections as it is minimally processed and mostly consumed raw (Tyler & Triplett, 2008). *Escherichia coli*, especially *E.coli* O157:H7 has been the leading cause of the outbreaks: >73 000 illnesses, >2 000 hospitalisations and >60 deaths annually (Mead *et al.*, 1999). Regardless of the seriousness of the outbreaks, there are also major costs involved. It has been estimated that the cost per illness in the USA ranges between \$1 000 and \$1 500, with the fresh produce related illnesses costing about \$ 39 billion annually (Scharff, 2010).

Once fresh produce gets contaminated, microbes can survive and grow very well on the produce (Mandrell, 2009). There are three routes a pathogen can contaminate fresh produce: the surface; by internalisation through the root system; and through injured tissue (Suslow *et al.*, 2006). Once pathogens are internalised it is nearly impossible to remove with techniques such as washing and sanitizers (Gerald & Perkin, 2003). The ideal growth conditions for survival of potential pathogens are warmer temperatures, high moisture, humidity conditions and nutrient rich environments (Brandl, 2006).

Irrigation water has been found to be a major source of faecal contamination and many outbreaks have been linked to contaminated irrigation water (Söderström *et al.*, 2008; Gelting *et al.*, 2011). Various studies have identified different irrigation water sources to have faecal coliform levels above the WHO recommend guideline of a 1 000 faecal coliforms per 100 mL (WHO, 1989). With faecal coliform levels higher than the recommended guideline, there is an increase in the risk of carry-over to produce (Ackermann, 2010).

In South Africa the annual water usage for irrigation purposes is 59% of the total water requirements (Backeberg, 2005) with river water being the main irrigation source (Tarver, 2008). However, many rivers in South Africa have been reported to have high levels of faecal pollution (Bezuidenhout *et al.*, 2002; Barnes & Taylor, 2004; Paulse *et al.*, 2007; Paulse *et al.*, 2009). There are numerous factors contributing to this problem, including: failing sewage systems; surface runoff water; illegal dumping; poor sanitation of informal settlements; overcrowding; inadequately treated sewage as well as industrial and agricultural effluents being dumped into water sources (Suslow *et al.*, 2006). It was already estimated in 2008 that in South Africa about 5 million people

lacked inadequate and safe water supplies (Haldenwang, 2009). Taking these factors into consideration, it is clear that good quality water sources for irrigation purposes are scarce and there is increasing evidence of irrigation water being a major source of fresh produce contamination (Pachepsky *et al.*, 2011).

Escherichia coli need to survive in the water source before it is carried over to fresh produce. There are numerous environmental factors that may play a role in the survival of *E.coli* in river water, including temperature, organic and inorganic compounds, pH, salinity, UV-radiation and competing microorganisms (Nwachuku & Gerba, 2008; Sela & Fallik, 2009). Low pH values, strong UV-radiation and competing microorganisms can limit microbial growth, whereas moisture, sufficient nutrients and higher more optimal temperatures normally lead to growth increases (Pachepsky *et al.*, 2011).

Microbes require warmer temperatures for growth, with the optimum growth temperature of *E.coli* being from 35° - 37°C (Palumbo *et al.*, 1995). In contrast, cooler temperatures have been shown to be more favourable for the survival of *E.coli* in water. Wang & Doyle (1998) showed *E.coli* O157:H7 survived for >91 days at 8°C and about 84 days at 25°C in water. When temperatures are too low for example, 4°C, *E.coli* cannot grow (Karapinar & Gönül, 1991).

Regardless of the temperature of the environment, microbes cannot grow without sufficient nutrient sources. Organisms require nutrients (carbon, nitrogen and phosphate) in a balanced ratio of at least 106:12:1 for optimal bacterial growth (Goldman *et al.*, 1987). The Goldman ratio is indicative of a nutrient sufficient status to support near optimal growth rates (Hecky *et al.*, 1993). Carbon (organic compound) is the most important energy source necessary for microbial growth, with bacteria being able to utilise carbon from readily available substrates, such as carbohydrates, with an efficiency of up to 90% (Goldman *et al.*, 1987; Sela & Fallik, 2009). Therefore when the carbon source is limited it could result in minimal growth and lower survival rates (Vital *et al.*, 2008).

The overall objective of the study was to determine the impact of selected environmental factors on *E.coli* growth in river water as well as investigating the carry-over of *E.coli* from contaminated irrigation water to fresh produce. Since temperature and carbon/nutrient levels have major impacts on *E.coli* growth, it is important to know what are the impact of different carbon concentrations, temperature and incubation time on *E.coli* growth in river water. The same parameters will also be evaluated using a “pure” culture of *E.coli* inoculated into sterile river water and into MRS broth (Merck) to try and eliminate the impact of other organisms “naturally” present in the river water.

In the second phase of the study, the carry-over of *E.coli* from contaminated river water (Plankenburg River) to green beans cultivated under “controlled conditions” was monitored. The effect of “daily” irrigation compared to “once-off” irrigation, was included. *E.coli* strains isolated from the river water and from the green beans will be identified and then the strains from both

sources (irrigation water and green beans) will be linked based on phenotypical and molecular methods, so as to positively show that carry-over did take place.

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CHAPTER 2

LITERATURE REVIEW

A. WATER SITUATION

South Africa is a water scarce country as 60% is classified as semi-arid to arid (Nomquphu *et al.*, 2007). The average rainfall level is less than 500 mm per year, with 21% of the country receiving less than 200 mm per year (Perret, 2002; Ilemobade *et al.*, 2009). It is estimated that 65% of the country does not receive enough rainfall to undertake successful crop production, therefore irrigation systems are essential for economic crop production (WRC, 1996; FAO, 2005). Water resources are obtained from surface water (77%), groundwater (9%) and from return flows (14%) such as sewage and effluent purification waters (DWAF, 2009; Haldenwang, 2009). It is estimated that only 8.5% of South Africa's annual rainfall turns into runoff and eventually flows into rivers (WRC, 1996).

In 2010, South Africa had an estimated population of 49.99 million, while the total population of the Western Cape was projected to be about 5.22 million (Stats SA., 2010). In 2004, 42% of the population was already living in rural areas (FAO, 2005). About 14 million rural and suburban South Africans do not have access to running water in their homes (Perret, 2002). In 2008 there was still an estimated 5 million people who lacked adequate and safe water supplies, while 15 million still lacked basic sanitation (Haldenwang, 2009). Only 50% of the children have access to clean safe water and therefore as a result hospitalisation, even death occur frequently due to diarrheal complications (Wenhold *et al.*, 2007). DWAF (2005) estimated that approximately 15 diarrhoea-related deaths occur per 1 000 people, especially children under the age of five, due to the lack of good quality water and sanitation services. In 2000, the cost of poor quality water was estimated at R 2.2 billion in direct health costs, R 700 million in indirect health costs and R 64 million for water treatment costs (DWAF, 2005).

It was projected that the demand for water will have an annual growth of 1.5% between 1990 and 2010, ranging from 3.5% in urban and industrial areas and 1% for irrigation (FAO, 2005). It is expected that South Africa will exceed its water source availability in 2025 (Haldenwang, 2009).

B. IRRIGATION WATER

Water is an important resource in food production, which also makes it a critical factor in food security (Wenhold *et al.*, 2007). Irrigation provides a significant proportion of the world's food, including most of its vegetables, fruits and high value crops (Turrall *et al.*, 2009). The production of

food is dependent on water availability due to farming being very water-intensive (Wenhold *et al.*, 2007).

In South Africa, it is estimated that the annual water requirement for irrigation is 59% of the total water requirements (Backeberg, 2005; FAO, 2005), but can vary from 58% to as high as 93% (Backeberg, 2005). Under irrigated agriculture, about 30% of the water used for irrigation is lost during storage and conveyance, while the remaining 70% has further losses due to runoff and drainage (Qadir *et al.*, 2003). As determined in 2002, around 15.7 million ha in South Africa is cultivated, which is based on the sum of arable land and land under permanent crops (FAO, 2005). It has been estimated that the water that is available for irrigation can support 1.59 million ha of land, which is about 10% of the cultivated area (Backeberg, 2005). South Africa has approximately 1.3 million ha of irrigated land for both commercial and subsistence agriculture (WRC, 1996; Perret, 2002). It is estimated that another 200 000 ha of irrigated land will have to be developed in the future (Schreiner & Naidoo, 2000). Approximately 1.5 million ha in 2002 was equipped for full or partial control irrigation (FAO, 2005). This was divided into localised (178 000 ha), surface (500 000 ha) and sprinkler irrigation (820 000 ha) (FAO, 2005). More than a third of the rural households in South Africa engage in agriculture production, which makes it the most important source of income for the rural poor (Machethe, 2004). In 2000, 136 200 ha of vegetables were irrigated and harvested (FAO, 2005).

Irrigation methods can be grouped as restricted and unrestricted irrigation. Restricted irrigation is the irrigation of all crops, except for salad and vegetable crops that may be eaten uncooked, while unrestricted irrigation is the irrigation of salad crops and vegetables eaten uncooked (Mara *et al.*, 2007). According to Qadir *et al.* (2010) there are different factors to consider when choosing a specific irrigation method. These include: flood, furrow, drip and overhead:

- Flood irrigation is the cheapest method of irrigation, however the efficiency is low and cannot succeed if water is limited;
- Furrow irrigation is considered as providing better health protection, but needs favourable topography as well as land levelling;
- Drip irrigation is the most effective in protecting farmers and consumers, because of minimised exposure to humans and crops; and
- Sprinkler irrigation spreads water all over the crop surface. It has medium to high costs and medium water efficiency. Overhead sprinkler allows more control over the amount of water applied and leads to less soil saturation, which in return leads to harvest being completed quicker (Suslow *et al.*, 2006). Irrigation at night time and not irrigating when the wind is blowing are also important factors when sprinkler irrigation is used.

River and surface waters

Surface water such as rivers is the main source used for irrigation (Tarver, 2008). Surface waters also function as the receptors of wastewater (Tarver, 2008). If there is a malfunction of sewage systems, the sewage will contaminate the river and groundwater with potential faecal pathogens (Paul *et al.*, 1997). The availability of water, climate, sewage spills, runoff from animal production, storm-related contamination of surface waters as well as illegal discharge of waste will influence the quality of rivers and groundwater (Suslow *et al.*, 2006).

Runoff water is not usually absorbed by soil and flows to lower ground levels, where it eventually drains into a stream, river or any other water source (DWAF, 2005). This could lead to contamination of the surface water, due to the direct contact between surface runoff water along with pollutants (Tarver, 2008). Examples of runoff water are motor fluids, roadway grease, road salt, lawn pesticides, construction-site sediment and even trash. Crop production processes are also another way for surface water to get contaminated (Tarver, 2008).

Storm water originates from pervious and impervious areas during rainfall periods and can contain pollutants including pathogens (Arnone & Walling, 2007). Litter that is discarded in streets usually ends up in storm water systems, which eventually end up in the rivers (DWAF, 2005).

The use of polluted rivers for irrigation purposes is practiced all over the world (Pettersson *et al.*, 2001). The World Health Organization recommends the guideline of $\leq 1\ 000$ faecal coliforms per 100 mL in irrigation water to be used for crops (WHO, 1989). If this limit is exceeded it could lead to serious health hazards for the users. However, according to DWAF, the guideline for irrigation water should not exceed 4 000 faecal coliforms per 100 mL (DWAF, 2008).

Wastewater

Wastewater (including greywater) can originate from showers, baths, toilets, hand basins, kitchen sinks and washing machines (Eriksson *et al.*, 2002; Tarver, 2008). When it does not contain any sewage (or toilet water) and only household water from domestic activities such as bathing, washing and food preparation it is called "greywater" (Anon., 1999; Murphy, 2006). It can also be considered as a mix of domestic and industrial wastewater as well as storm water (Qadir *et al.*, 2010). Wastewater can also contain helminths (worms), protozoa, viruses and pathogenic bacteria which can be dangerous to human health (Eriksson *et al.*, 2002; Qadir *et al.*, 2010). Greywater usually contains less organic matter and nutrients than normal wastewater, because it does not include faeces, urine and toilet paper (Eriksson *et al.*, 2002). The wastewater that originates from industrial sources usually contains a lot of metals such as metalloids and volatile/semi-volatile compounds (Qadir *et al.*, 2010). Domestic wastewater contains a lot of chemicals such as phosphates and it can also contain sewage (Anon., 1999; DWAF, 2005). Domestic wastewater needs treatment before it is released back into the environment and used for

irrigation, because alongside pathogens it can contain food scraps, hair, human waste, soaps and grease (Tarver, 2008; Qadir *et al.*, 2010).

The use of wastewater has become a major source of irrigation water over the past two decades (Rai & Tripathi, 2007). Farmers in urban and peri-urban areas in almost all developing countries have to use wastewater for irrigation as there is no other alternative (Qadir *et al.*, 2010). The wastewater is normally undiluted, since it is inexpensive and believed to provide more nutrients (Qadir *et al.*, 2010). Kitchen greywater usually has the poorest microbial quality, while laundry greywater is the least suitable for irrigation due its high sodium concentrations and high pH (Qadir *et al.*, 2010).

The reuse of wastewater contributes to the conservation of water resources, recycling nutrients (nitrogen, phosphate, potassium and oligo-elements) that are good for crop growth and disposing of waste products in a low-cost and hygienic manner (Song *et al.*, 2006; Qadir *et al.*, 2010). The use of greywater for urinal and toilet flushing can be of great use, since a lot of countries use water of drinking quality to flush the toilets (Eriksson *et al.*, 2002). Wastewater also poses a microbial risk, therefore wastewater used for irrigation up to 500 m³ should not exceed 100 000 faecal coliforms per 100 mL (Anon., 1999). When irrigated up to 2 000 m³ of domestic and biodegradable industrial waste the faecal coliform count should not exceed a 1 000 per 100 mL (Anon., 2004b). When wastewater is discharged back into water resources like rivers, the faecal coliform count should not exceed a 1 000 per 100 mL (Anon., 1999). Also if irrigating with wastewater it should be more than 100 m from the edge of a water source or borehole which is used for drinking water (Anon., 2004b).

C. MICROBIAL POLLUTION OF SOUTH AFRICAN RIVERS

As stated previously, large parts of South Africa receive a low rainfall with an annual average of less than 500 mm. Thus, South Africa must rely on irrigation to supplement the rainfall in order to provide sufficient water for agricultural crops. Rivers traditionally serve as the main source of irrigation water.

Over the last decade, studies on the quality of the water of many of South Africa's rivers revealed an increase in pollution levels (DWAF, 2000; Bezuidenhout *et al.*, 2002; DWAF, 2002; Griesel & Jagals, 2002; Barnes & Taylor, 2004; Dalvie *et al.*, 2004; DWAF, 2004; Germs *et al.*, 2004; DWAF, 2005). It has also been reported that the microbiological pollution levels have reached unacceptable and dangerous levels (Barnes & Taylor, 2004). There have also been widespread public discussions over the last few years with media headlines such as: "Beware of badly polluted river" and "Groundwater badly polluted with faecal matter" (Barnard, 2008; Davids, 2008; Gosling, 2008).

Streams, rivers and other water sources are also used by humans for bathing, defecation and other domestic activities as well as by animals (Okafo *et al.*, 2003). Rivers also constantly get polluted due to poor sanitation, overcrowding, surface runoff, sewage effluents and failing of sewage systems of these informal settlements (Venter *et al.*, 1997; Barnes & Taylor, 2004; Pause *et al.*, 2009). The water can get directly or indirectly (subsurface water flow) contaminated with pathogens that are originally present in animal and human faeces (Arnone & Walling, 2007). In Africa urbanisation has been increasing which effects the quality of natural water resources, because of poor infrastructure and economic development (Amoah *et al.*, 2005; Carroll *et al.*, 2009). Approximately 35 000 people migrate annually to Cape Town, leading to the expansion of these informal settlements (DWAF, 2005).

The rivers in South Africa with the highest health risk are those where individuals have no access to treated water for domestic use and lack of sanitation infrastructure, especially where it is overpopulated (DWAF, 2000). Sewage discharges play an important role in water pollution, due to the contribution to oxygen demand and nutrient loading of the water sources (Morrison *et al.*, 2004). The storm water runoff, sanitary sewer overflows, wastewater treatment plant effluents, inadequately treated wastewater or collapsed sewers all contribute to the pollution of surface waters (DWAF, 2005; Arnone & Walling, 2007).

Western Cape Province

Stormwater and litter from urbanised areas surrounding the Lotus and Diep, Lourens, Hout Bay, Bokramspruit, Sir Lowry's Pass and Eerste River as well as wastewater discharges and spills from blocked sewage pump stations in the Kuils and Black Rivers have resulted in water quality problems of the Western Cape rivers (DWAF, 2005). The flow of the Kuils River has doubled due to wastewater discharges (DWAF, 2005). DWAF (2005) also reported that Cape Town had several pollution incidents during 2004, including 169 illegal discharges to storm water, 78 888 sewage blockages of which six resulted in serious overflows into the storm water system, as well as 43 pump station and rising sewer incidents due to electrical and mechanical failures. The discharges above contribute to poor quality of river water, especially in the Kuils River since it receives the highest volume of wastewater (DWAF, 2005).

Barnes & Taylor (2004) performed a study on the Plankenburg River from 1998 - 2002 to determine the level of faecal pollution present in the river using the multiple tube fermentation (MTF) method. The results indicated values ranging between 130 faecal coliforms per 100 mL and 130 *E.coli* per 100 mL to maximum values as high as 17.4×10^6 faecal coliforms per 100 mL and 12.99×10^6 *E.coli* per 100 mL respectively, with the majority of the results being above the WHO limit of a 1 000 faecal coliforms per 100 mL. The highest values were obtained during the summer months. The faecal pollution also increased after it had flowed past Kayamandi (the informal settlement of Stellenbosch) as observed when counts increased from 93 333 *E.coli* per

100 mL before Kayamandi to 1 192 422 *E.coli* per 100 mL below Kayamandi. Although the results increased drastically after the river flowed past Kayamandi, it was noted that considerable amounts of faecal pollution entered the river at other points below Kayamandi (Barnes & Taylor, 2004).

Paulse *et al.* (2009) reported that the highest counts for the Plankenburg River's most probable number (MPN), faecal coliforms (FC) and *E.coli* was 9.2×10^6 , 3.5×10^6 and 3.5×10^6 microorganisms per 100 mL, respectively. The study also included the evaluation of the Diep River, which had a MPN count of 5.4×10^6 and 1.6×10^6 for both FC and *E.coli*. The reason for the contamination at the Plankenburg River was ascribed to the informal settlements near the river banks, which had poor sanitary facilities and service delivery. For the Diep River it was due to the accumulation of waste effluents from the nearby residential and industrial areas (Paulse *et al.*, 2009).

It was reported that the Berg River (Paarl section) had MPN values as high as 3.5×10^7 microorganisms per 100 mL of which 1.7×10^7 per 100 mL were identified as *E.coli* (Paulse *et al.*, 2007). The conventional heterotrophic plate-count technique was used to indicate the level of culturable organisms and gave 1.04×10^6 cfu.mL⁻¹. Reasons for the high levels of contamination was ascribed to raw sewage spills and storm water effluent from the informal settlements in the Paarl and Wellington areas (Paulse *et al.*, 2007).

The malfunctioning of wastewater treatment works is also responsible for polluting South African rivers (Barnes & Taylor, 2004). Mkize (2006) evaluated the quality of the Eerste and Veldwachters Rivers, where numerous factors contributed to the river pollution. Factors that play a role included the solid waste site, illegal dumping and illegal agricultural waste dumping. Drainage from the nursery and vineyards in the vicinity as well as human faeces on the river banks was also observed during the study to contribute to the river pollution (Mkize, 2006).

DWAF (2000) ranked the Eerste River in the 20th position of rivers with the highest potential health risk. The low quality of the river water can have an impact on the community, wine industry and tourism. These are important sources of income and economic development for the area which in return would influence the municipality economically as investors and developers will look for alternative areas to set up businesses (Mkize, 2006).

Eastern Cape Province

In 2004 the microbiological quality of the domestic water used by selected rural communities (Gaga, Gqumashe and Gogogo) in the Eastern Cape was assessed (Zamxaka *et al.*, 2004). The Gaga and Gqumashe villages are situated in the semi-rural Nkonkobe area, while the Gogogo village is in a proper rural area. Zamxaka *et al.* (2004) found that the microbial quality of the water sites in Gogogo had the highest microbial risk to individuals compared to the Nkonkobe sites, which was due to surface runoff from poor sanitation facilities of the informal settlements as well

as human and animal activities at the water collection sites. Gogogo sites were higher in total and faecal coliform counts with the average and highest coliform count being 1 000 cfu.100 mL⁻¹ and 100 000 cfu.100 mL⁻¹, respectively. Overall the water in the Gogogo as well as the Nkonkobe sites is not fit for human consumption and needs prior treatment.

The water quality of the Umtata River catchment was also analysed and revealed that there is a health risk to rural communities (Fatoki *et al.*, 2004). Total coliform counts of up to 69 000 cfu.100 mL⁻¹ and faecal coliform counts reaching 21 000 cfu.mL⁻¹ were found. The river is mainly used by individuals for recreational purposes, domestic use and for the watering of livestock. It was concluded that the reason for the high pollution levels are as a result of domestic wastes, from informal settlements that are situated along the riverbank, being “dumped” into the river (Fatoki *et al.*, 2004).

KwaZulu-Natal

The Mhlathuze River in KwaZulu-Natal serves as a drinking, washing, recreation and agricultural water source for all the rural communities in the area (Bezuidenhout *et al.*, 2002). This river was monitored from March 1998 for a period of 21 months. The mean total and faecal coliform counts over the sampling period were generally very high with values of 125.2 x 10² and 36.2 x 10² cfu.100 mL⁻¹, respectively. The higher bacterial counts found in the summer were due to high surface water temperature and the surface runoff that resulted from the heavy rains. Some of the bacteria isolated included *E.coli*, *Pseudomonas* spp. and *Enterobacter* spp. The study indicated that the river can be a vehicle of infectious disease transmission to the rural communities using it.

Limpopo Province

Potgieter *et al.* (2006) determined the microbiological quality of privately owned and communal boreholes in the Limpopo Province of South Africa in 2002. A total of 97 boreholes were evaluated for the presence of total coliforms, faecal coliforms, faecal enterococci and *Clostridium perfringens*. Since the water is used mainly for drinking it is important that the values found to be 0 - 10 counts per 100 mL for total coliforms, while for faecal coliforms, faecal enterococci and *C.perfringens* values should be 0 counts per 100 mL (DWA, 1996). Overall the maximum values reported were 1 000 total coliforms per 100 mL, 900 faecal coliforms per 100 mL, 800 faecal enterococci per 100 mL and 1 000 *C.perfringens* per 100 mL. It was found that the private boreholes resulted in very high values, especially during rainy months because surface runoff gains access to the unclosed boreholes. The communal borehole results indicated the maximum values of 1 000 total coliforms per 100 mL, 250 faecal coliforms per 100 mL, 116 faecal enterococci per 100 mL and 100 *C.perfringens* per 100 mL. Although communal boreholes indicated lower values, they were still over the guideline limits (Potgieter *et al.*, 2006).

Germes *et al.* (2004) evaluated the microbial quality of the Chunies River, which runs through commercial farmland and rural communities, and found that the water quality was very poor. The faecal coliform count, measured by membrane filtration, reached levels as high as 40.5 per 100 mL, which makes the river unacceptable for human consumption and domestic use since the guideline is 0 faecal coliforms per 100 mL (DWAF, 1996).

Untreated surface water used for drinking and domestic purposes in rural areas of the Venda region was evaluated to determine the possible health risk (Obi *et al.*, 2004b). When taking all the sampling points of the Levubu, Vuwani, Mutale, Ngwedi, Tshinane, Makonde, Mutshindudi and Mudaswali rivers into consideration, the minimum and a maximum faecal coliform values were 1.5×10^3 cfu.mL⁻¹ and 6.3×10^4 cfu.mL⁻¹, respectively. The total coliforms had minimum and maximum values of 6.0×10^2 cfu.mL⁻¹ and 3.7×10^4 cfu.mL⁻¹, respectively (Obi *et al.*, 2004b). Both the minimum and maximum values of the faecal coliforms and total coliforms were above the DWAF recommended limit of 0 faecal coliforms per 100 mL and 5 total coliforms per 100 mL (Obi *et al.*, 2004b).

Free State Province

In 2009, the river/stream water at the Mautse and Motouleng heritage sites in eastern Free-State Province were monitored for three months to determine the quality (Vos & Cawood, 2010). Mautse reportedly has a maximum faecal coliform count of 1 203 cfu.100 mL⁻¹ of which 921 cfu. 100 mL⁻¹ were *E.coli*. Although the Motouleng sites were less contaminated, they still had an average faecal coliform value of 100 cfu.100 mL⁻¹ which also consisted mostly of *E.coli*. The desirable value according to DWAF is 0 cfu.100 mL⁻¹ for human consumption, while above 20 cfu.100 mL⁻¹ possess a risk of infection and concentrations above 200 cfu.100 mL⁻¹ possess an infectious risk for young livestock (Vos & Cawood, 2010).

Gauteng Province

Müller *et al.* (2001) tested the presence of *E.coli* O157:H7 in different South African water sources intended directly or indirectly for human consumption. Of the 204 samples obtained from 15 different sites in the Vaal Barrage Reservoir drainage basin in the Gauteng region, eight of the samples showed one or more *E.coli* virulence factors (*stx* 1, *stx* 2 and haemolysin plasmid) that is characteristic to *E.coli* O157:H7, while there was no evidence of *E.coli* O157:H7 in the rest of the samples. No sample gave a full range of virulence factors needed to confirm *E.coli* O157:H7 presence. It indicated that the probability of infections of *E.coli* O157:H7 in these sources are low and that the water containing some of the EHEC virulence factors might hold a health risk if consumed.

Another study, performed by Venter *et al.* (1997), monitored the river in the Rietspruit catchment. The river receives inputs from sewage treatment plants, effluent from manufacturing industries and mines as well as inputs from informal settlements from agricultural activities. The individuals of the informal settlements use the river for drinking and washing purposes, while the river is also used for boating and fishing. It was found that the water in the Rietspruit area cannot be used for domestic purposes directly and is also not really suited for full-contact recreational purposes. The average for the faecal coliform count was $>2\ 000$ per $100\ \text{mL}^{-1}$, which is above the recommended guidelines. The main contribution to the microbial quality of the water included surface runoff of the informal settlements and the inputs of the wastewater treatment plants. The industrial and mining discharges did not affect the microbial quality of the water and even sometimes improved the water quality due the dilution effect. Thus this study indicated that the river in the Rietspruit catchment can pose a health risk if used by individuals for direct consumption, full-contact recreation or even irrigation purposes (Venter *et al.*, 1997).

D. FACTORS IMPACTING THE SAFETY OF FRESH PRODUCE

The survival and growth of pathogens in soil and water and on produce is influenced by many environmental conditions that can enhance or inhibit the survival of microorganisms once they have contaminated the fresh produce (Harris *et al.*, 2006). The major environmental factors that impact their survival are temperature, organic and inorganic compounds, UV radiation, rain water/moisture, pH and competing microorganisms (Nwachuku & Gerba, 2008).

Temperature

There is a general increase in foodborne illness and diseases during summer when temperatures are higher (Harris *et al.*, 2006; Thomas *et al.*, 2006; Franz & Van Bruggen, 2008). Temperature is important because it influences the survival and growth pattern of pathogens in water. For example vibrios are found to be higher in numbers during the warmer months of the year (Anon., 2004a). According to Harris *et al.* (2006) there is also an increase on the consumption of fresh produce during warm summer months.

When the irrigation water temperature is low, the cell metabolism of the pathogen is reduced and therefore its survival is enhanced. If there is a temperature increase, enzyme-catalysed reactions increase, followed by increased growth. The growth rate at temperatures above 20°C will double for every 10°C rise in temperature (Prescott *et al.*, 2005). After a certain point if the temperature is increased, the growth will slow down (Prescott *et al.*, 2005). It is important to note that increases in temperature leads to increases in growth rate, but the growth starts declining rapidly after the optimum growth rate is reached (Sela & Fallik, 2009).

Escherichia coli is known to have a minimum and maximum survival level at 10°C and 45°C, respectively, with an optimum growth rate at 35° - 37°C (Palumbo *et al.*, 1995; Prescott *et al.*, 2005). Some studies have reported that *E.coli* is able to grow at temperatures ranging from 8° - 48°C, while more recent studies reported a maximum growth temperature of 48.5°C (Theron & Cloete, 2002; Rudolph *et al.*, 2010). *E.coli* is able to grow over this wide range of temperatures, because it has the ability to adjust its cytoplasmic membrane's fatty acid composition so it can maintain a degree of fluidity which is necessary for optimum solute transport and membrane-associated functions (Cebrián *et al.*, 2008).

In a study to determine the survival of *E.coli* O157:H7 in municipal water that had been filtered and autoclaved, reservoir water and lake water incubated at temperatures 8°, 15° and 25°C (Wang & Doyle, 1998), it was found that *E.coli* O157:H7 had the best survival potential in the filtered autoclaved municipal water and the lowest in lake water. Regardless of the source of the water, the *E.coli* O157:H7 had the best survival rate at 8°C and the lowest at 25°C. The study also showed that *E.coli* O157:H7 can survive for long periods in water even at low temperatures (Wang & Doyle, 1998).

The survival of *E.coli* in sterile spring water was also investigated for 64 weeks at 4°C (Karapinar & Gönül, 1991). The *E.coli* numbers started to decline after one week and by the end of 13 weeks no *E.coli* were detected. The study also revealed that if *E.coli* is present in a mixed culture, the die-off is higher than when present as a "pure" culture (Karapinar & Gönül, 1991).

A study on the effect of temperature and sand on the survival of *E.coli* in a lake water microcosm revealed that the decline of *E.coli* was the slowest at 4°C and the highest at 14°C, while the presence of sand, which probably provided protection and nutrients, increased the survival time regardless of the temperature (Sampson *et al.*, 2006).

Another study determined the survival of *E.coli* in non-sterile river water and found *E.coli* to survive for 6 days at 37°C, 8 days at 20°C and for 12 days at 4°C, which proves that the lower the temperature the longer *E.coli* will survive (Bogosian *et al.*, 1996). The survival of *E.coli* in farm water was also investigated and revealed that *E.coli* can survive for 14 days at temperatures lower than 15°C, while the organism was still detectable after 31 days in the water at 15°C (McGee *et al.*, 2002).

Post-harvest storage temperature also plays an important role in the survival of *E.coli*. McEvoy *et al.* (2009) investigated the effect of different storage temperatures on field-cored lettuce. Results showed that if the lettuce was stored at 5°C, the *E.coli* was still viable after 8 h, but no significant growth had occurred. Lettuce stored at 30°C showed an increase of 100 cfu.g⁻¹ in *E.coli* levels after 8 h (McEvoy *et al.*, 2009). Others studies done on the effect of abusive storage temperatures on damaged leaves of spinach and lettuce showed that *E.coli* O157:H7 grew on the spinach leaves that were stored at 8° and 12°C for three days and increased by 15 and 120 cfu per leaf, respectively (Khadil & Frank, 2010). However, on the lettuce there was no growth

observed at the two temperatures, but only a slight increase of <10 cfu per leaf after 8 h stored at 15°C. The reason for the difference in supporting the pathogen growth is probably the natural high antioxidant level at the damaged area of the spinach, compared to the low level of antioxidants present at the damaged area of the lettuce (Khadil & Frank, 2010). In a study done by Luo *et al.* (2010), *E.coli* O157:H7 survived, but did not grow on lettuce stored at 5°C and started to decline 79 cfu.g⁻¹ by day 10. Lettuce stored at 12°C supported a 100 cfu.g⁻¹ increase after three days. This study proves that if produce is stored at 5°C or lower it will have better quality and safety, since low temperatures according to Luo *et al.* (2010) lead to the die-off of *E.coli*.

The temperature samples are kept before analyses are also of importance. It is generally recommended that samples should be analysed within 8 h after sampling, while kept at <10°C. However, a study reviewed this recommendation and concluded that if samples are kept at 10°C or lower they can be analysed beyond 8 h and the results will still be comparable to those analysed within 8 h of sample collection (Pope *et al.*, 2003). Samples stored at 4°C showed no difference in cell density after 48 h probably due to temperatures being too low. While *E.coli* increased during the first 8 h at 10°C, but only at a low growth rate (Pope *et al.*, 2003).

Rain water/moisture

Waterborne disease outbreaks are most likely to peak during extreme rainfall and in combination with warmer temperatures (Thomas *et al.*, 2006). Extreme rainfall contributes to the contamination of river water due to faecal matter that enters the river via surface runoff water (Rai & Tripathi, 2007; Nwachuku & Gerba, 2008). The survival of pathogens such as *Salmonella* are also higher during rainy and moist conditions, which can be why disease outbreaks peak during extreme rainfall periods (Zaleski *et al.*, 2005; Rai & Tripathi, 2007). It can also be debated that during rainy seasons the microbial counts in rivers should be lower, due to the volume of water, self-purification, dilution and dispersion of pathogens (Okafo *et al.*, 2003).

When it is raining the microbial count on vegetables can also be lower due to the cleansing of the vegetables by rain water (Okafo *et al.*, 2003). In contrast, a study indicated that microbial counts on vegetables are higher in rainy seasons than in drier seasons, possibly due to the better survival in humid conditions experienced during the rainy seasons (Suslow *et al.*, 2006). Another study also revealed faecal coliforms to be more prominent on lettuce during rainy seasons, due to the splashing on lettuce of already contaminated soil during heavy rains (Amoah *et al.*, 2005).

A moist environment is very favourable for microorganisms to grow, therefore if the surface of the produce gets injured it releases cell fluid which is favourable for microorganisms, because of the nourishing environment it provides (Harris *et al.*, 2006). An additional source of moisture that can favour microbial growth is water that is added to the produce after it has been harvested. For example in Australia, harvested lettuce is kept fresh by adding water to the cut surfaces of the

produce. If potential pathogens are present they can penetrate the cut surfaces and move into the tissue of the produce (Hamilton *et al.*, 2006). Once inside the produce, the pathogens will survive and grow as they are also protected from sunlight (Hamilton *et al.*, 2006). It was therefore concluded by Hamilton *et al.* (2006) that vegetables with less moisture on their surfaces will have a lower microbial risk.

UV radiation

Bacteria are more susceptible to UV-rays than protozoa and viruses (Johnson *et al.*, 1997). Contaminated lettuce has led to many disease infections and outbreaks in the past (Pettersen *et al.*, 2001). A further reason for the increases is the protection the leaves of the lettuce gives from light and desiccation (Pettersen *et al.*, 2001). UV radiation from the sun can destroy microorganisms due to the short wavelength (100 - 400 nm) and high energy (Prescott *et al.*, 2005).

The exposure to sunlight is believed to reduce the growth of contaminating organisms on surfaces. A wavelength of 260 nm is the most destructive, because the waves are absorbed by the DNA of the microorganism. Absorption leads to the formation of thymine dimers in the DNA which causes the inhibition of DNA replication and function (Prescott *et al.*, 2005). UV radiation wavelengths below 300 nm reach the earth's surface, but near-UV radiation between 325 - 400 nm can damage microorganisms. The near-UV radiation breaks down tryptophan to toxic photoproducts which along with the near-UV rays break the DNA strands of the microorganism (Prescott *et al.*, 2005). For example in a sunlight experiment, a study was done by Minhas *et al.* (2006) to monitor the faecal coliform counts on vegetables after being irrigated with sewage water (1.5×10^8 MPN.100 mL⁻¹). The data indicated that after 4 h of sunlight exposure the faecal coliform counts were reduced to levels ranging between <2 and 9×10^5 MPN.100mL⁻¹, while during the evening minimum reduction was observed. This showed that UV rays were responsible for the decrease of coliforms.

pH

pH can be defined as a measurement of the activity of hydrogen ions in a solution (Buck *et al.*, 2002). The pH of natural surface waters can be neutral (pH = 7) due to the contribution of base salts such as ammonia and nitrates (Mkize, 2006). In contrast the pH of soil is slightly higher when applying organic culturing techniques, compared to conventional systems (Mäder *et al.*, 2002). The optimum growth pH for most bacteria is near neutral (pH 6.5 - 7.5), others are acid tolerant and able to survive at pH levels of 4.5 (Sela & Fallik, 2009).

The majority of the vegetables have a pH higher than 4.6, which is suitable for the growth of most pathogenic bacteria (Buck *et al.*, 2003). Laboratory studies revealed that *Salmonella* can

grow on damaged tomatoes at pH 4.1 - 4.5 stored at temperatures between 20° - 30°C (Harris *et al.*, 2006). In contrast when fully ripe tomatoes are undamaged, the pH ranges between 3.9 - 4.5, which is unfavourable for the growth of pathogens such as *E.coli* O157:H7 and *Shigella* (Buck *et al.*, 2003).

Escherichia coli has been shown to develop acid-resistance especially when they are cultivated under acidic pH conditions. It is also known that once they are introduced to a low pH such as found in gastric juices (pH 1 - 2) they will be less sensitive to the low pH (Benjamin & Datta, 1995; Diez-Gonzalez *et al.*, 1998; Harris *et al.*, 2006). *E.coli* O157:H7 strains can also adapt to low pH conditions (pH 3) when induced by organic acids such as citric, malic, lactic and acetic acid. It was observed that *E.coli* O157:H7 acid resistance was dependent on the specific acid as well as the strain of the *E.coli* isolate (Buchanan & Edelson, 1999). Conner & Kotrola (1995) also determined the survival of *E.coli* O157:H7 in acidic conditions. If the pH of the environment drops below 6, *Salmonella typhimurium* and *E.coli* synthesise an array of new proteins as part of their acidic tolerance response (Prescott *et al.*, 2005). The acidic tolerance response is based on the principle of pumping protons out of the cell or by making more ATP (Prescott *et al.*, 2005). If the pH decreases to 4.5 or lower it leads to acid shock proteins being synthesised, making the pathogens resistant to low pH conditions (Prescott *et al.*, 2005).

Organic and Inorganic compounds

Microorganisms need specific nutrients in higher concentration for optimum metabolism. If only a limiting concentration is available it will lead to the inhibition of bacterial growth (Sela & Fallik, 2009). The substrates that are necessary for balanced bacterial growth need to contain carbon, nitrogen and phosphate in the Redfield ratio of 106:12:1 (Goldman *et al.*, 1987) The ratio is indicative of a nutrient sufficient status for optimal growth rates (Hecky *et al.*, 1993). Vidovic *et al.* (2007) found the highest die-off rate for *E.coli* O157:H7 was in low carbon soil (1% organic carbon and 0.1% total nitrogen) compared to high carbon soil (2.1% organic carbon and 0.198% total nitrogen), proving that nutrient limitation can affect the survival of a pathogen.

Carbon (organic compound) is an essential energy source for microbial growth, with bacteria being able to utilise the carbon from readily available substrates, such as glucose, with an efficiency of up to 90% (Goldman *et al.*, 1987; Sela & Fallik, 2009). Vital (2008) reported that less carbon is consumed by *E.coli* at lower temperatures and that there is a positive growth correlation between carbon utilisation and growth temperature. This study investigated the ability of *E.coli* O157 to grow and survive at low carbon concentrations in fresh water. Results showed that *E.coli* can grow at very low assimilable carbon levels of 184 - 534 $\mu\text{g}\cdot\text{L}^{-1}$. From the carbon available *E.coli* was able to utilise between 20 - 66%. Although it was able to utilise the majority of the carbon, the natural bacteria present in the water still had a growth rate 2 - 4 times higher than that of *E.coli* at 30°C and resulted in the final *E.coli* cell concentration being five times lower than that

of the natural bacteria. The reason given was that the natural bacteria were able to grow at even a lower assimilable organic carbon level of $10 \mu\text{g.L}^{-1}$ (Vital *et al.*, 2008).

Nitrogen is required by microorganisms for amino acid and protein synthesis. Some organisms use inorganic nitrogen as the main source for all their nitrogen requirements (Sela & Fallik, 2009). Nitrogen-fixing bacteria have the ability to convert atmospheric nitrogen into ammonia available for cellular metabolism, while other bacteria require inorganic nitrogen in the form of ammonium or nitrate (Sela & Fallik, 2009). Particular microbes may even require organic nitrogen such as preformed amino acids and peptides (Sela & Fallik, 2009). Nitrogen is really important for the survival of *E.coli* in unfiltered lake water, as proved by Lim & Flint (1989). In their study *E.coli* did not survive without an external nitrogen source in the presence of competitors. The addition of ammonium sulphate and amino acids increased the survival times of *E.coli* proportionally to the concentration of the nitrogen source added.

Phosphate is also a necessary element in metabolism and for stable cell structure, but was found to have little effect on the survival of *E.coli* in unfiltered and filtered-autoclaved lake water (Lim & Flint, 1989). Another study showed that if there is an increase in phosphate there will be an increase in bacterial growth in drinking water (Miettinen *et al.*, 1997). Miettinen *et al.* (1997) reported that $1 \mu\text{g.L}^{-1}$ phosphate increased the microbial growth in drinking water, while the addition of magnesium, potassium and calcium had no effect on the microbial growth. The addition of nitrogen also had negligible effects. This study proved that phosphate plays a role in the growth of microorganisms.

Fresh produce contains sufficient carbohydrates to support the growth of different microorganisms (Sela & Fallik, 2009). Pathogens such as *E.coli* O157:H7 are classified as copiotrophic, which means that they are usually present in environments with high nutrient levels (Koch, 2001; Franz & Van Bruggen, 2008). When enteric pathogens are found to be higher in younger new produce leaves, it was ascribed to the younger leaves being richer in total nitrogen and carbon (Brandl & Amundson, 2008). In their study ammonium-nitrogen was added to the older leaves, which increased *E.coli* O157:H7 population, while the addition of glucose had no effect on *E.coli* O157:H7 growth, suggesting that low levels of nitrogen can limit the growth of pathogens (Brandl & Amundson, 2008).

Competing microorganisms

In river water or on fresh produce there will always be natural bacteria present that compete for food and space. A competitive advantage is gained by an organism if it can grow faster, therefore establishing both prevalence and dominance when nutrient levels are high or can still grow when nutrients are limited (Sela & Fallik, 2009). A few studies have been done to show how pathogens interact with these organisms. Some organisms can even produce metabolic by-products or secondary metabolites to inhibit and outgrow their competitors (Sela & Fallik, 2009).

In a study where lettuce was planted in soil containing bovine manure that had been inoculated with *E.coli* O157:H7, no *E.coli* was detected on the outer leaves, edible parts or the roots of the lettuce. It was concluded that no carry-over took place due to the presence of *Pseudomonas* spp. in the soil. These organisms are very well adapted to soil environment and were present in abundance, which out competed the *E.coli* which was present in low concentrations. (Johannessen *et al.*, 2005).

Bogosian *et al.* (1996) evaluated the survival of an inoculated *E.coli* strain in sterile and non-sterile river water. In the sterile river water, *E.coli* lasted for longer than 50 days at 37°C. In the non-sterile river water the inoculated samples were kept at temperatures 4°, 20° and 37°C and the *E.coli* survived for 12, 8 and 6 days, respectively. This indicated that *E.coli* persisted for much longer in the sterile river water where no competing microorganisms were present and thus suggests that *E.coli* could not compete very well in the presence of natural bacteria. Another possible reason for the short persistence of *E.coli* in the non-sterile river water could be that the bacteria have been consumed by the indigenous inhabitants present in the samples (Bogosian *et al.*, 1996).

Vidovic *et al.* (2007) also investigated the effect of indigenous microorganisms in soil as well as temperature, soil composition and environmental conditions on the survival of *E.coli* O157:H7. The results indicated that the highest deaths rate in sterile soil occurred in the low-carbon soil at 4°C, which was probably due to the low temperature and nutrient limitation. Whereas in non-sterile soil the highest death rate was observed in the low-carbon soil at 22°C, probably due to competing microorganisms and/or starvation (Vidovic *et al.*, 2007).

Vital *et al.* (2008) also reported there is strong competition between *E.coli* and the bacteria naturally present in fresh water with low carbon sources. The natural bacteria have faster growth rates, resulting in the cell concentrations being almost five times higher than that of *E.coli*.

In a study done on field-cored lettuce, it was found that *E.coli* numbers increased on the lettuce at 30°C and, although the natural bacteria were 10 cfu.g⁻¹ higher than the *E.coli* population, the *E.coli* O157:H7 still grew well in the presence of the other organisms (McEvoy *et al.*, 2009). This is an indication that even though natural bacteria are present in higher numbers, it will not inhibit the growth of *E.coli*.

E. FRESH PRODUCE

If there are numerous factors that play a role in the growth and survival of *E.coli* on fresh produce. If the fresh-produce is undamaged on the outer surface, the organisms can still survive, but they will be unable to grow (Harris *et al.*, 2006). This is probably due to the presence of natural protective barriers, like cell walls and wax layers the plant uses against pathogens (Harris *et al.*,

2006). Undamaged produce surfaces lead to lower moisture and nutrient levels, which are released once the tissue is damaged (Harris *et al.*, 2006). If there is physical damage like punctures and bruising or by direct degradation by plant pathogens, the protective epidermal barrier is broken and could lead to the growth of pathogens especially when the temperature is favourable (Harris *et al.*, 2006). Under these conditions the pathogens are protected from UV-rays, have access to nutrients and moisture. Many pathogens have the ability to form biofilms which can protect them from the environment (Harris *et al.*, 2006; Harapas *et al.*, 2010). Harapas *et al.* (2010) evaluated the persistence of *E.coli* on injured vegetables like lettuce, celery and chives. They reported that all the injured vegetables proved to have a higher persistence of *E.coli* when compared to uninjured vegetables. The mean counts of 2.5×10^5 cfu.g⁻¹ on uninjured lettuce decreased to undetectable levels after one week, while injured lettuce with initial counts of 1.3×10^5 cfu.g⁻¹ still had counts of 6.3×10^2 cfu.g⁻¹ after one week. This illustrates that the persistence of *E.coli* is higher in injured than uninjured tissue.

Bacteria tend to accumulate in certain areas of plants on structures such as the stomata (Buchanan, 2006). It is also important to keep in mind that although some vegetables are eaten raw, some are peeled before being consumed. In a study done by Jackson *et al.* (2009), onions and carrots were peeled after irrigation, and found the risk of contamination being reduced on the exterior surface. Vegetables with a rough external skin texture will favour microbial growth due to the ideal niche it provides (Jackson *et al.*, 2009). The type of produce influences the persistence of *E.coli* O157:H7 on the produce itself (Islam *et al.*, 2004b). In a study where carrots and green onions were planted in soil fertilised with contaminated manure, *E.coli* O157:H7 showed a higher survival rate during the growth of the carrots. *E.coli* only decreased by 200 cfu.mL⁻¹ in soil and only by 50 cfu.g⁻¹ on the carrots in 84 days. Similarly *E.coli* decreased by 1 000 cfu.g⁻¹ in soil and by more than 100 cfu.g⁻¹ on the green onions within 64 days (Islam *et al.*, 2004b).

Pu (2009) demonstrated that the internalisation of *E.coli* O157:H7 in spinach leaves is not that common in contrast to the surface frequent contamination. It was found that spinach leaves of four to five weeks had the highest contamination, but after five weeks the contamination decreased. In addition there was no contamination when spinach leaves were younger than three weeks which suggests a plant defence mechanism was active during the plant development phase (Pu, 2009). This is in contrast to the work of Brandl & Amundson (2008) who demonstrated that the population of *E.coli* O157:H7 is about 10-fold higher in the younger inner leaves than the older middle leaves of lettuce. The growth rate was also higher in the younger leaves if compared to the older middle leaves. After elemental analysis of exudates on the surface of leaves of different ages it was found that they were 2.9 and 1.5 times richer in total nitrogen and carbon, respectively, when compared to the middle leaves, proving that leaf age has an effect on pathogen colonisation.

A study was done by Abong'o *et al.* (2008) in the Eastern Cape on vegetables purchased at shops, supermarkets as well as open air markets to determine whether *E.coli* O157:H7 are

present. The vegetables included carrots, onions, spinach, cucumber and cabbage and all had counts of presumptive *E.coli* O157:H7 ranging between 1.3×10^3 - 1.6×10^6 cfu.g⁻¹. Four (10.3%) out of 39 samples tested positive for *E.coli* O157:H7, which increases the risk that it would be pathogenic to humans if consumed (Abong'o *et al.*, 2008).

Commercially available vegetables (carrots, cabbage, wild parsley, tomatoes, cucumber etc.) from Malaysia were examined for the persistence of *Listeria* spp. and *Listeria monocytogenes* (Ponniah *et al.*, 2009). Out of the 306 samples, 102 were contaminated with *Listeria* spp. and 69 with *L.monocytogenes*. The counts determined by MPN-PCR indicated that most of the samples had a microbial load of between 3 and 1 100 MPN.g⁻¹. Of these samples 96.4% had microbial loads less than 100 MPN.g⁻¹. *L.monocytogenes* was also mostly detected in Japanese parsley and beans.

In another study *E.coli* was isolated from 8.2% (55 of 673 samples) of fresh produce samples obtained from farmer's markets in Alberta, Canada (Bohaychuk *et al.*, 2009). The vegetables included lettuce, spinach, carrots and green onions. The counts varied between 3 and 1 096 MPN.g⁻¹. No *E.coli* was detected on tomatoes and strawberries, while no *E.coli* O157:H7 strains, *Salmonella* or *Campylobacter* were isolated from the samples (Bohaychuk *et al.*, 2009).

In Turkey, the bacterial quality of raw salad vegetables (lettuce, iceberg lettuce, "cos" lettuce, parsley, dill and carrots) was examined (Aycicek *et al.*, 2006). The outer leaves of the lettuce, iceberg lettuce and "cos" lettuce had the highest coliform counts ranging between 10^3 - 7.9×10^6 cfu.g⁻¹. Coliforms were also detected in the inner leaves, but at lower levels compared to the outer leaves. *E.coli* counts reached maximum values up to 6.3×10^3 cfu.g⁻¹ on the lettuce outer leaves, while *E.coli* was mostly detected on the parsley (21/30) and dill samples (12/30) (Aycicek *et al.*, 2006).

F. MICROBIAL INDICATORS

Pathogens

Pathogens are organisms that can lead to food infections or poisoning (Arnone & Walling, 2007). There are more than 1 400 potential food-contaminating species of which 58% are zoonotic; pathogens that infect other hosts (Woolhouse & Gowtage-Sequeria, 2005). About 177 species are regarded as emerging or re-emerging pathogens (Woolhouse & Gowtage-Sequeria, 2005). The WHO estimated that around 13 million people die each year from waterborne infections (Theron & Cloete, 2002; Arnone & Walling, 2007), while the CDC estimated foodborne infections result in 76 million illnesses, 325 000 hospitalisations and 5 000 deaths in the United States annually (Mead *et al.*, 1999). Known pathogens cause an estimated 14 million illnesses, 60 000 hospitalisations and 1 800 deaths annually in the United States (Mead *et al.*, 1999).

Pathogens that are most likely to be associated with fresh produce are *Salmonella*, *Shigella*, *Escherichia coli* and *Klebsiella*. *Klebsiella pneumoniae* occurs naturally in the environment and is not associated with faecal contamination (Cahoon & Song, 2009). Organisms such as *Staphylococcus*, *Clostridium perfringens*, *Clostridium botulism* and *Bacillus cereus* are most likely to occur when food is contaminated by the environment (Rai & Tripathi, 2007). *Cryptosporidium*, *E.coli* O157:H7, *Giardia lamblia*, *Listeria* and *Salmonella* are all harmless in livestock, but can lead to serious health risks if they are present in water sources and on fresh produce (Tarver, 2008). Pathogens can be shed by humans and animals in the absence of any sign of illness (Harris *et al.*, 2006).

E.coli is naturally present in the gastrointestinal tract of humans and animals (Warriner & Namvar, 2010). The majority of *E.coli* are non-pathogenic and are essential for human health, with benefits that include probiotic functions and vitamin K production for cell repair (Warriner, 2011). The pathogenic *E.coli*, also known as diarrhoea *E.coli* due to the potential to cause diarrhoea in humans at low infectious doses, has been divided into six groups based on the mode they cause illness, which include virulence properties, mechanisms of pathogenicity and clinical syndromes (Pu, 2009; Warriner, 2011). The six classes of *E.coli* are recognised as: enterohaemorrhagic (EHEC); enterotoxigenic (ETEC); enteroinvasive (EIEC); enteroaggregative (EAEC), enteropathogenic (EPEC); diffusely adherent (DAEC); and enteroaggregative (EA_gEC) (Buchanan & Doyle, 1997).

Enterohaemorrhagic (EHEC) also known as *E.coli* O157:H7, designated as somatic (O) and flagellar (H) antigens, is one of the most frequently occurring pathogenic *E.coli* that is responsible for causing illness (Buchanan & Doyle, 1997; Nwachuku & Gerba, 2008). *E.coli* O157:H7 can survive in the gastric acid of the stomach, after which it will eventually bind to a receptor on the epithelial cells of the gastro-intestinal tract of the colon (Warriner, 2011). *E.coli* O157:H7 harbours a plasmid that gets encoded by the *stx* gene to produce Shiga toxin 1 (*stx* 1) or Shiga toxin 2 (*stx* 2), which is known to cause Haemorrhagic colitis (HC) and can lead to haemolytic uremic syndrome (HUS) (Buchanan & Doyle, 1997; Thomas *et al.*, 2006; Nwachuku & Gerba, 2008; Cahoon & Song, 2009). HUS causes blood in the urine as well as diarrhoea, which eventually leads to kidney failure and could even result in death (Warriner, 2011).

Warriner (2011) explained the ability of STEC (*E.coli* O157:H7) to cause such dangerous illness, was due to its virulence factors, *stx* 2, attachment protein (*eae*) and haemolysin. However, the past five years showed that HUS can also be caused by non-O157 STEC even though these strains do not contain *stx* 2 or the attachment protein (*eae*). These strains can produce *stx* 1, although it has lower pathogenicity towards humans and will only result in viral gastroenteritis for about 24 h. Hyper production of Shiga toxin and the production of a toxin that shuts down the protein factory of the host, called subtilase, is the reason why non-O157 can cause HUS (Hughes *et al.*, 2006; Warriner, 2011). Non-O157 STEC serotypes that have caused clinical infections

include O26, O111, O103, O121, O45 and O145. However the chances of HUS developing from non-O157 STEC infections is less than 2%, compared to 8% associated with *E.coli* O157 (Warriner, 2011).

E.coli can also be divided into four groups according to phylogenetic analysis, namely A, B1, B2 and D (Clermont *et al.*, 2000). Pathogenic *E.coli* causing extra-intestinal diseases (urinary tract infections, meningitis and septicemia) belongs mainly to group B2 and to a lesser extent group D, while commensal (non-pathogenic) *E.coli* to group A or B1 (Clermont *et al.*, 2000).

Indicator organisms

It is difficult to monitor each and every pathogen in water or food as the presence of certain pathogens can be rare, difficult to culture, have a patchy distribution and are highly infectious even at low doses (Field & Samadpour, 2007). In many cases large assays are needed to monitor all organisms present, which can be very expensive and technically complicated (Field & Samadpour, 2007). Therefore, indicator organisms are used to evaluate the quality and safety of raw or processed food products and water as well as validating the effectiveness of microbial control measures (Busta *et al.*, 2006).

An indicator organism is used as a water quality parameter (Leclerc *et al.*, 2001). The quantity of indicator organisms indicates the sanitary quality of the water (Okafo *et al.*, 2003). The presence of these indicator organisms in food will also give an indication of inadequate processing (Jay, 1997). An indicator organism is therefore considered to be a non-pathogenic, low risk microorganism or a group of organisms that are indicative that food has been exposed to conditions that pose an increased risk that the food may be contaminated with a pathogen or held under conditions conducive for pathogen growth (Busta *et al.*, 2006; Cahoon & Song, 2009). If the indicator organism is from faecal origin and if they are present in the water it is taken as an indication of faecal pollution and possibly enteric pathogens (Savichtcheva & Okabe, 2006).

When the indicator organism is absent or present in low concentration it means that the food has not been exposed to conditions that could lead to contamination by a specific target pathogen or present the opportunity for its growth (Busta *et al.*, 2006). The indicator bacteria should correlate with the presence of pathogens in other words it should have a survival profile similar to the pathogens whose presence it indicates (Field & Samadpour, 2007). The indicator organism does not indicate the presence or concentration level of any specific pathogen, but is mainly used to indicate the potential of faecal contamination (Pachepsky *et al.*, 2011).

The use of indicator organisms do not identify the source of contamination, that is why faecal source tracking is used (Field & Samadpour, 2007). However, an indicator organism or group would give an indication of the type of pathogens that might be present and at what expected concentration they might be present.

When choosing an indicator organism, one needs to identify the role of the specific indicator in the control of microbial hazards in fresh produce (Busta *et al.*, 2006). Factors that are influential are the specific produce item, harvesting area and other environmental growth conditions (Busta *et al.*, 2006). Requirements of an indicator bacteria, as suggested by Busta *et al.* (2006), are that an appropriate indicator will continue to be present in foods at any time that the target pathogen or toxin might be present. Concentrations before and after handling should directly relate to those of the target. The indicator must be absent from food when the target is not present, or absent from the process that is supposed to eliminate the target. The growth or increase of the indicator organism must be equivalent or slightly greater, but not less than the target under processing and storage conditions of food as well as in analytical situations (Busta *et al.*, 2006). It should also be resistant to cell injury and not decrease in concentration from stress conditions during handling, processing and storing, unless it will be equivalent to the effect that will happen to the target (Busta *et al.*, 2006). The indicator should also be easy to enumerate, quick and inexpensive to measure and should be non-pathogenic or non-hazardous to testing personnel (Scott *et al.*, 2002; Busta *et al.*, 2006).

Naturally occurring indicators can also be used to determine apparent product safety or treatment effectiveness (Busta *et al.*, 2006). The most common indicator organisms used in water quality and health risk assessments are total and faecal coliforms, *E.coli*, faecal enterococcus and *Clostridium perfringens* (Meays *et al.*, 2004; Field & Samadpour, 2007; Cahoon & Song, 2009). These organisms are used as indicators, because it is simple and less costly to enumerate than the pathogens themselves (Meays *et al.*, 2004).

Total coliforms are mostly harmless bacteria that live in soil, water and the gut of animals (Arnone & Walling, 2007). Total coliforms are an indication of the general sanitary quality of water, since it includes bacteria of faecal origin (Barnes & Taylor, 2004). Faecal coliforms (*E.coli*) are a subgroup of the coliform group and are mostly used as a faecal indicator (Arnone & Walling, 2007).

Faecal indicator bacteria will give a clear indication whether harmful pathogens might be present. The coliform and enterococcus groups are most suitable as indicator organisms since they can monitor the state without forming spores, which means if they are present it is indicative of recent contamination (Cahoon & Song, 2009).

There is not one standard detection method available for pathogenic *E.coli* in water, but due to the behaviour of non-pathogenic *E.coli* being very similar to pathogenic *E.coli*, it ensures a margin of safety (Nwachuku & Gerba, 2008). When *E.coli* levels are determined to monitor the quality of the water it is done with the MUG test, where 4-methyl-umbelliferone glucuronide (MUG) is hydrolysed by β -glucuronidase, the enzyme possessed by *E.coli*, to yield a fluorogenic product (Nwachuku & Gerba, 2008). *E.coli* is also a good indicator of enteric pathogens, because it will be present at concentrations a lot higher than the pathogens it predicts (Scott *et al.*, 2002). The

presence of *E.coli* will give an indication that virulent pathogens such as *Salmonella*, *E.coli* O157 and *Shigella* might be present (Warriner & Namvar, 2010).

Enterococci has been successfully used as an indicator of faecal pollution in water (Scott *et al.*, 2002). *Enterococcus* spp. includes five species: *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus gallinarum* and *Enterococcus avium* (Scott *et al.*, 2002). *Enterococcus faecalis* and *Enterococcus faecium* are most frequently found in humans (Scott *et al.*, 2002)

Clostridium perfringens is an enteric, Gram-positive, anaerobic, spore forming, pathogenic bacterium found in human and animal faeces (Scott *et al.*, 2002). *Clostridium perfringens* is an indication of older faecal contamination as the spores can survive for a long period (Cahoon & Song, 2009).

Microbial indicators have remained the primary way to monitor faecal contamination in water, because they are inexpensive, simple and easy to use and have the ability to indicate faecal contamination by humans (Arnone & Walling, 2007). Although faecal coliforms have been the indicator of choice, they do not always correlate well with the incidence of disease. There are many bacteria that fit the description of faecal coliforms, but do not come from the gastrointestinal tract of humans and animals, therefore raising the question whether these bacteria are good faecal indicators (Arnone & Walling, 2007).

G. OUTBREAKS ASSOCIATED WITH FRESH PRODUCE

Poor sanitation and contaminated water leads to water and food diarrheal diseases which kills around 1.6 million children each year, making diarrheal disease the leading cause of death for children under 15 years (Cohen, 2008). Contaminated water used for irrigation is another reason why disease outbreaks linked to fresh produce have increased during the past few years and are continuing to increase (Harris *et al.*, 2006). This is also because of better reporting systems, changing life styles, demographics, travelling, decreasing water supplies to certain countries and importation of foods can influence the growing number of disease outbreaks (Barnes & Taylor, 2004). Since there is a greater concern nowadays to live a healthy life style, the consumption of fresh fruit and vegetables are on the increase. This leads to the demand for fresh produce to be available all year round (Buck *et al.*, 2003; Franz & Van Bruggen, 2008). A number of pathogens have been isolated from a wide variety of fresh fruits and vegetables, sometimes at high levels (Harris *et al.*, 2006).

The faecal coliform count of raw vegetables and fruit has to be less than $200.g^{-1}$ of which *E.coli* should be $0.g^{-1}$ (DoH, 2010). If the count is more than the stipulated limit, it can lead to disease infections. A lot of people and farmers are not aware of the risk associated with contaminated vegetables (Rai & Tripathi, 2007).

In the USA the estimated cost-of-illness ranges between \$1 000 and \$1 500, with the fresh produce related illnesses costing about \$ 39 billion annually (Scharff, 2010). A total of 190 outbreaks, 16 058 illnesses, 598 hospitalisations and 8 deaths, regarding fresh produce were reported between 1973 - 1997 (Sivapalasingam *et al.*, 2004). Fresh produce also caused the second highest number of foodborne disease outbreaks (22%) and the highest number of disease cases among the five main food categories between 1999 - 2004 (Franz & Van Bruggen, 2008). Fresh produce commodities that are the cause of the majority of outbreaks are leafy greens (lettuce and spinach), tomatoes, cantaloupes, herbs (basil and parsley) and onions (Buchanan, 2006; Mandrell, 2009).

Between 1986 - 1995 leafy green consumption increased with 17.2% compared to the previous decade, along with 59% increase in outbreaks (Herman *et al.*, 2008). The leafy green consumption increased 9% between 1996 - 2005 while the leafy green associated outbreaks increased with 38.6% (Herman *et al.*, 2008). During 1973 - 2006 it was determined that the USA leafy green consumption resulted in 502 outbreaks, 18 242 illnesses and 15 deaths (Herman *et al.*, 2008). The CDC reported that in 2006, that of a single commodity there was around 1081 reported cases and 17% outbreaks due to leafy greens (CDC, 2009). *Salmonella* was responsible for the majority of the outbreaks during the same period (35 outbreaks) (Herman *et al.*, 2008).

Over the years *E.coli* and *Salmonella* were responsible for the majority of fresh produce outbreaks (Mandrell, 2009). *E.coli* O157:H7 and *Salmonella* are dangerous and causes the majority of the outbreaks, because they have the ability to grow prior to consumption and have low infectious doses (Buck *et al.*, 2003).

E.coli

It is estimated that *E.coli* O157:H7 causes 73 480 illnesses, 2 168 hospitalisations and 61 deaths in the USA annually (Mead *et al.*, 1999). In the USA, leafy green vegetables contaminated with *E.coli* O157:H7 have been responsible for 20 foodborne outbreaks, 600 illnesses and 5 deaths since 1995 (Mandrell, 2009) (Table 2). It was also determined that between 1998 - 2006 leafy greens counted for 30% of the total produce outbreaks (Buchanan, 2006). Between 1973 - 2006 *E.coli* O157:H7 was responsible for 30 outbreaks (Herman *et al.*, 2008).

Escherichia coli has a low infective dose (10 - 1 000 cells), which is why even a few of these pathogens pose a risk to consumers when contaminated vegetables are consumed or cross-contamination takes place (Anon., 2004a; Johannessen *et al.*, 2005). It has also been reported that *E.coli* O157:H7 has a lower infectious dose than the other *E.coli* pathotypes and can cause illness at the low infectious dose of 2 - 2 000 cells, the reason being its ability to survive the gastric acid of the human stomach (Buchanan & Doyle, 1997; Steyert *et al.*, 2011).

In 2005, *E.coli* O157 (VTEC) infections also resulted in a large outbreak in Sweden. About 135 cases were reported, which included 11 cases of haemolytic uremic syndrome. The source of

the outbreak was locally produced lettuce that were irrigated with a contaminated stream water (Söderström *et al.*, 2008).

The biggest outbreak of *E.coli* O157:H7 (Table 2) in the USA occurred in 2006, when packaged baby spinach resulted in 206 infections and three deaths (Buchanan, 2006; Lynch *et al.*, 2009; Mandrell, 2009; Wendel *et al.*, 2009). Irrigation water was found to be the source of the contamination (Gelting *et al.*, 2011).

Table 2 Selected outbreaks associated with *E.coli* O157:H7 and leafy green vegetables in the the USA (Mandrell, 2009)

| Month-Year | Known or suspected vehicle | No. cases |
|------------|-----------------------------|-----------|
| Jul-95 | Lettuce, Romaine | 74 |
| Sep-95 | Lettuce, Iceberg | 30 |
| Sep-95 | Lettuce, Romaine | 20 |
| Oct-95 | Lettuce | 11 |
| May-96 | Lettuce, Mesclun mix | 61 |
| Jun-96 | Lettuce, Mesclun | 7 |
| Sep-98 | Lettuce | 4 |
| Feb-99 | Lettuce, salad | 65 |
| Sep-99 | Lettuce, Romaine | 8 |
| Sep-99 | Lettuce, Romaine | 6 |
| Oct-99 | Lettuce, salad | 47 |
| Oct-99 | Lettuce, Romaine hearts | 3 |
| Oct-99 | Lettuce, Romaine | 41 |
| Jul-02 | Lettuce, Romaine | 29 |
| Nov-02 | Lettuce | 24 |
| Sep-03 | Lettuce, Iceberg/Romaine | 57 |
| Sep-03 | Lettuce, mixed with Romaine | 5 |
| Oct-03 | Spinach | 16 |
| Nov-04 | Lettuce | 6 |
| Sep-05 | Romaine, also vegetables | 11 |
| Aug/Sep-05 | Lettuce, Ice berg | 135 |
| Aug/Sep-06 | Spinach, baby, bagged | >200 |
| Nov-06 | Lettuce, Iceberg | 71 |
| Nov/Dec-06 | Lettuce, Iceberg | 81 |
| May-08 | Lettuce, Romaine | 10 |

More recently, in 2010 *E.coli* O145 caused a multistate outbreak in the USA which resulted in 26 confirmed and 7 probable illnesses. The cause of the outbreak was due to the consumption of shredded romaine lettuce (CDC, 2010). The most recent outbreak occurred in Germany, where

more than 852 patients with HUS and 32 deaths associated with HUS have been reported (CDC, 2011). The cause of the outbreak was sprouts that were contaminated by *E.coli* O104:H4. *E.coli* O104 is a non-O157 STEC that has been rarely associated with foodborne outbreaks (Warriner, 2011). The genes of the strain were found to be 93% similar to EAaggEC, but it also tested positive for *stx 2* which is associated with the EHEC group although it tested negative for the *eae* gene that is also associated with the EHEC group (Warriner, 2011). The majority of the reported cases were also among adult women (Gault *et al.*, 2011). A reason for this might be due to the use of birth control, which results in increasing receptors necessary for the attachment of the pathogen or the involvement of the pathogen in urinary tracts infections (Warriner, 2011).

So far the largest international reported outbreak of *E.coli* O157:H7 occurred in Japan. In 1996 6 000 Japanese children were infected (Michino *et al.*, 1999). The source for the outbreak was due to contaminated radish sprouts (Buchanan & Doyle, 1997). This outbreak was one of the 16 outbreaks that happened between May and December of 1996 in Japan. There were 9 451 cases, 7 900 hospitalisations and 12 deaths in total (Michino *et al.*, 1999).

Salmonella

Salmonella is asymptotically carried by various agricultural animals (Franz & Van Bruggen, 2008). *Salmonella* consists of about 2 000 serotypes that cause salmonellosis (Anon., 2004a). Enteric fever (typhoid fever) is primarily caused by *S.Typhi* and *S.Paratyphi*, while nausea, vomiting, abdominal cramps and diarrhoea can also be caused by other *Salmonella* spp. (Anon., 2004a).

The infective dose of *Salmonella* is about 10 000 cells, however there have been outbreaks where the infective dose was about 10 - 100 cells (Franz & Van Bruggen, 2008). *Salmonella* infection depends on its ability to survive in the environment outside the digestive system, its ability to survive in the gastric acid of the stomach and the ability of the pathogen to attach and enter the intestinal cells.

Fresh produce can be a vehicle of transmission of *Salmonella* (Anon., 2004a). *Salmonella* contaminated tomatoes is the major commodity responsible for outbreaks (Table 3). *Salmonella* contamination of tomatoes has resulted in 12 outbreaks and more than 1 600 cases since 2 000 (Mandrell, 2009). Tomatoes were also responsible for 17% of the total produce outbreaks between 1998 - 2006 (Buchanan, 2006).

Table 3 Selected outbreaks associated with *Salmonella* spp. and tomatoes in the USA (Mandrell, 2009)

| Pathogen | Month-Year | No. cases |
|------------------------------|-----------------|-----------|
| S.Javiana | Jun/Aug-90 | 176 |
| S.Montevideo | Jun/Aug-93 | 100 |
| S.Baildon | Des-98 - Jan-99 | 86 |
| S.Javiana | Jun/Jul 02 | 141 |
| S.Newport | Sep/Oct-02 | 510 |
| S.Braenderup | Jul-04 | 125 |
| S.Javiana and other serovars | Jul-04 | 429 |
| S.Newport | Jul/Nov-05 | 72 |
| S.Braenderup | Nov/Dec-05 | 82 |
| S.Newport | Jul/Nov-06 | 115 |
| S.Typhimurium | Sep/Oct-06 | 190 |

Reporting systems

Foodborne illnesses are of great concern, especially in South Africa where the number of HIV individuals is increasing. In 2010, it was estimated that the total number of individuals living with HIV is about 5.24 million, while 410 000 were infected in 2010 alone of which 40 000 were children (Stats SA, 2010). Due to the low immune systems of HIV infected individuals they can usually suffer from health problems such as cirrhosis, hepatitis, haemochromatosis etc. (Anon., 2004a). These individuals are also more susceptible to infections from pathogens with low infectious doses such as *E.coli*. There is little information available on *E.coli* 0157:H7 infection in most of the African countries, most probably due to the lack of surveillance systems (Pu, 2009). Also the lack in reporting food borne diseases makes it difficult to estimate the exact number and source of the outbreaks (Franz & Van Bruggen, 2008).

Only a few reports are available regarding vegetables being contaminated with pathogenic *E.coli* in Africa (Abong'o *et al.*, 2008). In contrast to South Africa, a lot of information is available regarding the outbreaks in the United States. The European Union has less data available, since the reporting system was only implemented in 2005 (Franz & Van Bruggen, 2008). In 2005 and 2006, 38 and 23 alert notifications, respectively, for the presence of human pathogens associated with fruits and vegetables was reported by the Rapid Alert system of the European Union (Franz & Van Bruggen, 2008). Foodborne diseases are still underreported and therefore an accurate

estimate cannot really be determined (Anon., 2004a). The failure to screen *E.coli* O157:H7 by laboratories can also lead to a lot of EHEC infections not being reported (Müller *et al.*, 2001).

H. CARRY-OVER DURING PRE-HARVEST PROCESSES

Pathogens can contaminate fresh produce at any point during the production system. According to Suslow *et al.* (2006) there are three major routes for a pathogen to contaminate a fresh produce: the surface of the edible part of the produce; transfer via an injury to the produce tissue; or by root system transport. Contamination of the surface can also take place via splash dispersal from the soil surface or directly by irrigation (Franz & Van Bruggen, 2008). Edible portions can additionally get contaminated without direct exposure to *E.coli* O157:H7, indirectly via wind or rain (Islam *et al.*, 2004a). The concentration of pathogens in the original contamination source plays a role in the extent of contamination of the vegetables (Johannessen *et al.*, 2005).

The main pre-harvest points of contamination (Buck *et al.*, 2003; Harris *et al.*, 2006; Franz & Van Bruggen, 2008), include: irrigation water as source of contamination; manure as source of contamination; soil as source of contamination; sewage informal settlements and inadequate sanitation; and sewage system failure.

Irrigation water as source of contamination

Faecal contamination of irrigation water is an important source of fresh produce contamination (Suslow *et al.*, 2006) and a number of outbreaks was directly traced to contaminated irrigation water (Franz & Van Bruggen, 2008). Worldwide, 800 million farmers are engaged in urban agriculture of which 200 million practice market oriented farming on open spaces with low quality irrigation water (Qadir *et al.*, 2010). This could result in contaminated fresh produce and also possible infections and diseases. As vegetables are also in demand all year round it leads to farmers using any source of water to irrigate, whether it is clean or not, especially by farmers in urban areas (Amoah *et al.*, 2006). Alternative irrigation water sources would include wastewater, greywater and sewage water.

The use of wastewater has become a common source to irrigate fresh produce (Rai & Tripathi, 2007), even though consumers of wastewater irrigated produce are at risk for infections and diseases (Qadir *et al.*, 2010). In a study by Rai & Tripathi (2007) on treated municipal wastewater used to irrigate the crops, it was found that the irrigation water had total coliform counts between 2.1×10^4 - 1.3×10^6 cfu.mL⁻¹, while the vegetables had counts ranging between 1.5×10^3 - 1.3×10^6 cfu.mL⁻¹. The results indicated that the main source of contamination was the irrigation water.

Since irrigating with wastewater poses a high risk for containing harmful pathogens, it has been suggested to use subsurface drip irrigation as an alternative to wastewater irrigation. The reason is due to the minimum exposure of the water to the people and fresh produce (Song *et al.*, 2006). Buck *et al.* (2003) suggested that to lower the pathogens found in irrigation water, the history of the land, origin and distribution should be known and occasional monitoring of the irrigation water for human pathogens should be known. The storage of reclaimed water in reservoirs also improves the microbial quality of water (Qadir *et al.*, 2010).

Song *et al.* (2006) found that furrow irrigation led to greater contamination of crops than drip irrigation and suggested it was due to the direct application of the irrigation water. Direct application causes an even distribution of microorganisms on fresh produce, while with drip irrigation water needs to penetrate through the soil barrier. The penetration can lead to selective transport of some microorganisms due to the non-homogeneity of soil. The selective transport and filter, the soil provides, leads to the less contamination with drip irrigation (Song *et al.*, 2006).

The use of greywater for irrigation was studied in KwaZulu-Natal, South Africa, to determine whether this water can be used as an irrigation source rather than viewing it as waste (Jackson *et al.*, 2009). The vegetables were irrigated with greywater, tap water and hydroponic solution water. The mean values of the greywater was the highest out of the three sources, but still acceptable according to WHO guidelines (WHO, 1989). The *E.coli*, Enterococcus and total coliform counts were 35, >999 and 400 million cfu.100 mL⁻¹, respectively. While the potting soil of the vegetables had a high level of total coliforms, it had non-detectable levels of the other organisms. It was concluded that greywater irrigated vegetables are not likely to cause diseases (Jackson *et al.*, 2009).

Minhas *et al.* (2006) observed that irrigation with sewage water would result in high counts of faecal coliforms on vegetables such as cabbage. The mean faecal coliform values for the vegetables ranged between <2 and 9 x 10⁵ MPN.100 g⁻¹, with cabbage having a maximum value of 22.6 x 10⁵ MPN.100 g⁻¹. It is suggested that the coliform level should be lower than 10 cfu.g⁻¹ for vegetables to not cause foodborne diseases, while food containing more than 5 x 10⁷ cfu.mL⁻¹, considered unsafe for consumption (Minhas *et al.*, 2006). It was also found in the study that the coliform level reduced dramatically after the two outer leaves were removed after irrigation. It is important to note, since it can help the consumer to avoid hazards by not consuming the outer parts of the cabbage (Minhas *et al.*, 2006). What was also noted is that the vegetables that grew on the sides of the “bed” and “ridges” had higher coliform counts than those grown in the inside of the “beds” (Minhas *et al.*, 2006).

The occurrence of *E.coli* O157:H7 in a river used for fresh produce irrigation was examined over a 10-month period. The faecal coliform count during the dry season had a mean value of 2.8 x 10⁴ MPN.100 mL⁻¹ and 3.5 x 10³ MPN.100 mL⁻¹ during the rainy season, which indicates that the river is not safe for irrigation and might be a potential health hazard (Chigor *et al.*, 2010).

In Nigeria, a study was done by Okafo *et al.* (2003) on the transfer of pathogens from contaminated irrigation streams (two drains and a river) to vegetables via surface irrigation. It was found that of the 196 water samples taken from the three irrigation sources, the faecal coliform count ranged between 2.5×10^5 - 2.5×10^6 cfu.mL⁻¹. Of the 326 irrigated vegetables, which included tomatoes, lettuce, and cabbage, the faecal coliform counts varied between 2.5×10^3 - 2.4×10^6 cfu.mL⁻¹ (Okafo *et al.*, 2003). The highest counts were found during the dry seasons, while the lettuce had the highest contamination due to the bigger area exposed to effluent in the irrigation water (Okafo *et al.*, 2003). *Salmonella*, *Vibrio* and *E.coli* were isolated from the irrigation water and vegetables, while the latter had 39 isolates of which 15 was toxigenic (Okafo *et al.*, 2003).

A similar study by Amoah *et al.* (2005) in Ghana on the sources of contamination was performed as samples of water were taken from shallow wells, piped water, drains and streams. While it was found that all the irrigation sources were higher than the WHO guideline of 1 000 cfu.100 mL⁻¹ (WHO, 1989), it indicated that the faecal coliform counts were the highest in samples collected from the streams. Results were between 4×10^3 - 4×10^8 MPN.100 mL⁻¹. This is an indication that the quality of the water used in urban areas is very low. The study included measuring the level of contamination on lettuce that had been irrigated with these water sources. The faecal coliform counts were all more than a 1 000 per 100 g lettuce. Although lettuce irrigated with piped water proved to have less coliforms, in 95% of the samples it was still more than 1 000 faecal coliforms per 100 g wet weight. This is a clear indication that fresh produce irrigated with low quality water, could lead to serious infectious diseases.

In 2006, a study done by Amoah *et al.* (2006) on pathogen contamination on locally bought fresh produce on the urban market of Ghana found that none of the samples had values lower than 4 000 faecal coliforms.g⁻¹. The lettuce had faecal coliform counts between 4.0×10^3 to 9.3×10^8 g⁻¹ wet weight. The other vegetables, cabbage and spring onions also had high coliform counts, but not as high as the lettuce probably due to the smaller area exposed to irrigation water. These faecal coliform values exceeded the values consumers can safely be exposed to. Amoah *et al.* (2006) suggested the high faecal coliform counts are due to polluted irrigation water, fresh poultry manure and unsanitary market-related handling.

Manure

Animal manure are often used as an organic fertilisers to provide nutrients for the crops and to improve the quality of the soil, but it can also be a major source of fresh produce contamination (Franz & Van Bruggen, 2008). The use of untreated or fresh manure may lead to the contamination, as it might harbour *E.coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* (Johannessen *et al.*, 2005; Loncarevic *et al.*, 2005). Animal manure has been used for many years, because it makes the soil fertile due the nitrogen present. (Suslow *et al.*, 2006).

The isolation of *E.coli* O157:H7 from organic produce could be a result of the use of cow manure as fertilisation (Nwachuku & Gerba, 2008). In a study performed on beef and dairy cattle, *E.coli* O157:H7 was isolated in 8.3% and 16% of the dairy and beef cattle samples, respectively (Faith *et al.*, 1996). *E.coli* O157:H7 and *Salmonella* spp. might be present in the intestinal tract of animals and therefore also in the manure (Loncarevic *et al.*, 2005; Suslow *et al.*, 2006). These pathogens might be present in the manure depending on animal source. For example, *E.coli* O157:H7 is found in the faeces of ruminants such as cattle, but normally not in poultry (Suslow *et al.*, 2006). Whereas *Salmonella* are more frequently found in the manure of cattle as well as poultry (Suslow *et al.*, 2006). These organisms are then shed asymptotically in the faeces therefore it might still be present in the manure if it is not properly treated (Solomon *et al.*, 2002). *E.coli* O157:H7 can survive in animal faeces and slurries for weeks, even if it is applied to the crop area (Nwachuku & Gerba, 2008). It is important that manure gets properly managed and stored to reduce pathogens that might be present, this includes anaerobic digestion, aeration of sludge and composting (Suslow *et al.*, 2006). Because conditions are not favourable for pathogens once excreted outside the gut, the risk of contamination is depending on the survival of the pathogen in manure and manure amended soils (Franz & Van Bruggen, 2008).

Johannessen *et al.* (2005) investigated the carry-over of *E.coli* O157:H7 from contaminated manure to fresh produce. *E.coli* O157:H7 was not detected in the edible parts of the lettuce, outer leaves or roots, therefore it was concluded that no transmission from the contaminated manure to the fresh produce took place (Johannessen *et al.*, 2005). Another study evaluated the bacteriological quality of organically grown iceberg lettuce and found that *E.coli* O157:H7 could only be isolated in low numbers from the bovine manure and not from the lettuce (Johannessen *et al.*, 2004).

Bohaychuk *et al.* (2009) also found that the occurrence of *E.coli* in organically and conventionally grown produce had no statistical difference. In contrast, *E.coli* was not detected 100 days after manure has been applied on the lettuce, but sporadic *E.coli* was still detected on lettuce after 120 days regardless whether manure was applied. Low levels of *E.coli* was also still detected in the enriched soil, 132 - 168 days after the manure has been applied (Ingham *et al.*, 2004). It was also found that manure should rather be applied during cooler conditions (<10°C), than warmer conditions (>20°C) to the vegetables to ensure it does not get contaminated with *Salmonella enterica* Serovar Typhimurium. It was found if manure was applied during the warmer conditions, *Salmonella enterica* Serovar Typhimurium might be present on vegetables even after the recommended 120 days elapsed between manure application and vegetable harvest (Natvig *et al.*, 2002).

The quality of organically grown lettuce produced in Norway was also investigated by Loncarevic *et al.* (2005) who identified 8.9% positive *E.coli* O157:H7 samples, with some samples having counts of up to 5 000 cfu.g⁻¹. The results indicated that contamination of pathogenic

bacteria on lettuce occur occasionally. The reason why no *E.coli* were detected might be due to competing bacteria that grew and reduced the chances of contamination (Franz & Van Bruggen, 2008). Organically fertilised soils usually have a higher microbial diversity and activity, which results in contaminating bacteria being unable to compete, resulting in less contaminated fresh produce (Mäder *et al.*, 2002).

Soil

Contamination can also occur when crops are grown in contaminated soil (Franz & Van Bruggen, 2008). The contamination of crops grown in soils enriched with contaminated manure will largely depend on the survival capabilities of the pathogen in the manure and manure-amended soils, while the manure and soil characteristics will determine the persistence of the pathogens (Franz & Van Bruggen, 2008). Moist soil support the survival of the pathogens (Zaleski *et al.*, 2005). The type of soil also plays an important role in pathogen survival, with clay soil supporting pathogen survival for the longest period of time, due to greater moisture retention than sandy soils (Zaleski *et al.*, 2005).

The transmission of *E.coli* O157:H7 to lettuce from contaminated irrigation water and contaminated manure incorporated in the soil was studied (Solomon *et al.*, 2002). They irrigated the soil of the 15 lettuce and applied manure slurry to 10 lettuce. *E.coli* O157:H7 was detected on eight of 15 lettuce that had been irrigated with contaminated water. Seven out of the 10 lettuce treated with manure slurry tested positive for *E.coli* O157:H7. This showed that *E.coli* O157:H7 is capable of entering the root system and can be transported to the “upward” locations within the edible portions of the lettuce (Solomon *et al.*, 2002). There was no direct contact necessary between the leaves and the contamination source for the pathogen to become incorporated into the edible part of the lettuce tissue (Solomon *et al.*, 2002).

Agriculture land is usually tilled to cultivate the land before planting the crops, which usually changes the arrangement of aggregates of the soil particles and pores (Artz *et al.*, 2004). Artz *et al.* (2004) determined whether the soil structure would influence the leaching of *E.coli* O157:H7 through the soil. It was observed that the leaching rates decreased with increased dry bulk density and were also increased by the presence of earthworm burrows in repacked cores.

The persistence of *E.coli* O157:H7 in soil and on leaf lettuce and parsley was proven when contaminated irrigation water and manure was applied as treatment (Islam *et al.*, 2004a). The pathogen concentration of the irrigation water and manure were 10^5 cfu.mL⁻¹ and 10^7 cfu.mL⁻¹ respectively. Islam *et al.* (2004a) observed that *E.coli* O157:H7 persisted on the lettuce and parsley between 77 - 177 days and between 154 - 217 days in amended soils. In their study it was also found that *E.coli* survived longer in soil protected by vegetation, such as in the soil with parsley compared to the soil where lettuce was grown. Another study also indicated that *E.coli*

O157:H7 survived for eight weeks in soil, but was not detected after 12 weeks (Johannessen *et al.*, 2005).

Sewage, informal settlements and inadequate sanitation

Individuals who live in rural areas usually lack basic needs such as a clean safe water supply. Informal settlements are usually overcrowded with poor drainage systems, inadequate or non-existent sanitation and piles of uncollected refuse (Alder, 1995). As a result, the available fresh water resources are used for all basic needs such as drinking and domestic use (Fatoki *et al.*, 2004; Germs *et al.*, 2004).

It was revealed that in South Africa about 5 million urban residents live more than a quarter of a kilometre away from their nearest available water source (Goldblatt, 1999). Although the water used for consumption should be safe and free from contamination, the microbial quality of these water resources are poor and could contain pathogens such as *E.coli* (Obi *et al.*, 2004a; Obi *et al.*, 2007b). When sewage from informal settlements enters the rivers, it is untreated and therefore poses a serious health hazard to individuals who consume the water “as is” (Obi *et al.*, 2007a). This can lead to serious infections and diseases in the rural community as well as for other individuals that make use of the water downstream (Lötter, 2010). The same water source that is used for domestic and drinking purposes is sometimes also used by the informal settlement’s livestock as well as other grazing animals (Fatoki *et al.*, 2004). Surface runoff and sewage effluents can further contribute to the contamination of the surface water passing through informal settlements (Venter *et al.*, 1997; Paulse *et al.*, 2009). The impact of informal settlements on the pollution of river water was shown when Barnes & Taylor (2004) reported that the faecal pollution increased drastically after the Plankenburg River passed through the Kayamandi informal settlement. Other studies also revealed that informal settlements influence the quality of river water (Venter *et al.*, 1997; Bezuidenhout *et al.*, 2002; Fatoki *et al.*, 2004; Germs *et al.*, 2004; Obi *et al.*, 2004b; Zamxaka *et al.*, 2004; Paulse *et al.*, 2007; Paulse *et al.*, 2009; Vos & Cawood, 2010).

The other problem with water quality resources in informal settlements is that the majority of HIV/AIDS infected individuals live in these rural areas. This is a problem because these individuals are more susceptible to pathogens and resulting diseases (Obi *et al.*, 2007b). These individuals are in need of safe clean water because it is required for anti-retroviral medications, drinking and to prepare the formula feeds that is necessary for infected babies (Obi *et al.*, 2007b).

Sewage system failure

In most developing countries there are few treatment facilities available for municipal or industrial wastewater (Fatoki *et al.*, 2004). Usually in rural and peri-urban areas water sources are treated in small water treatment plants (SWTP) (Obi *et al.*, 2007a). These SWTPs are usually not well

serviced and do not fall within the limits of urban areas (Obi *et al.*, 2007a; Obi *et al.*, 2008). The failing or malfunction of these SWTP systems are becoming a serious concern and play an important role in the pollution of rivers. Once untreated sewage enters the river, it can still contain virulent pathogens that can lead to infections and diseases if consumed by individuals.

A recent survey revealed that the sewage treatment facilities of 30% of the municipalities in the Western Cape Province do not function properly (Peyper, 2010). It was also revealed that out of the 449 of 852 sewage treatment systems evaluated, 55% did not operate effectively (Peyper, 2010). This means that there can even be more sewage treatment systems not operating correctly. The Kuils River, one of the rivers that receive the most treated wastewater and as a result has very poor water quality (DWAF, 2005). In 2004, there were 78 888 sewage blockages and 43 pump station and rising sewer malfunctions due to mechanical failures in Cape Town alone (DWAF, 2005).

The microbial quality of raw water, treated water and water in the distribution systems of SWTP in the Limpopo and Mpumalanga Provinces were evaluated by Obi *et al.* (2007a). The study revealed that the microbial quality of raw water was very poor, but the water treatment level was efficient at the majority of the SWTP. The treated water from 69% and 85% of the water treatment plants was below the recommended guideline for total coliforms ($0 - 10 \text{ cfu.mL}^{-1}$) and faecal coliforms (0 cfu.mL^{-1}), but the study showed that most plants need regular monitoring.

Another study reported *Listeria* in the final effluents of a South African wastewater treatment facility, which proves that SWTP effluents can be sources of *Listeria* pathogens (Odjadjare & Okoh, 2010). They found free-living *Listeria* to be the most abundant (96%) compared to plankton associated *Listeria* (58-67%). The free-living *Listeria* reached levels of $3.2 \times 10^3 \text{ cfu.mL}^{-1}$, while the plankton associated *Listeria* reached $1.1 \times 10^4 \text{ cfu.mL}^{-1}$ after treatment. Although there was an improvement after the treatment, the wastewater effluent did not reach the recommended guideline levels (Odjadjare & Okoh, 2010).

In Switzerland, the malfunction of a wastewater treatment system led to gastroenteritis in a village of 3 500 people with more than a 50% infection level (Häfliger *et al.*, 2000). When investigated it was revealed that the drinking water contained high levels of faecal coliforms due to a defective pump in the wastewater treatment system, which caused the sewage to block and infiltrate into the ground water that was later collected for drinking purposes (Häfliger *et al.*, 2000). It is therefore important that sewage systems is working properly as it could result in serious infections when untreated sewage is released into surfaces waters intended for drinking and irrigation purposes.

I. CONCLUSIONS

Fresh produce outbreaks all over the world are increasing and are becoming a serious concern. Irrigation water has been identified as one of the major factors for the carry-over of dangerous pathogens onto fresh produce. Scientific reports published by different institutions regarding the safety of irrigation water for fresh produce made it clear that there is definitely a problem.

In South Africa it is clear that the safety of our surface waters cannot be guaranteed. The river water quality is decreasing due to industrialisation, sewage system malfunctions, overcrowding and poor sanitation. The faecal contamination of the river need to be continually monitored and tested to determine what the outcome will be on fresh produce. Since the main purpose of water is mainly for drinking, the quality of the water used for irrigation is not of such major concern. This leads to fresh produce being irrigated with low quality water which in return may lead to infections and diseases. By doing research in this area one should be able to determine the level of pathogen carry-over from irrigation water to fresh produce as well determining the original source of the faecal pollution in the irrigation water. The main reason why pathogens are increasing in water should be determined as well as if environmental factors are contributing to their survival. This will enable researchers and environmentalists to develop a strategy to decrease the faecal pollution of our surface waters. This will result in cleaner safer water as well as a safer final agricultural product, used for various purposes.

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CHAPTER 3

IMPACT OF ENVIRONMENTAL FACTORS (TEMPERATURE AND COD:N:P) ON THE GROWTH AND SURVIVAL OF *ESCHERICHIA COLI* IN RIVER WATER

SUMMARY

The impact of environmental factors including temperature, carbon, nitrogen and phosphate levels on *E.coli* and ACC growth was determined. Plankenburg River water was sampled and incubated at 10°, 15°, 20°, 25°, 30° and 35°C and the *E.coli* and ACC loads determined at 0, 6, 12 and 24 h. The impact of differential COD:N:P ratios on growth were also evaluated by altering the river water values that were originally found. The growth of a “pure” *E.coli* 58 culture in sterile river water and in MRS (Merck) was also determined, to see how *E.coli* would grow without other competing microorganisms. It was observed that increasing temperature leads to an increase in growth. At 10° to 20°C limited growth was observed, but from 25° to 35°C lead to the largest growth increases. It was found that after 12 h the *E.coli* started to die-off at the higher temperatures, possibly due to nutrient limitation. The ACC was always found to be higher and appeared to limit the *E.coli* growth. The *E.coli* “pure” culture reached higher population values in the absence of other competitors, suggesting that the organisms naturally present in the river water compete more efficiently for nutrients. When the COD:N:P was increased to higher rates the *E.coli* had longer growth increases and survived longer. When the COD:N:P values were adjusted above the optimum ratio of 106:12:1, the *E.coli* showed no die-off at any of the temperatures. It was therefore concluded that under specific conditions, carbon is a major limiting factor on microbial growth.

INTRODUCTION

Surface water is the most important water resource in South Africa, with irrigation annually requiring 59% of the total water requirements (Backeberg, 2005). Besides irrigation, untreated surface water is also used for drinking, domestic and recreation purposes (Venter *et al.*, 1997).

Over the last decade the quality of many South African rivers has decreased and pollution levels are way above recommended guidelines (DWAF, 2000; WHO, 1989; Bezuidenhout *et al.*, 2002; DWAF, 2002; Griesel & Jagals, 2002; Barnes & Taylor, 2004; Dalvie *et al.*, 2004; DWAF, 2004; Germs *et al.*, 2004; DWAF, 2005). The causes include: failing sewage systems; inadequately treated and direct discharge of sewage; poor sanitation of informal settlements; surface runoff water; as well as illegal dumping (Venter *et al.*, 1997; Barnes & Taylor, 2004; Paulse *et al.*, 2009). Many researchers have also isolated faecal coliforms, especially *E.coli* from contaminated river water (Bezuidenhout *et al.*, 2002; Vos & Cawood, 2010). Certain *E.coli* strains are pathogenic and can cause serious human illnesses at low infective doses (Muniesa *et al.*, 2006). *E.coli* is a natural inhabitant of the digestive tract and can also adapt very well to

environments that are low in nutrients (Leclerc *et al.*, 2001; Beuchat, 2002). Human or animal faeces can end up in rivers and lead to *E.coli* contaminated river water. Various factors are known to influence the growth, survival and decay of *E.coli* in rivers. These include temperature, pH, competing microorganisms as well as the presence or absence of essential organic and inorganic compounds (Sela & Fallik, 2009). Temperature is one of the most important factors impacting the growth and survival of enteric pathogens in water (Gerba, 2009). It is known that *E.coli* grows better at higher temperatures, with an optimum of about 35° - 37°C (Leclerc *et al.*, 2001). However, lower temperatures (10°C) have also been shown to support *E.coli* growth (Pope *et al.*, 2003). Similar studies have shown that lower temperatures enhance the survival of *E.coli* mainly due to decreases in cell metabolism (Wang & Doyle, 1998; Gzajkowsk *et al.*, 2005).

Even though temperature has a major influence, nutrient availability is also a key factor in facilitating microbial growth (Vital *et al.*, 2010). If the available nutrients are limited, the cell metabolism is slowed and this can lead to lengthening in the survival of the organism (Roszak & Colwell, 1987; Terzieva & McFeters, 1991). Bacteria usually require carbon, nitrogen and phosphate levels in a ratio of 106:12:1 for balanced growth, with organic carbon being the most important energy source (Goldman *et al.*, 1987). When wastewater is untreated or inadequately treated and released into rivers, it can lead to an increase in nitrogen and phosphate levels (Nhapi & Tirivarombo, 2004), which will result in more nutrients being available for microbial growth. These inorganic compounds in the presence of sufficient carbon have also been shown to enhance the growth of *E.coli* (Miettinen *et al.*, 1997; Brandl & Amundson, 2008).

Only a few studies, as referenced above, have been done on the effect of environmental factors on the growth and survival of *E.coli* in river water. Since there is limited knowledge available regarding the impact of environmental factors, temperature and nutrient levels (COD:N:P) on *E.coli* growth and survival specifically in river water, it is essential to determine the impact of these factors.

This study will focus on the impact of different environmental factors like carbon levels, temperature, incubation time and initial microbial load on the growth of *E.coli* and “naturally” occurring microbes (in terms of viable aerobic microbial load) in river water. The study will be done by sampling Plankenburg river water at site 3, which is an irrigation source site. The same environmental factors will also be monitored using a “pure” *E.coli* culture in sterile river water and a nutrient rich medium (MRS broth), to eliminate the impact of competing microorganisms.

MATERIALS AND METHODS

Site selection

Plankenburg River, site 3 (Lötter, 2010), situated in the Stellenbosch area of the Western Cape, was selected specifically where the Eerste River merges with the Plankenburg River (Fig. 1). This

site was selected as it is an irrigation water source point where water is drawn daily by local farmers to irrigate fresh produce and fruit downstream. An informal settlement about 2 km upstream also uses the river for domestic purposes. The specific site was shown by Lötter (2010) and Kikine (2011) to have high levels of faecal pollution.



Figure 1 Site 3, situated where the Plankenburg and Eerste Rivers converge and water is drawn for irrigation purposes downstream.

Sampling

The water was sampled on a weekly basis from September - November 2010 at $\pm 07:00$ according to the SANS 5667-6 (2006) method. The water was sampled in the centre of the river using a 2 L sterile container, which was held 30 cm below the surface facing the direction of the flow. The sample was then placed on crushed ice until the laboratory analyses started.

Environmental parameter determination

Temperature

The temperature of the river water was measured at the point of sampling using a digital thermometer (Crison).

Conductivity

Conductivity ($\text{mS}\cdot\text{m}^{-1}$) is an indication of the salinity of the water and was measured using a HI 8711 conductivity meter (Hanna Instruments).

pH

The pH was measured, according to Standard Methods (APHA, 2005), by using a hand held pH meter (WTW).

Chemical Oxygen Demand

The chemical oxygen demand (COD) was determined according to Standard Methods (APHA, 2005) using a DR 2000 HACH spectrophotometer set at 585 nm, and measured the following ranges: 4 - 40; 10 - 150; and 100 - 1500 mg.L⁻¹. To facilitate adjusting the carbon concentration to the required carbon concentrations as given in Table 1, the required COD values was provided by the addition of glucose. The COD levels were used as the carbon value in the C:N:P ratio.

Nitrogen

The total nitrogen value was determined using the Nitrogen (total) Cell Test kit (Merck) with range between 0.5 – 15 mg.L⁻¹ according to the SANS 11905-1 (SANS, 1997) method. The nitrogen value was measured using the Merck Spectroquant® Nova 60 spectrophotometer.

Phosphate

The Phosphate Cell Test kit (Merck) was used to determine the phosphate (PO₄-P) concentration in the range between 0.05 - 5 mg.L⁻¹ according to SANS 6878 (SANS, 2004). The phosphate value was measured using the Merck Spectroquant® Nova 60 spectrophotometer.

Microbiological analysis

E.coli enumeration

The *E.coli* count was determined according to the method prescribed in Standard Methods (APHA, 2005). A dilution series in duplicate was prepared in sterile physiological saline solution (PSS). Violet Red Bile Agar (VRBA) (Merck) was used for the enumeration. The VRBA plates were incubated for 24 h at 30 °C as described in the Merck Manual (2005). Colonies that were red, surrounded by reddish precipitation zones were counted as *E.coli* as described in the Merck Manual (2005).

Aerobic colony count (ACC)

In this study the ACC was taken as representative of the competing organisms “naturally” present in the river water. The enumeration method was performed according to the SANS 4833 (2007) method. A dilution series in duplicate was prepared in sterile PSS. Plate Count Agar (PCA) (Merck) was used to determine the aerobic colony count and the PCA plates were incubated for 48 h at 30 °C.

Experimental design

To determine the impact of different COD:N:P levels, temperature, incubation time and initial cell concentrations on *E.coli* and ACC growth (Table 1). The COD:N:P of the river samples was adjusted with a stock solution to obtain values representing low to high COD concentrations. The second part of the study was to determine the growth of a “pure” culture, *E.coli* 58 (ATCC 11775), in sterilised river water as well as in MRS broth (Merck).

Table 1 Experimental setup used in the growth studies

Design

Plankenburg River (Plank-3 site)

Sampled weekly at ±07:00 for 10 weeks (Sep - Nov 2010)

Enumeration of *E.coli* and ACC using VRBA and PCA, respectively

“Pure” culture studies using *E.coli* 58 (ATCC 11775) as reference and control strain (sterile river water and MRS broth)

Studies

A. River

1. Determine the impact of incubation temperature (10°, 15°, 20°, 25°, 30° and 35°C) and time (0, 6, 12, 24 h) on *E.coli* and ACC.
2. Determine the impact of different COD:N:P ratio changes (COD values = 54, 83, 108, 150, 208 and 350 mg.L⁻¹) on *E.coli* and ACC. The COD values were used as basis to adjust the N and P values to as near possible to the Goldman *et al.* (1987) ratio of 106:12:1. The required COD was provided by glucose addition.

B. “Pure” culture

1. Sterile river water + *E.coli* 58 (ATCC 11775)
 2. MRS broth (Merck) + *E.coli* 58 (ATCC 11775)
 3. The same parameters: temperature; incubation time and COD:N:P changes described in section A, were applied to the water samples inoculated with the “pure” culture.
-

Adjustment of river water to obtain different COD:N:P ratio's

The sampled river water was adjusted to obtain the required COD:N:P ratio, ranging from low to high (Table 1). To adjust the sampled river water to the required carbon, nitrogen and phosphate levels, a stock solution was prepared, where the COD level was adjusted with glucose (Saarchem), the nitrogen level with urea (Saarchem) and the phosphate level with di-potassiumphosphate (ACE). An aliquot of the adjusted river water solution (140 mL) was placed in sterile containers and incubated at the different temperatures and incubation times as described in section A of Table 1.

Incubation temperatures and time of analysis

To determine the impact of incubation temperature on the growth of *E.coli* and “natural” competing organisms (ACC), the containers (140 mL) were incubated in waterbaths set at different temperatures (10°, 15°, 20°, 25°, 30° and 35°C). The samples were analysed at 0, 6, 12 and 24 h to determine the changes in *E.coli* and ACC numbers.

Preparation of “pure” *E.coli* culture in sterile river water

To determine the growth of a reference culture in sterile river water, *E.coli* 58 (ATCC 11775) was prepared in nutrient broth (Merck) for 24 h at 37°C and the optical density (OD) measured (Spectronic 20 Genesys™ spectrophotometer). The averaged OD value was used in the *E.coli* 58 growth curve equation ($y = 2.3x + 7.533$) to determine the cfu.mL⁻¹ of the *E.coli* in the broth. The *E.coli* broth was diluted and added to sterile river water that had been adjusted with the stock solution to give specific COD values of 100, 150 and 350 mg.L⁻¹. The temperatures and incubation times were the same as used for the incubation of the river water.

E.coli 58 was also inoculated into MRS broth (Merck) as an alternative to the sterile autoclaved river water. The COD of the MRS broth was adjusted to give values representing below optimum and extreme carbon levels (90 and 450 mg.L⁻¹). The *E.coli* 58 was prepared as described above and inoculated into the sterile MRS broth (Merck). The incubation temperatures and times of analysis were also the same as above.

RESULTS AND DISCUSSION

River characteristics

Temperature

The river temperature at site 3 during the sampling period was found to range between 10.1° - 18.2°C (Table 2). A previous study by Lötter (2010) reported that between September and

December 2007 the temperature at site 3 ranged between 15.7 - 18.7°C. These temperatures indicate that this river is not a very “warm” river, even during the hotter summer months. The lowest temperature was observed in winter (August), while the highest temperature was observed in summer (November). This could be due to the river water being exposed to more sunlight during the warmer summer months, resulting in increasing river temperature (Zamxaka *et al.*, 2004).

pH

Throughout the sampling period no major pH variation was seen with values varying between, 6.27 - 6.76 (Table 2). It is recommended by DWAF that water intended for domestic use, should range between pH 6 - 9 (DWAF, 1996b), while the “target water quality” pH range for irrigation should be between 6.5 - 8.5 (DWAF, 1996d). The pH for this site was thus acceptable for domestic use, but only 50% of the time acceptable for irrigation purposes. A previous study by Lötter (2010) showed that between September 2007 and February 2008 the pH values ranged between 6.03 - 7.33. Unlike the pH and temperature data found by Lötter (2010), no relationship was found between the pH and the temperature in this study.

Conductivity

The conductivity is an indicator of the salinity of the water (Morrison *et al.*, 2004). Domestic sewage, municipal stormwater drainage and industrial effluents contain high amounts of dissolved salts, which can enter rivers and increase the salinity content of the water. Irrigation water with high salinity content is undesirable, since it has a negative impact on soil and crops. For conductivity not to have any health, aesthetic and treatment effects, it should range between 0 and 70 mS.m⁻¹ (DWAF, 1996b; Koning *et al.*, 2000). The conductivity for the 10 samples from this study ranged between 28 - 41 mS.m⁻¹ (Table 2). The lowest two conductivity values, 28 and 31 mS.m⁻¹, were found when it rained on the day of sampling. The rain could be the reason for the lower values as it was previously reported that rainfall has a dilution effect in river water and resulted in lower conductivity levels (McLain & Williams, 2008). A previous study showed that between September - December 2007 and February 2008, the conductivity values for the same site varied between 49 and 72 mS.m⁻¹ (Lötter, 2010). These values were all higher than what was found during the 10 week sampling period of this study and indicates that this site could reach higher values that is above the recommended guideline.

Table 2 The chemical parameters, the initial *E.coli* and ACC at t_0 of the Plankenburg River (site 3) samples

| Nr. | Sample date (2010) | Temperature (°C) | pH | Conductivity (mS.m ⁻¹) | COD* (mg.L ⁻¹) | Nitrogen* (mg.L ⁻¹) | Phosphate* (mg.L ⁻¹) | <i>E.coli</i> * (cfu.mL ⁻¹) | ACC* (cfu.mL ⁻¹) |
|-----|-----------------------|---------------------|------|---------------------------------------|-------------------------------|------------------------------------|-------------------------------------|--|---------------------------------|
| 1. | August | 10.1 | 6.37 | 35 | 12 | 1 | 0.2 | 165 | 25 100 |
| 2. | September | 12.7 | 6.27 | 40 | 7 | 4 | 0.2 | 145 | 2 245 |
| 3. | September | 14.2 | 6.52 | 41 | 37 | 2 | 0.2 | 70 | 120 000 |
| 4. | September** | 14.8 | 6.29 | 31 | 44 | 3 | 0.4 | 1 870 | 22 150 |
| 5. | September | 12.7 | 6.36 | 36 | 36 | 4 | 0.2 | 930 | 10 300 |
| 6. | October | 16.8 | 6.52 | 32 | 14 | 1 | 0.2 | 155 | 6 950 |
| 7. | October | 12.7 | 6.76 | 36 | 30 | 3 | 0.3 | 1 665 | 26 250 |
| 8. | October | 14.7 | 6.58 | 37 | 24 | 2 | 0.3 | 885 | 19 400 |
| 9. | November** | 14.8 | 6.51 | 28 | 8 | 2 | 0.3 | 1 180 | 13 350 |
| 10. | November | 18.2 | 6.41 | 32 | 13 | 2 | 0.3 | 1 580 | 44 500 |

* Average values of duplicates; ** Raining on day of sampling (September = 6mm rainfall and November = 4mm rainfall)

Chemical Oxygen Demand (COD)

The COD over the sampling period (Table 2) ranged between 7 and 44 mg.L⁻¹. The highest COD was obtained when it was raining, therefore it could be that surface runoff have contributed towards the higher COD value. It is recommended by DWAF that wastewater effluents discharged into rivers should not exceed a COD level of 30 mg.L⁻¹ (DWAF, 1999). Taking this limit into consideration, the recommended upper value was exceeded 40% of the time during the sampling period.

COD is used to classify water according to its quality for industrial and agricultural uses (DWAF, 1996c): the first category is the best quality water ranging between 0 - 10 mg.L⁻¹; the second category ranging up to 15 mg.L⁻¹; the third category up to 30 mg.L⁻¹; and the fourth category up to 75 mg.L⁻¹. Two of the samples from this study can be classified as category 1 water (7 and 8 mg.L⁻¹). Three samples classified as category 2 (12, 13 and 14 mg.L⁻¹), while two samples can be classified as category 3 (24 and 30 mg.L⁻¹). Three of the samples (36, 37 and 44 mg.L⁻¹) can be classified as category four. This indicated that based on the COD level, only 20% of the river samples during this study could be considered of a superior quality, compared to 30% of the samples, classified under the worst water quality.

It was previously found by Lötter (2010) that during September - December 2007 the COD of the Plankenburg River (site 3) ranged between 81 - 193 mg.L⁻¹ and then dropped to 53 mg.L⁻¹ in February 2008. Even though the COD in this study was much lower than the data reported by Lötter (2010), it is still an indication that the COD values of the Plankenburg River can reach levels higher than 106 mg.L⁻¹, which is the minimum required for optimum microbial growth as recommended by Goldman *et al.* (1987).

Nitrogen and Phosphate

The nitrogen levels (total nitrogen present) found in this study ranged between 1 - 4 mg.L⁻¹ (Table 2). The "target quality water range" for nitrogen in water intended for irrigation should not exceed 5 mg.L⁻¹ (DWAF, 1996d). The total nitrogen levels found in this study did not exceeded the nitrogen guideline for irrigation during the 10 week sampling period.

Miettinen *et al.* (1997) reported that phosphorus can be a limiting factor for microbial growth. The phosphate levels from the river site ranged between 0.2 - 0.8 mg.L⁻¹ (Table 2). Phosphate is also a growth limiting factor for eutrophication and therefore to reduce algal and unwanted plant growth in water resources, DWAF recommends that phosphate should not exceed 5 µg.L⁻¹ (0.005 mg.L⁻¹) (DWAF, 1996a). This guideline was thus exceeded during the whole sampling period of this study.

Impact of COD:N:P levels in the river water on *E.coli* growth kinetics

Based on the COD:N:P data for the Plankenburg River as given in Table 2, the next trial was conducted to determine the impact of temperatures and different COD:N:P levels on *E.coli* growth in river water. For this study the *E.coli* growth kinetics were specifically considered as cell number changes representing growth, survival and decay. The impact of increasing temperature and time on *E.coli* growth/die-off was evaluated based on the COD, nitrogen and phosphate levels that occurred naturally in the river water during the sampling period. For the first phase of the study, the microbes (*E.coli* and ACC) in the river water samples were exposed to the selected temperatures (Table 1) over a 24 h period with no adjustments to the COD:N:P levels.

“Low” carbon (COD) level - growth profile

In Fig. 2 the growth pattern of *E.coli* at a low COD level (7 mg.L⁻¹) is illustrated. This COD level was taken as representative of a “fairly clean” river at that point of time. The initial cell concentration (sampling time = t_0) of the *E.coli* in the sampled river water was 145 cfu.mL⁻¹ (Table 2). The reason for the low cell concentration might be ascribed to the low COD value. The data in Fig. 2 show that at all the temperatures a large cell number increase was found during the first 6 h, with the highest increase at 35°C (1 200 cfu.mL⁻¹) and the lowest at 10°C (655 cfu.mL⁻¹). At all the temperatures, except at 10°C, no major growth changes occurred after 6 h and the *E.coli* started to die-off after 12 h, possibly due to nutrient limits. Dutka & Kwan (1980) reported that minimal polluted water ensures a faster die-off for organisms, because of nutrient shortage.

An explanation for the lack of die-off at 10°C may be that at lower temperatures less nutrients are consumed due to a slower metabolism (Terzieva & McFeters, 1991; Easton *et al.*, 2005). This in turn could lead to a more stable growth profile over a longer period of time. Easton *et al.* (2005) reported that, besides the COD level, a lower temperature (9°C) showed a slower die-off rate in sewage contaminated water when compared to higher temperatures (23°C). This was similar to a study by Borst & Selvakumar (2004) who found the die-off of *E.coli*, faecal coliforms and total coliforms can be twice as fast at 20°C when compared to 10°C.

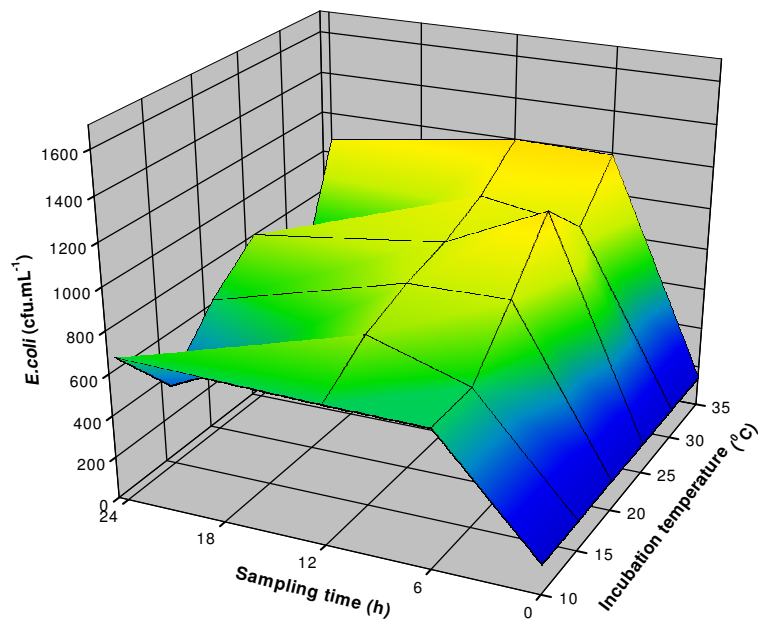


Figure 2 Growth pattern of *E.coli* naturally present in the river water when exposed to a “low” COD (7 mg.L⁻¹). The initial cell concentration was 145 cfu.mL⁻¹.

“Medium” carbon (COD) level - growth profile

In the river water containing a COD of 12 mg.L⁻¹, the initial *E.coli* cell concentration was 165 cfu.mL⁻¹ (Table 2). In Fig. 3 the same profile was observed as in Fig. 2, with the highest growth occurring at the highest temperature and the slowest at the lowest temperature. The largest growth increases were found in the first 6 h at 35 °C and after 6 h at 30 °C. At 20 ° and 25 °C after 6 h there were no major changes in growth increases or die-off. The *E.coli* did however grow to higher microbe levels at 20 ° and 25 °C compared to those at 10 ° and 15 °C. This might be due to more nutrients being available and utilisable at the higher temperatures. With the exception of 10 ° and 15 °C, the *E.coli* began to show die-off after 12 h possibly due to the nutrients becoming limited at the higher temperatures where more growth took place. The reason why the *E.coli* increased after 12 h at 15 °C might be ascribed to the higher COD (12 mg.L⁻¹) and the slower growth that occurred at the low temperature. Therefore, the slow growth observed confirms that fewer nutrients were utilised leading to nutrients available for a longer period of time. Easton *et al.* (2005) also found that die-off occurs slower in low temperature waters compared to higher temperature waters.

In this trial after 6 h, 30 °C was the only temperature which still showed a high growth increase. This corresponds to a recent study by Vital *et al.* (2008), where the highest growth for *E.coli* O157 was observed at 30 °C and not 35 °C. Vital *et al.* (2008) concluded that such

differences might be due to different *E.coli* strains having different physiological properties or having probably undergone acclimatisation.

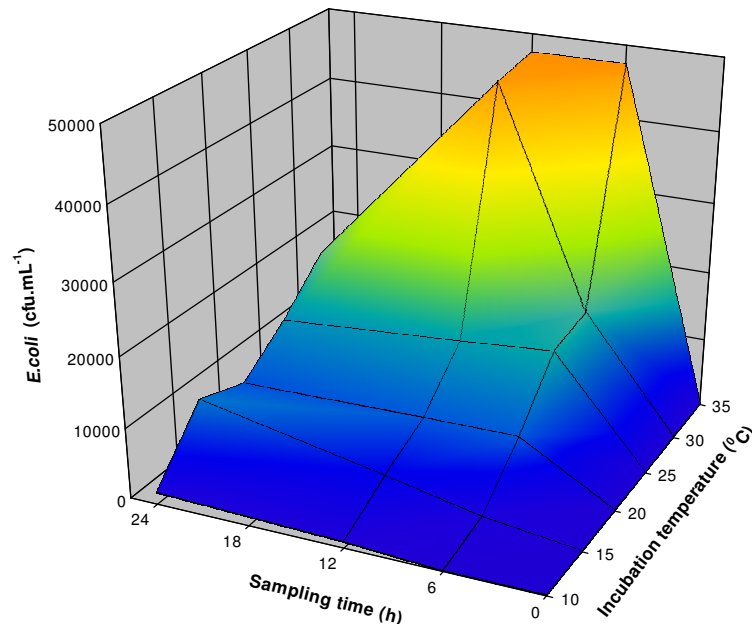


Figure 3 Growth pattern of *E.coli* naturally present in the river water when exposed to a “medium” COD (12 mg.L⁻¹). The initial cell concentration was 165 cfu.mL⁻¹.

“High” carbon (COD) level - growth profile

The highest COD level of the river water found during the 10 week sampling period was 44 mg.L⁻¹ (Table 2), with an initial *E.coli* cell concentration of 1 870 cfu.mL⁻¹. What was noticeable in this trial (Fig. 4) was that although the COD is higher than in the previous two trials, slow growth still occurred at 10° and 15°C, which indicated that although the COD is higher, temperature has a major influence on growth. At 20°C no large growth increases were observed after 12 h. At 25°C after 6 h there was also no major growth increase, but already signs of population die-off due to nutrient limitation. At 35°C, because of a more optimum growth temperature and more available nutrients (due to the higher COD), a larger increase in the population was seen in the first 6 h, reaching 235 000 cfu.mL⁻¹. However, after 6 h the population started to die-off at 35°C, which indicated that 44 mg.L⁻¹ is still too low for sustained population growth longer than 6 h. At optimum temperature according Prescott *et al.* (2005), the metabolism of organisms increase, leading to a faster consumption of nutrients and subsequent increase in numbers. Since the initial population was also higher than in Fig. 2 and Fig. 3, the nutrients could thus have been used at a faster rate and exhausted faster.

From the data it is clear that even if the temperature is at an optimum (35°C), if the COD is too low, the population will start to die-off after 6 h. The question then arises as how the growth pattern would change if *E.coli* had access to sufficient nutrients and the temperature were at an optimum for growth.

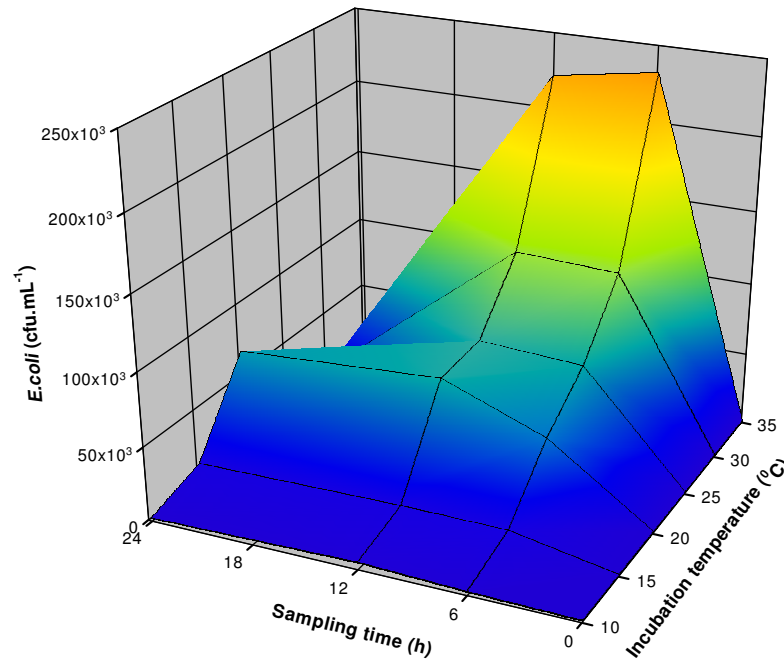


Figure 4 Growth pattern of *E.coli* naturally present in the river water when exposed to a “high” COD (44 mg.L⁻¹). The initial cell concentration was 1 870 cfu.mL⁻¹.

Expected growth under ideal conditions - hypothetical

Fig. 5 is an illustration of what the expected growth profile of *E.coli* could be under ideal environmental conditions (high COD and optimum growth temperature) in comparison to the *E.coli* growth profile found at a low COD (7 mg.L⁻¹). The growth profile with an initial cell concentration of 145 cfu.mL⁻¹ found at COD 7 mg.L⁻¹ (Fig. 2) was used for the illustration in Fig. 5. Since *E.coli* has the ability to double every 20 min under ideal circumstances (Buchanan & Klawitter, 1992), it can be argued that it will have a generation time (T_d) value of 72 in 24 h. This value can only be achieved if the carbon and nutrient content is high enough and the temperature is “ideal” (35° - 37°C). Under these “ideal” conditions, the initial population of 145 cfu.mL⁻¹ would have reached a final cell concentration of 3.4×10^{23} cfu.mL⁻¹ (T_{d72}). However, under the conditions as detailed for Fig. 2, the population did only reach a T_d value of 3 in 24 h. This was because the *E.coli* was only able to double every 6 h and reach a final cell concentration of 1 160 cfu.mL⁻¹ (T_{d3}) after 24 h. This is a clear indication that temperature is not always the major growth factor and that carbon levels must under certain conditions be considered the major growth limiting factor. The question to be

answered now is how will higher carbon (COD) levels impact the growth of *E.coli* at different temperatures?

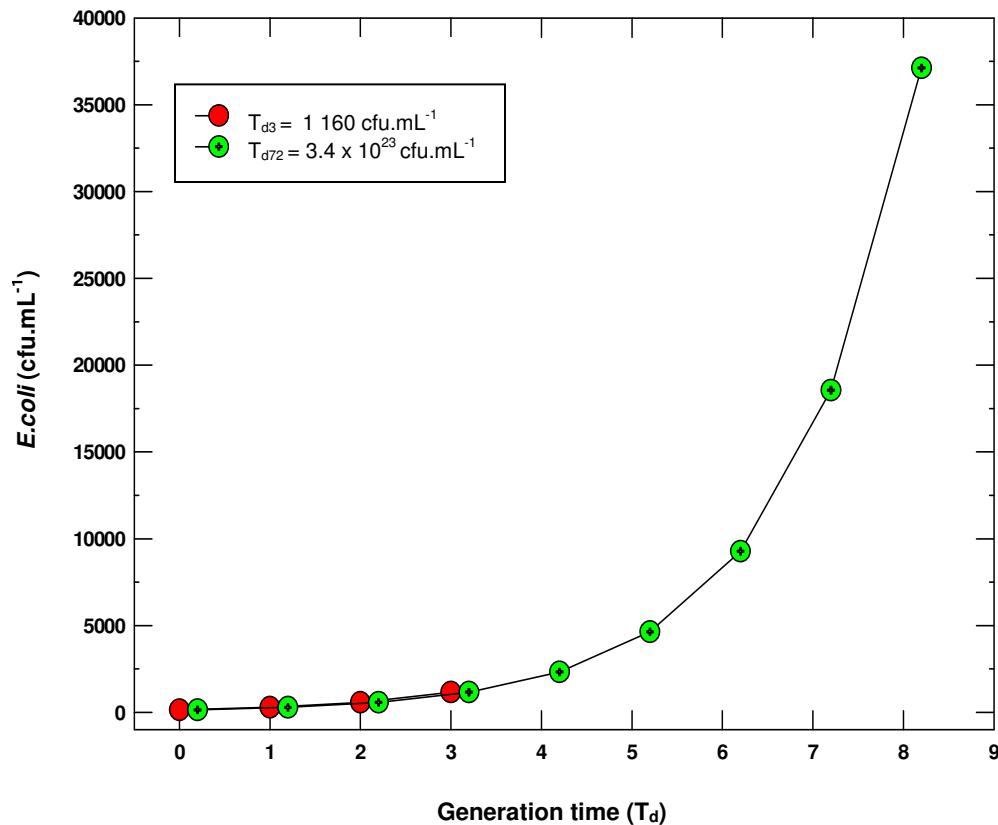


Figure 5 The *E.coli* generation time (T_{d72}) at optimum temperature ($35^{\circ} - 37^{\circ}\text{C}$) and under “optimum” ($>1\ 000\ \text{mg.L}^{-1}$ COD) carbon conditions (green line). The *E.coli* would reach a cell concentration of $3.4 \times 10^{23}\ \text{cfu.mL}^{-1}$, compared to low carbon ($7\ \text{mg.L}^{-1}$ COD) conditions as “naturally” found in the river water (red line), which with an initial cell concentration of $145\ \text{mg.L}^{-1}$ the *E.coli* reached a T_{d3} value of $1\ 160\ \text{cfu.mL}^{-1}$ in 24 h.

***E.coli* growth after COD:N:P ratio adjustments**

To determine the impact of increased carbon (COD) values, the COD:N:P ratio was adjusted to determine the growth differences at low, optimum and above optimum levels (Table 3). Although the levels of the adjusted COD ratios were never found in the Plankenburg River samples from this study, it will illustrate what might happen to the population at above optimum COD levels.

Table 3 COD:N:P values originally found in the river water samples and values after adjustment to higher values

| Sample date (2010) | Normal COD:N:P (mg.L ⁻¹) | Initial cell concentration (cfu.mL ⁻¹) | Adjusted COD:N:P (mg.L ⁻¹) |
|-----------------------|---|---|---|
| September (1) | 36:4:0.2 | 930 | 54:6:0.3 |
| October (1) | 14:1:0.2 | 155 | 108:10:1.0 |
| October (2) | 30:3:0.3 | 1 665 | 208:24:2.4 |
| October (3) | 24:2:0.3 | 885 | 350:38:3.0 |
| November (1) | 8:2:0.3 | 1 180 | 83:8:0.8 |
| November (2) | 13:2:0.3 | 1 580 | 150:15:1.5 |

“Below optimum” carbon (COD) level - growth profile

The COD:N:P of November (1) was adjusted from a low initial COD of 8 mg.L⁻¹ to 83:8:0.8 (Table 3), with an initial cell concentration of 1 180 cfu.mL⁻¹. In this trial the *E.coli* showed no major growth increases during the first 12 h for the temperature range 10° to 20°C (Fig. 6). This was probably due to the low temperatures and the data agrees with Jones *et al.* (1987), who reported that growth is slower below 20°C. At 24 h, the population at 20°C reached a value of 153 000 cfu.mL⁻¹. At 24 h there were major increases at 25° and 30°C, reaching final concentrations of 284 500 and 1 290 000 cfu.mL⁻¹, respectively. At 35°C, during the first 6 h, there were not large growth increases, but the highest count was observed at 12 h, reaching a value of 1 800 000 cfu.mL⁻¹. After 12 h the population at 35°C showed a 30% decrease from 1 800 000 to 1 250 000 cfu.mL⁻¹. This suggests that the carbon source may not have been sufficient to continuously

support the optimum growth. From this trial it was concluded that the COD:N:P ratio may still not have been sufficient to sustain optimum growth at the optimum temperature (35°C). It was necessary to increase the COD:N:P ratio to optimum level.

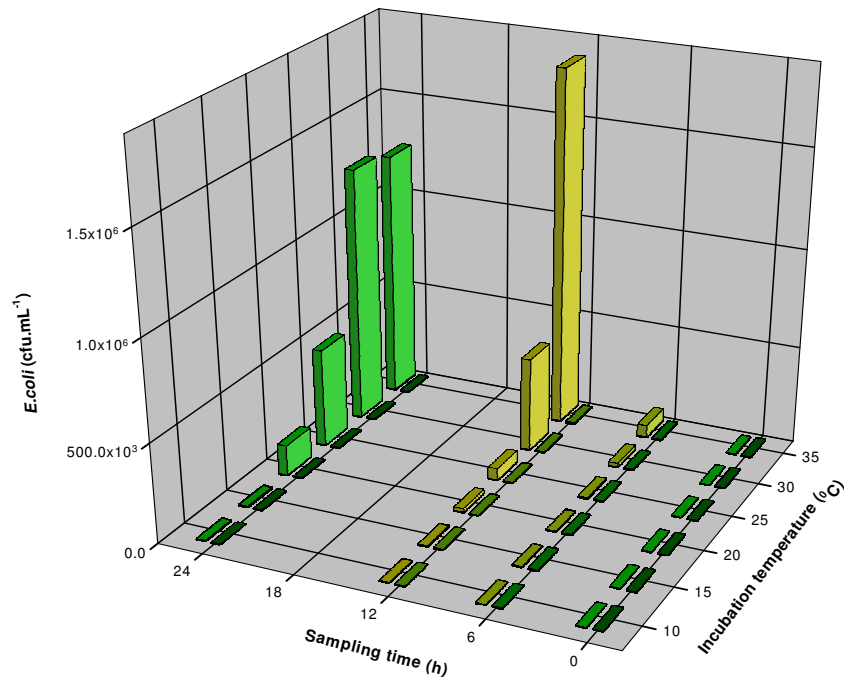


Figure 6 Growth of *E.coli* at the COD:N:P ratio of 8:2:0.3 found in the original river water (right columns of each row), compared to the adjusted COD:N:P ratio of 83:8:0.8 (left columns of each row). The initial cell concentration was 1 180 cfu.mL⁻¹.

“Optimum” carbon (COD) level - growth profile

For the next trial the COD:N:P ratio was adjusted from 14:1:0.2 with a low initial cell concentration of 155 cfu.mL⁻¹ to 108:10:1.0 (Table 3), which was near the optimum ratio of 106:12:1 (Goldman *et al.*, 1987). This was done to determine whether sufficient carbon was available to continuously (over 24 h) support optimum microbial growth. The data in Fig. 7 shows that for the first 6 h very little growth took place at the lower temperatures (10°, 15° and 20°C). For the adjusted COD samples at 10°C growth was slow and resulted in the lowest increases, but in time it still reached a population value of 16 950 cfu.mL⁻¹ after 24 h. After 24 h, the adjusted COD samples at 15° and 20°C reached values of 29 600 and 98 000 cfu.mL⁻¹, respectively.

According to Ng (1969), lower temperatures will usually result in lower growth. They found that *E.coli* grown at 10°C used 37% of the carbon from glucose to form cellular material, whereas the *E.coli* grown at 30°C used 53%. It was also found that the *E.coli* cells grown at 10°C, had a higher glycogen-to-protein ratio compared to those grown at 37°C. Ng (1969) explained the above

in terms of a temperature decrease, which led to an uncoupling of energy production from energy utilisation taking place. The growth rate is slower at lower temperatures, which results in biosynthesis (anabolism) taking place at a much slower rate than catabolism, with the excess energy then being stored in the form of glycogen. Since there was a high nutrient availability (optimum ratio), the *E.coli* population in this trial was still able to increase after 12 h at 25°, 30° and 35°C. At 35°C, the population reached the highest value of 495 000 cfu.mL⁻¹ after 24 h. The increase in *E.coli* numbers corresponds to previous research by Dukta & Kwan (1980), which showed that water that is more polluted contains more nutrients than unpolluted water, resulting in an increased survival/growth of *E.coli* over time.

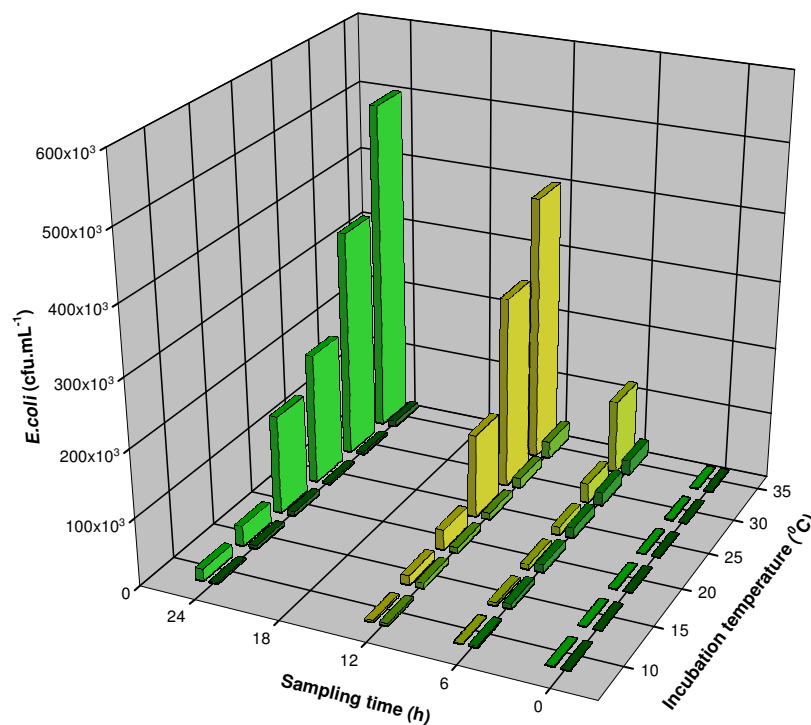


Figure 7 Growth of *E.coli* at the COD:N:P ratio of 14:1:0.2 found in the original river water (right columns of each row), compared to the adjusted COD:N:P ratio of 108:10:1.0 (left columns of each row). The initial cell concentration was 155 cfu.mL⁻¹.

“Double optimum” carbon (COD) level - growth profile

After determining the growth profile of *E.coli* at an “optimum” nutrient level (Fig. 7), the next trial was done to determine the growth profile of *E.coli* when the nutrient ratio is nearer “double” that of the optimum ratio. The COD:N:P ratio was therefore adjusted from 30:3:0.3 to 208:24:2.4, which was almost double that of the recommended Goldman *et al.* (1987) optimum ratio at 106:12:1. The growth profile in this trial (Fig. 8) is very similar to the growth profile found in Fig. 7. During the 24 h period no large growth increases occurred at the two lower temperatures (10° and 15°C).

At 20°C the *E.coli* population increased from 12 150 cfu.mL⁻¹ at 12 h to 175 000 cfu.mL⁻¹ at 24 h. At 30°C the highest growth increase occurred from 12 to 24 h, to reach a final population count of 945 000 cfu.mL⁻¹. The highest population value of 1 045 000 cfu.mL⁻¹ was found after 24 h at 35°C, even though the growth increase after 12 h at 35°C was not as large as found at 30°C. This might be due to a higher metabolism at 35°C and the nutrients were used much faster than at 30°C. Since the nutrients were probably becoming more limited, the growth is also slowed down after 12 h. The initial cell concentration (1 665 mg.L⁻¹) was also higher than found in the other river samples (Table 2), which could have resulted in the population increasing during the first few hours until the growth rate started to decrease, due to the nutrients present becoming more limited (Easton *et al.*, 2005).

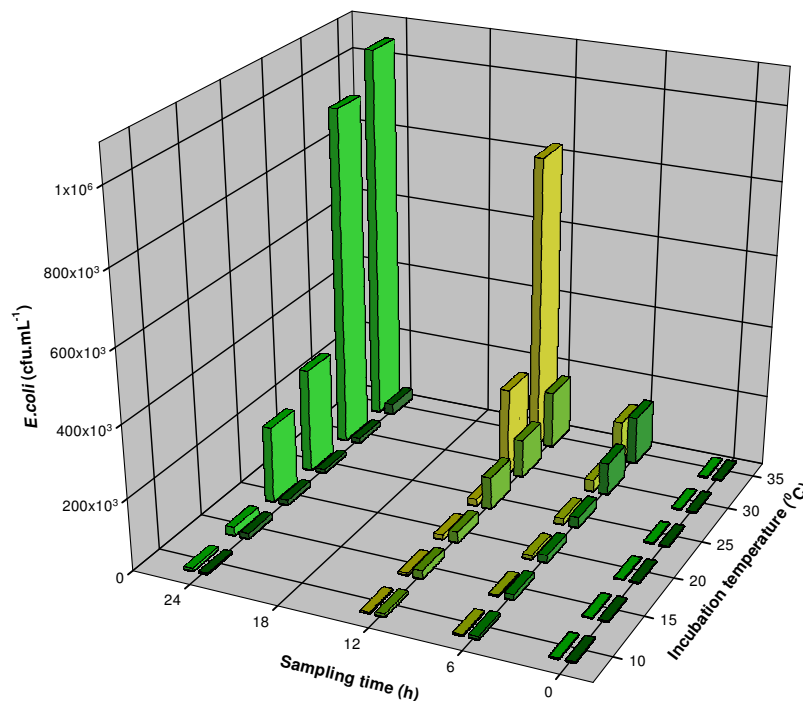


Figure 8 Growth of *E.coli* at the COD:N:P ratio of 30:3:0.3 found in the original river water (right columns of each row), compared to the adjusted COD:N:P ratio of 208:24:2.4 (left columns of each row). The initial cell concentration was 1 665 cfu.mL⁻¹.

“Extremely high” carbon (COD) level - growth profile

In this trial the COD:N:P ratio was adjusted from 24:2:0.3 to an “extremely high” ratio of 350:38:3.0 (Fig. 9). This was done to determine how the growth profile would change if there is a lot more carbon available than what *E.coli* requires in the 24 h growth period. The growth profile was similar to that in Figs. 7 and 8. Again, the lowest growth was at 10° and 15°C, reaching 22 400 and 50 500 cfu.mL⁻¹, respectively, after 24 h. Even though these were the lowest *E.coli* values observed at all the temperatures, they were still higher in comparison to the population values at

all the temperatures when the COD was only 7 mg.L^{-1} (Fig. 2). This indicated that although the temperature was low, when the COD:N:P level is more than what was required, the population would still reach higher values after 24 h. A large growth increase was observed after 12 h at 30°C , growing from a population of $2\,420\,000 \text{ cfu.mL}^{-1}$ at 12 h to $4\,800\,000 \text{ cfu.mL}^{-1}$ at 24 h. The largest growth increase was observed after 12 h at 35°C , growing from a population of $2\,760\,000 \text{ cfu.mL}^{-1}$ at 12 h to $7\,400\,000 \text{ cfu.mL}^{-1}$ at 24 h. It was found by Vrede *et al.* (2004) that when bacteria were exposed to nutrients having C:N and C:P ratios much higher than what it requires, the microbes had a lower growth efficiency for carbon metabolism which at first will result in a lower growth rate. As expected the highest population increases were after 24 h at 35°C , with no die-off observed, probably since enough nutrients were available to continue growth after 12 h.

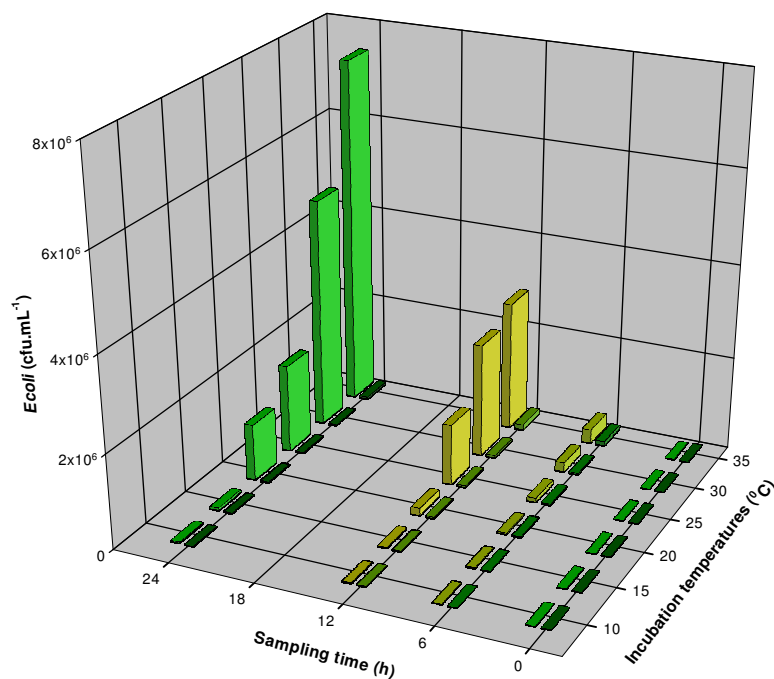


Figure 9 Growth of *E.coli* at the COD:N:P ratio of 24:2:0.3 found in the original river water (right columns of each row), compared to the adjusted COD:N:P ratio of 350:38:3.0 (left columns of each row). The initial cell concentration was 885 cfu.mL^{-1} .

Competing microorganisms

After determining the impact of different COD:N:P ratios on *E.coli* growth, it was necessary to determine if the organisms “naturally” in the river water have an effect on the growth of *E.coli* at these different COD:N:P ratios. In river water there are organisms “naturally” present and *E.coli*, which is not normally present in unpolluted waters, has to constantly compete with these other organisms for survival. These “natural” organisms can be considered to be well adjusted to their habitat unlike *E.coli*, which is a “natural” inhabitant of the gastrointestinal tracts of animals and humans (Warriner & Namvar, 2010). In this study it was assumed that when *E.coli* enters the

rivers it is to a new environment that differs in terms of the nutrient status and temperature and can thus then be considered to be a stressed environment for *E.coli*.

Impact of competing organisms at a “lower” carbon (COD) level - growth profile

It was firstly necessary to determine the growth profile of the competing organisms and *E.coli* at a “low” COD level of 36 mg.L^{-1} (Fig. 10). The *E.coli* had the lowest growth at 10°C and reached the highest population at 35°C . At 12 h, the *E.coli* reached its highest population value of $380\,000 \text{ cfu.mL}^{-1}$, after which the population started to die-off probably due to nutrient limitation and the inability to compete with the “natural” organisms. In comparison the ACC also had the lowest growth at 10°C and the highest at 35°C . After 12 h has passed, the ACC also reached its highest population value of $1.27 \times 10^6 \text{ cfu.mL}^{-1}$, at 35°C . However, after 12 h a drastic decline was observed, especially at 30° and 35°C . A possible reason for this might be a nutrient limitation, as microbes die-off faster until the carrying capacity of the environment is reached (Easton *et al.*, 2005). Quorum sensing, where organisms have the ability to control their own numbers in a population, might also be another explanation for the drastic die-off (Easton *et al.*, 2005). It was therefore concluded that the ACC have the ability to grow to higher population levels than *E.coli* at all the temperatures, especially at 35°C , and that major die-off also occurs when the COD:N:P levels is below the optimum level.

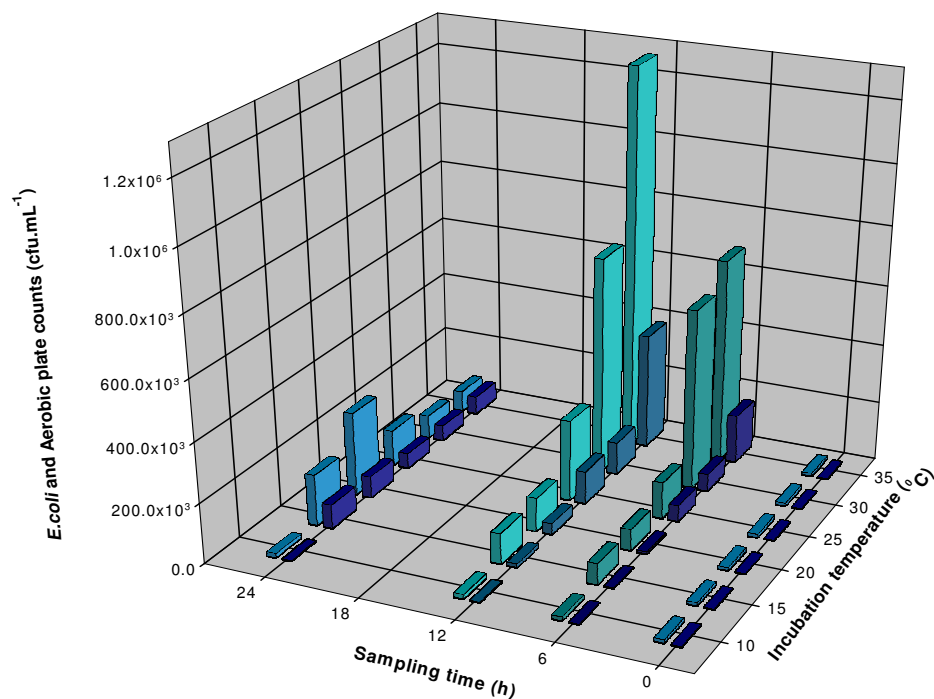


Figure 10 Comparison of the *E.coli* (right columns of each row) and Aerobic plate counts (left columns of each row) growth at a COD:N:P ratio of 36:4:0.2. The initial cell concentrations of *E.coli* and ACC were 930 and $10\,300 \text{ cfu.mL}^{-1}$, respectively.

Impact of competing organisms at “optimum” carbon (COD) level - growth profile

After it was found in the previous trial that the COD was too low to sustain optimum microbial growth, the COD was adjusted to an “optimum” level (108 mg.L^{-1}) to see whether the ACC will die-off after 12 h or whether growth can be sustained for in 24 h. (Fig. 11). The initial ACC and *E.coli* counts were $6\,950$ and 155 cfu.mL^{-1} , respectively, before being exposed to the various temperatures for 24 h. *E.coli* increased at all the temperatures, even after 12 h. The reason that there was no decline is that the nutrient levels available for the *E.coli* was probably at an optimum level. At the optimum temperature of 35°C , the *E.coli* reached a value of $495\,000 \text{ cfu.mL}^{-1}$ after 24 h. This is a large *E.coli* population, but in contrast the ACC was almost 14 times higher and reached a population value of $6\,900\,000 \text{ cfu.mL}^{-1}$. It was concluded that the ratio 108:10:1.0 is the ratio necessary for optimum growth of *E.coli* and ACC, with no die-off occurring at any of the temperatures in 24 h. It was again found that the ACC was higher than the *E.coli* population and that for both microbial groups, higher population levels are reached with an increased COD value.

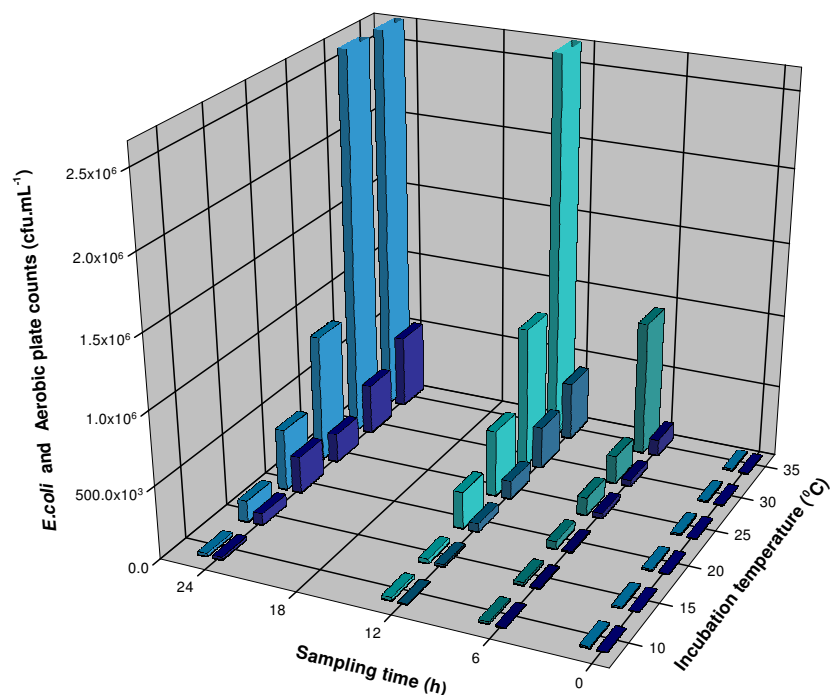


Figure 11 Comparison of the *E.coli* (right columns of each row) and Aerobic plate counts (left columns of each row) growth at a COD:N:P ratio of 108:10:1.0. The initial cell concentration of *E.coli* and ACC were 155 and $6\,950 \text{ cfu.mL}^{-1}$, respectively.

Impact of competing organisms at “extremely high” carbon (COD) level - growth profile

This trial was done to determine how an “extremely high” COD level will impact the population of the ACC in comparison to *E.coli*, as it was previously found to increase with increasing COD levels. The COD:N:P ratio of 24:2:0.3 was adjusted to 350:38:3.0. The growth profile for the *E.coli* and ACC in Fig. 12 is similar to the growth profile in Fig. 11, with no die-off occurring after

12 h probably due to the higher concentration of nutrients ($\text{COD} = 350 \text{ mg.L}^{-1}$) available. The data showed that the ACC and *E.coli* population in Fig. 12 reached higher levels compared to Fig. 11, which can be ascribed to the higher COD level. The highest values for the *E.coli* and ACC occurred at 35° after 24 h, reaching values of 7×10^6 and $12 \times 10^6 \text{ cfu.mL}^{-1}$, respectively. This shows that there were enough nutrients (carbon, nitrogen and phosphate) available and that the *E.coli* was able to grow and survive well in the presence of other organisms under these conditions.

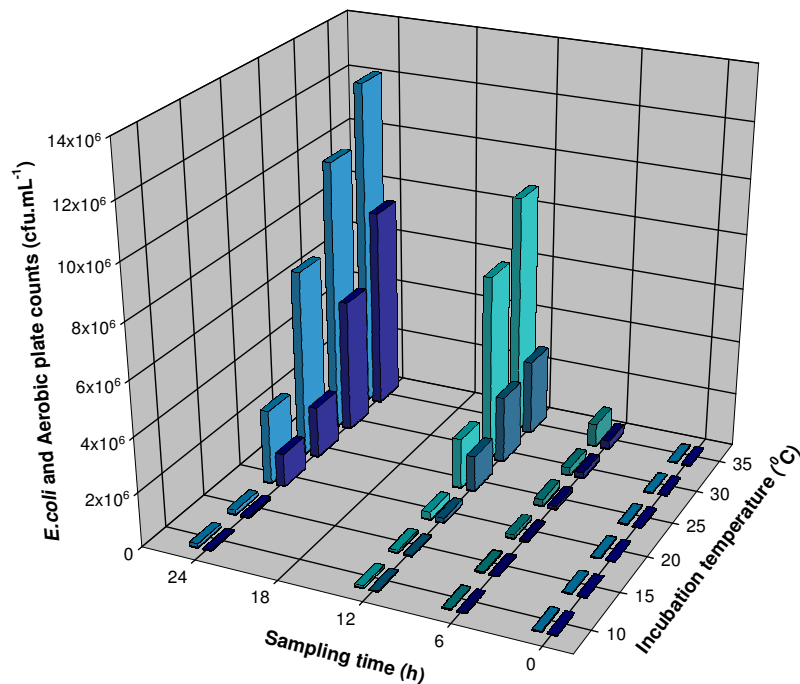


Figure 12 Comparison of the *E.coli* (right columns of each row) and Aerobic plate counts (left columns of each row) growth at a COD:N:P ratio of 350:38:3.0. The initial cell concentration of *E.coli* and ACC were 885 and 19 400 cfu.mL^{-1} , respectively.

There are a number of possible reasons why the ACC will normally be higher than the *E.coli* in polluted river water, firstly because *E.coli* must constantly compete for nutrients with the “natural” organisms. Since the “natural” organisms are mostly in abundance in this sort of environment, *E.coli* is not able to utilise the majority of the nutrients and therefore starts to die-off. Lim & Flint (1989) reported that *E.coli* is unable to compete successfully with the “natural” bacteria and therefore they start to die-off.

Another reason could be that the “natural” organisms might have utilised the available carbon better than *E.coli*. *E.coli* might also be consumed by the “natural” bacteria, as they could have a predator/prey relationship (Bogosian *et al.*, 1996; Easton *et al.*, 2005). When the *E.coli* starts to die-off the cells could provide more nutrients for the natural bacteria to grow, leading to higher growth increases in the population (Bogosian *et al.*, 1996).

Vital *et al.* (2008) reported that at 30°C “natural” bacterial counts were two to four times higher than that of *E.coli*. It was explained that the cell size of *E.coli* in the stationary phase is bigger than the “natural” organisms, therefore requiring more nutrients (carbon) to form new *E.coli* biomass. Vital *et al.* (2008) found that this will lead to the *E.coli* cell yield being five times lower than that for the “natural” organisms. Although *E.coli* utilised 20 - 66% of the assimilable organic carbon (AOC) it was still lower than the “natural” bacteria, because *E.coli* might have used the majority of the available carbon for maintenance energy production and less for biomass synthesis. Taking these findings into consideration it could be the explanation why the *E.coli* was always a lot lower than the ACC at the higher temperatures.

Vital *et al.* (2008) also reported that when exposed to very low assimilable organic carbon (AOC) levels such as 10 µg.L⁻¹, *E.coli* was unable to grow; where as the natural bacteria were able to grow to 1 x 10⁵ cfu.mL⁻¹. This is another explanation why the ACC were always higher, even though the nutrients available were limited. The ACC could have been able to utilise the carbon sources more effectively, resulting in larger numbers.

Lim & Flint (1989) found that nitrogen (ammonium sulphate and amino acids) can increase the survival of *E.coli* in the presence of other organisms. Although phosphate plays an important role in the cell structure and metabolism of *E.coli*, it has been found not to increase the survival of *E.coli* in fresh water.

It is therefore concluded that the “natural” bacteria will always be present in higher levels compared to *E.coli* and can grow to higher population levels. The presence of these organisms could explain the *E.coli* die-off after 12 h at lower COD values. From the above data it is clear that carbon is a major limiting growth factor of *E.coli* and “natural” organisms, with the highest growth increases occurring at 35°C. However die-off is more prominent at 35°C after 12 h at lower carbon values, but when increased to optimum and higher values, the numbers of *E.coli* and “natural” organisms carried on increasing after 12 h. If the *E.coli* is able to grow without the “natural” organisms constantly competing for the nutrients, it is possible that they will grow to higher numbers as there will be more nutrients available for utilisation.

Growth of the “pure” *E.coli* culture

After studying the impact of competing organisms on the growth of *E.coli*, a trial was done to determine the growth of *E.coli* without the influence of other organisms. To determine how *E.coli* would grow and survive without the inference of competing microorganisms, a standard reference *E.coli* 58 (ATCC 11775) strain was inoculated into a sterile carbon (COD) adjusted river water sample. The COD of the river water was adjusted from 12 mg.L⁻¹ to the COD values required (102, 160 and 300 mg.L⁻¹). It was found that the *E.coli* 58 did not show any growth increases in the river water at any of the three adjusted COD:N:P levels or different temperatures (results not

shown). These results were unexpected, since a similar trial done by Hendricks (1972), with the same *E.coli* strain (ATCC 11775) also inoculated in sterile river water with sufficient nutrients showed the highest growth at 30 °C and only little growth at 20 °C (Hendricks, 1972).

An explanation for the results obtained for this trial might be that the *E.coli* reference strain used had been in the laboratory for more than 30 years and probably lost its ability to grow under these more severe environmental conditions. To compensate for this problem, the *E.coli* 58 strain was then inoculated into MRS broth (Merck) which was adjusted to COD values from 90 mg.L⁻¹ to 420 mg.L⁻¹. MRS broth is considered a rich growth medium (Merck Manual, 2005).

In Fig. 13 the growth pattern of the reference *E.coli* 58 strain in 90 mg.L⁻¹ COD MRS is illustrated. From 10° - 30 °C there was a gradual increase with no decline in numbers after 12 h. Major growth increases occurred after 6 h at both 30° and 35 °C. The largest growth increase was found at 35 °C, which is the optimum growth temperature for *E.coli*. The *E.coli* strain grew from an initial cell count of 465 cfu.mL⁻¹ to reach 9 x 10⁶ cfu.mL⁻¹ after 24 h at 35 °C, with no die off. This was ascribed to the presence of no competing organisms and sufficient nutrients available for utilisation.

A similar growth profile (Fig. 14) where the COD had been adjusted to 420 mg.L⁻¹ was found. The initial cell concentration of 515 cfu.mL⁻¹ reached a maximum population value of 182 x 10⁶ cfu.mL⁻¹ at 12 h at 35 °C. Thus, for these high carbon conditions it was concluded that the COD had a major impact on the *E.coli* growth, as the *E.coli* numbers were higher (20 times) at a COD of 420 mg.L⁻¹ compared to the numbers at the lower COD of 90 mg.L⁻¹. However, major die-off occurred at 35 °C from 12 to 24 h, as *E.coli* reached a final value of 5 350 000 cfu.mL⁻¹.

The growth profile (Fig. 14) showed that *E.coli* can grow well in the absence of other competing microorganisms, since more substrate is available for utilisation. These findings were similar to a trial done by Bogosian *et al.* (1996), who reported that *E.coli* survived better in sterile compared to non-sterile river water at 37 °C. Lim & Flint (1989) also found that *E.coli* has a rapid decline in unfiltered lake water compared to that of filtered lake water.

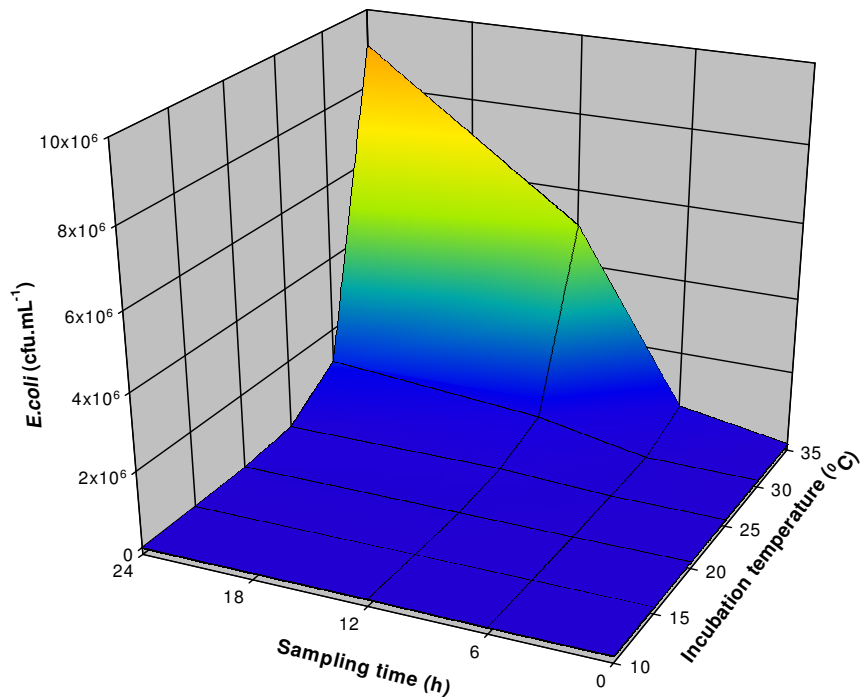


Figure 13 Growth of the “pure” culture *E.coli* 58 (ATCC 11775) in MRS broth with a high COD:N:P ratio of 90:9:1.6. The initial cell concentration was 465 cfu.mL⁻¹.

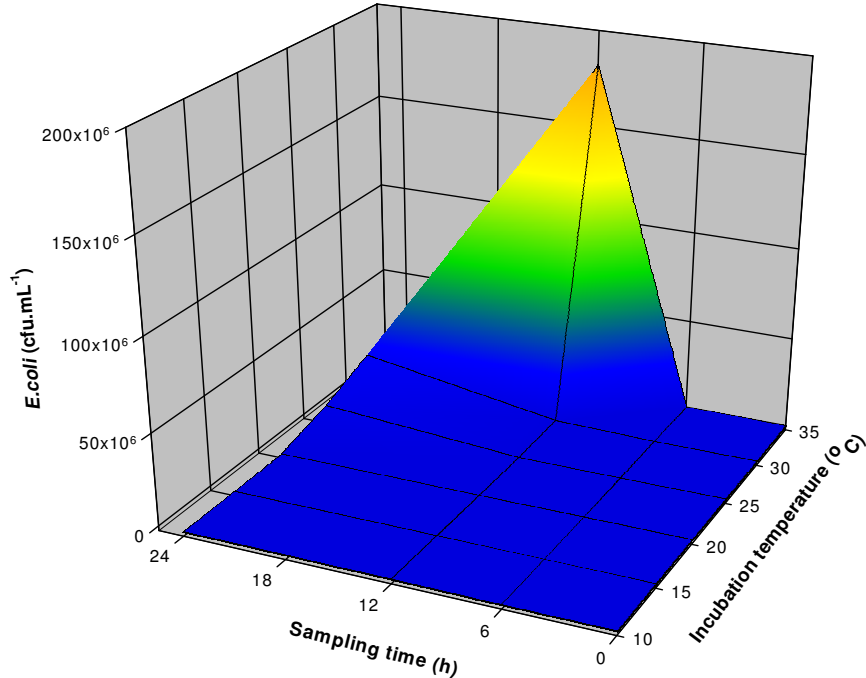


Figure 14 Growth of the “pure” culture *E.coli* 58 (ATCC 11775) in MRS broth with a very high COD:N:P ratio of 420:41:4.3. The initial cell concentration was 515 cfu.mL⁻¹.

CONCLUSIONS

From the results in this trial it is clear that an increase in temperature leads to an increase in the growth of *E.coli* in non-sterile river water. At temperatures of 10° - 20°C, limited growth was observed, which indicates that lower temperatures do not lead to major growth impacts. However, if there are enough nutrients available, the *E.coli* will grow to relatively high values in a 24 h period at these temperatures. No die-off was observed at the lower temperatures of 10° and 15°C after 12 h, even when the COD was low. This was due to the slower metabolisms and growth rate at the lower temperatures, leading to a longer survival period. Temperatures of 25° - 30°C were found to lead to major growth increases, with the optimum temperature of 35°C giving the highest numbers.

It was also clear that after 12 h *E.coli* started to die-off at higher temperatures when there were nutrient limitations. This is a clear indication that the carbon (COD) is a major limiting factor for *E.coli* growth. The higher COD values resulted in major growth increases, with no die-off occurring during the 24 h period. When the COD:N:P ratio was adjusted to the optimum level the lowest value showed no die-off, indicating the significance of the Goldman optimum ratio (Goldman *et al.*, 1987; Enriquez *et al.*, 1993).

Competing microorganisms, which are “naturally” present in river water will limit the growth of *E.coli* as there is a constant competition for nutrients. These organisms were more successful competitors than *E.coli* and utilised the available substrates more efficiently. In this trial it was found that competing microorganisms were always in abundance and grew to much higher numbers compared to *E.coli*. However, *E.coli* did grow very well in river water, even though higher population values were found when there were no other competing microorganisms present. The growth of the “pure” culture gave a clear indication what a major influence optimum temperature and especially nutrients had on the growth of *E.coli*.

It became clear from this trial that carbon (COD) has a major impact on microbial growth as the data showed that COD levels can limit or increase the growth of *E.coli* and other organisms. It was also found that a general increase in river temperatures will impact the microbial load, especially when the water is polluted (high microbial loads vs. high COD), which will have an impact on food safety if the water is to be used for irrigation of fresh produce.

From the results found it is clear that environmental factors do have an impact on the growth of *E.coli* in river water, especially the nutrient status of the water. *E.coli* can multiply to significantly high numbers in the water when there are enough nutrients available and temperatures are high (25° - 35°C). With water temperatures increasing due to global warming, rivers getting more polluted and general water sources becoming scarcer, a shortage of good quality water for domestic and irrigation purposes might be the result in the future if situations do not change.

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CHAPTER 4

DETERMINATION AND CONFIRMATION OF *E.COLI* CARRY-OVER FROM CONTAMINATED IRRIGATION WATER TO GREEN BEANS

SUMMARY

Contaminated fresh produce related outbreaks have increased over the years, due to various factors including contaminated irrigation water. Faecally polluted irrigation water could contain pathogenic *E.coli* that could be transferred to fresh produce and possibly lead to serious infections and diseases. To determine the carry-over and linking of *E.coli* from irrigation water to fresh produce, green beans were planted in a tunnel and irrigated. In the first trial the green beans were irrigated on a daily basis (14 days) with Plankenburg River water and a “pure” culture *E.coli* at a higher concentration of 10^6 cfu.mL⁻¹. Minimum carry-over was observed during the first few days of irrigation, followed by larger *E.coli* numbers of up to 87 000 cfu.mL⁻¹ and decreases from day 8 onwards. The increase in the *E.coli* concentration (10^6 cfu.mL⁻¹) of the irrigation water and the elimination of competing organisms resulted in higher carry-over loads, up to 4.5×10^5 cfu.mL⁻¹. In the second trial the green beans were irrigated once with a “pure” culture *E.coli* at 10^3 and 10^4 cfu.mL⁻¹. *E.coli* only survived at very low concentrations (<70 cfu.mL⁻¹) for a day after being irrigated with 10^3 cfu.mL⁻¹ and 2 days with 10^4 cfu.mL⁻¹.

Colonies were selected from VRBA plates of the river irrigation water (75 isolates) and green beans (67 isolates) and subjected to further phenotypical analysis. Based on the API 20E the species that were found most frequently were *E.coli*, *Enterobacter cloacae* and *Enterobacter asburiae*. These results represented the first evidence of linking. PCR confirmed 52 strains as *uidA* positive, indicating these strains are all *E.coli*. These strains included two strains previously identified as *Citrobacter freundii* with API 20E. Multiplex PCR amplification of intestinal virulence genes confirmed the presence of four EPEC strains isolated on various days from the river water, indicating the river water is a potential source for infections and diseases. Phylogenetic group identification was performed on selected *E.coli* strains from the river water and green beans to determine what the possible source of the faecal pollution might be. The majority of the strains clustered in phylo-group B2₃, which could be an indication of human faecal pollution.

Neighbour-joining tree analysis of *oriC*-locus sequences showed no bootstrap support and was unable to support linking. The *dnaJ* sequence clustering had significant bootstrap values, identifying different clades of *E.coli*, *E.cloacae* and *Klebsiella pneumoniae*. Linking of the environmental strains based on the *dnaJ* sequences could not be conclusively confirmed, however, carry-over of *E.coli* from the river water to the green beans appear to be highly probable. This study showed the importance of performing phenotypical characterisation in combination with molecular analysis to indicate whether carry-over did indeed occur.

INTRODUCTION

Fresh produce, leafy greens, tomatoes, herbs and cantaloupes are the leading source of *E.coli* outbreaks and the second highest source of food borne disease outbreaks (Franz & Van Bruggen, 2008; Mandrell, 2009). *E.coli* O157:H7 (EHEC) outbreaks are also common, especially as the result of consuming leafy greens (Mandrell, 2009). The largest outbreak of bacterial enteric disease occurred in 1996 when *E.coli* O157:H7 contaminated radish sprouts resulting in 9 451 cases, 7 900 hospitalisations and 12 deaths in Japan (Michino *et al.*, 1999). The biggest outbreak regarding *E.coli* O157:H7 in the USA occurred in 2006, when contaminated, packaged baby spinach resulted in 206 infections and three deaths (Buchanan, 2006). The most recent *E.coli* outbreak occurred in Germany, with >4 000 cases, 852 patients with hemorrhagic uremic syndrome (HUS) and 32 deaths due to *E.coli* O104:H4 contamination on raw sprouts (CDC, 2011).

There are numerous sources that could lead to pre-harvest contamination of fresh produce. These include faecally contaminated irrigation water, animal excretions, water used to apply fungicides and pesticides, inadequately composted manure, birds and insects as well as human handling (Bach & Delaquis, 2009). River water can easily get faecally contaminated, which has frequently been the cause of the fresh produce contamination as it is the main source used for irrigation purposes. Various studies on the quality of South African rivers indicated an increase in faecal pollution levels (Barnes & Taylor, 2004; Paulse *et al.*, 2009; Lötter, 2010). The WHO guideline states that if water is to be used for irrigation it should not exceed 1 000 faecal coliforms per 100 mL (WHO, 1989) as various studies have shown that *E.coli* at higher concentrations are carried over to fresh produce (Solomon *et al.*, 2002; Okafo *et al.*, 2003; Amoah *et al.*, 2005; Rai & Tripathi, 2007).

Many factors can affect the survival and growth of pathogens on fresh produce. Injured tissue could supply the pathogens with sufficient moisture and nutrients that are favourable for growth (Harapas *et al.*, 2010). The external skin texture can furthermore influence microbial growth, with hairy or rough skin textures providing more favourable conditions as growth environment (Jackson *et al.*, 2009). The age of the produce is also important, since younger produce has more nutrients present, which have shown to increase *E.coli* growth (Brandl & Amundson, 2008). Lastly, the area of the produce exposed to irrigation water is also a major factor, as a larger area can contain more pathogens (Amoah *et al.*, 2006).

It is important to identify the potential source of faecal pollution in river water, because if one can identify the source, it might be possible to eliminate it (Field & Samadpour, 2007). Even though standard methods have made it easier to identify whether irrigation water is faecally contaminated, it is still difficult to identify the specific contamination sources using conventional techniques (Carroll *et al.*, 2009). Combinations of microbial source tracking methods, based on phenotypic, genotypic or chemical characteristics (Moore *et al.*, 2005; Seurinck *et al.*, 2005) can facilitate identifying the sources of contamination.

Carlos *et al.* (2010) found that a triplex PCR method, where *E.coli* strains are classified into phylo-groups could be useful as a source tracking method, as each phylo-group could represent a potential source of contamination. Clermont *et al.* (2000) developed a genotyping method, where based on phylogenetic group analysis, *E.coli* can be divided into four main groups, A, B1, B2 and D with further sub-divisions A₀, A₁, B₁, B₂, B₂₃, D1 and D2. These groups differ in the presence of virulence factors, ecological niches and life-history (Gordon *et al.*, 2008; Carlos *et al.*, 2010). The strains will therefore also differ regarding phenotypic characteristics which include antibiotic-resistance profiles, growth rate-temperature relationships and their ability to utilise different carbohydrates (Gordon *et al.*, 2008). Commensal *E.coli* is usually associated with group A and B1. Intestinal pathogens also belong to group A, B1 as well as group D, while extraintestinal *E.coli* are mostly associated with group B2 and to a lesser extent group D (Clermont *et al.*, 2000; Houser *et al.*, 2008; Carlos *et al.*, 2010).

The main problem is that contaminated irrigation water can impact food safety and a study under controlled environmental conditions (outdoors) is needed to determine if *E.coli* is carried over to produce. It is also important to know at what level carry-over takes place when irrigated on a daily basis with contaminated irrigation water. Additionally the *E.coli* in the irrigation water should be confirmed to be identical to the *E.coli* strain present on the fresh produce in order to prove carry-over.

The main objective of the study is to determine the carry-over and linking of *E.coli* from contaminated irrigation water to fresh produce. The study will focus on: the effect of daily irrigation on carry-over; effect of "once-off" irrigation on the survival of *E.coli* on the produce; identifying different types of *E.coli* in the irrigation water and those present on the irrigated fresh produce; linking different *E.coli* types in the irrigation water to those present on the irrigated produce; and using the information from the above steps to identify the faecal pollution source of the irrigation water.

MATERIALS AND METHODS

Site selection

A suitable site for the plot studies was identified as a farm situated in the Devon Valley Stellenbosch, South Africa (Carinus, P.J., 2010, Manager, Champagne, Devon Valley, Stellenbosch, personal communication, 20 September). The selected site chosen was close to an irrigation source (tap - Teewaterskloof) that could provide the necessary water during the growth phase of the green beans. The area had no history of grazing animals.

Plot layout

A “hobby” vegetable tunnel of 18 m² (6 m x 3 m) (Rhino Plastics, Cape Town) was set up to minimise direct UV-rays, excessive winds and rain and other possible contaminants to come into contact with the beans (Fig. 2). Star 2000 green beans (Starke Ayres) were planted in four small sub-plots (1.5 m x 2.6 m) (Fig. 3a) consisting of four rows each (Fig. 3b). Spaces were left open between the sub-plots to minimise cross-contamination.



Figure 2 The hobby tunnel set up to minimising the influence of environmental factors.

(a)



(b)



Figure 3 Four small green bean plots (a) consisting of four rows each (b) were planted in the hobby tunnel. Spaces were left open between the four plots to minimise cross-contamination.

Design of experimental treatments

The experimental design for the two plot studies is shown in Fig. 4 and 5. The aim of study was to determine the level of *E.coli* carry-over if the green beans are irrigated on a daily basis. For the first plot study (Study I) two irrigation treatments were applied, which included “pure” *E.coli* culture irrigation water and Plankenburg River irrigation water. The *E.coli* numbers were enumerated for the “pure” culture and the river irrigation water as well as for the green beans from the plots. Colonies from the plates were purified, characterised and subjected to further molecular analysis (Fig. 5).

The aim of study II (Fig. 4) was to determine the survival of *E.coli* on green beans after “one-off” irrigation. Two of the sub-plots were irrigated only once with the “pure” culture *E.coli* irrigation water at a cell concentration of 10^3 cfu.mL⁻¹ and 10^4 cfu.mL⁻¹, respectively. The *E.coli* numbers of the irrigation waters and the green beans of the plots, were also determined, the isolates selected, purified, characterised and subjected to further molecular analysis (Fig. 5).

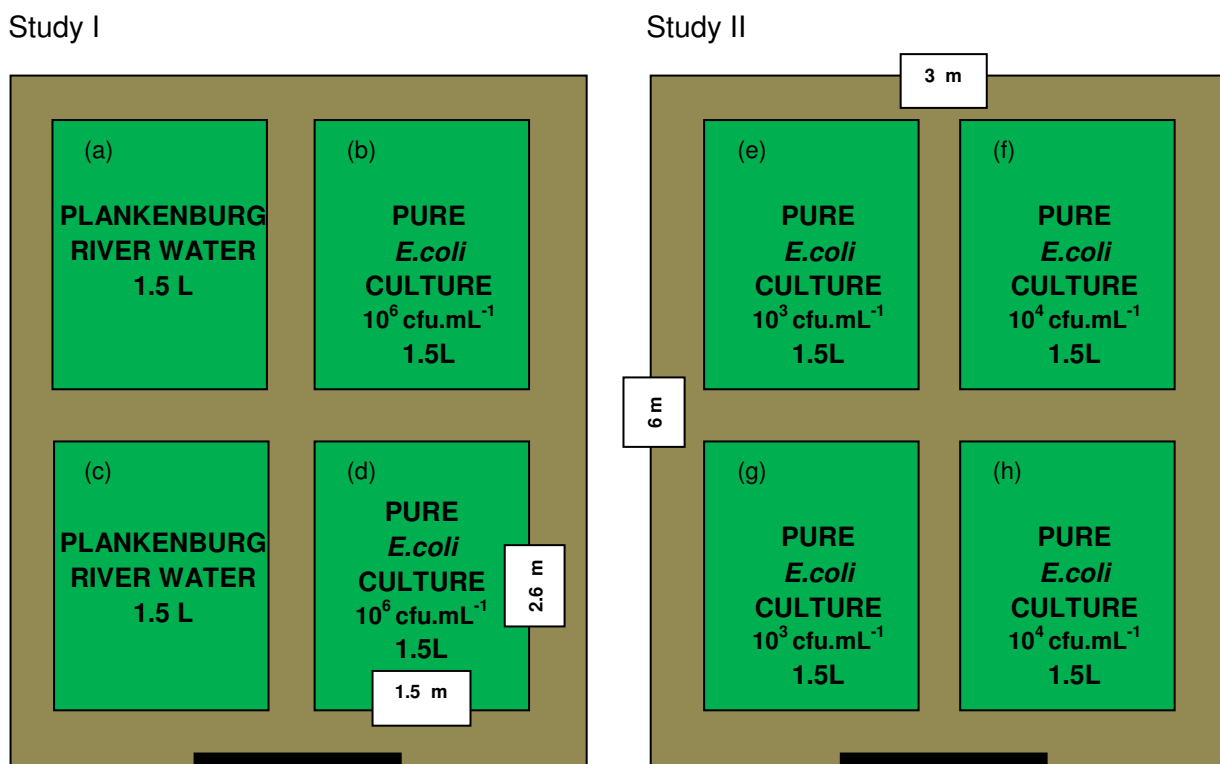


Figure 4 Schematic illustration of the plot layouts (Studies I and II). The sub-plots (a and c) of Study I received irrigation water treatments with Plankenburg River water and sub-plots (b and d) received the “pure” *E.coli* culture irrigation water (10^6 cfu.mL⁻¹). The sub-plots of Study II were “once-off” irrigated with “pure” *E.coli* culture irrigation water of 10^3 cfu.mL⁻¹ (e and g) and 10^4 cfu.mL⁻¹ (f and h), respectively.

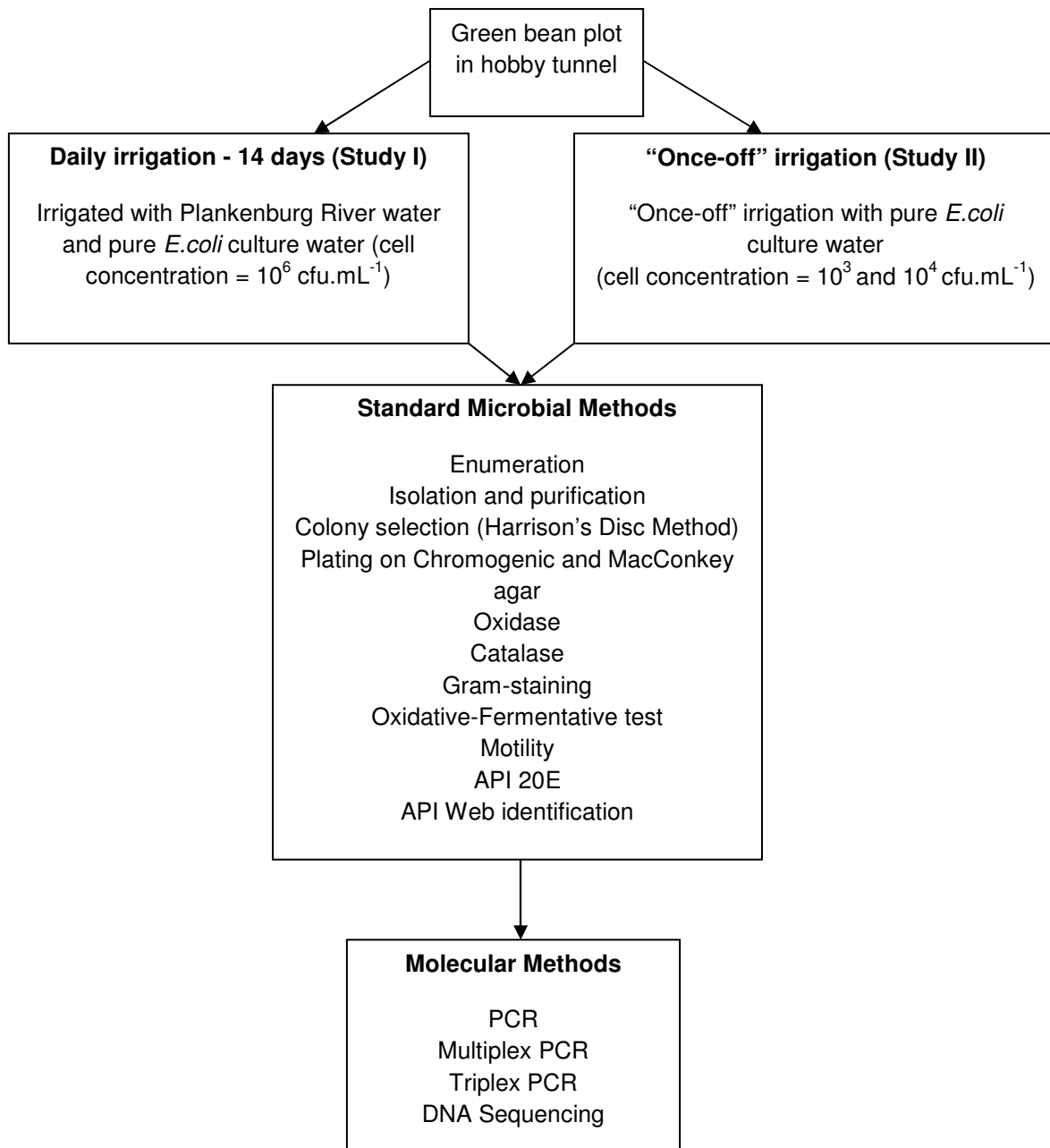


Figure 5 Experimental design of the two separate plot studies (Study I and II) and analyses done.

Irrigation before experimental treatments

Before the irrigation treatments for the study started, the green bean plots were drip irrigated (Fig. 6) daily for 1 h with clean uncontaminated water (0 cfu.mL⁻¹ *E.coli*). No form of overhead irrigation was used during this initial growth phase so as to prevent contact with the leaf and bean surfaces.



Figure 6 The drip irrigation system used to irrigate green beans before irrigation with *E.coli* count water began.

Plot irrigation of Study I and II

In Study I (14 day plot study) two irrigation treatments were used: The first was with river water from the Plankenburg Site 1 (Lötter, 2010) and the second, sterile physiological saline solution (PSS) inoculated with the “pure” *E.coli* strain (10^6 cfu.mL⁻¹). The layout of the irrigation treatments and volumes applied, are illustrated in Fig. 5. Special precautions were taken to prevent cross-contamination during the treatment sessions. Day 0 of the trial served as the control as the green beans were not irrigated with the *E.coli* water, followed by the daily irrigation sessions.

The river water samples (3 L) for the plots were applied using a sterile pressure sprayer. For microbial analysis, a further litre of river water was taken. The samples were taken in sterile Schott bottles, 30 cm below the surface facing the direction of the flow, according to the method described by SANS 5667-6 (2006). The samples were placed on crushed ice and laboratory analysis started within 2 h.

The “pure” non-pathogenic “natural” *E.coli* that was previously isolated from the Plankenburg River was obtained from the Department of Food Science, University of Stellenbosch, and used for the “pure” culture treatments. The culture was prepared on a daily basis and inoculated in MRS broth (Merck) for 24 h at 37°C to reach a final *E.coli* concentration of 10^6 cfu.mL⁻¹. The concentration was determined by measuring the broth turbidity using a Spectronic 20 Genesys™ spectrophotometer. The averaged turbidity value was used in the *E.coli* 58 growth curve equation ($y = 2.3x + 7.533$) to simulate the cfu.mL⁻¹ of the *E.coli* in the broth. A dilution series up in the MRS broth was prepared and added to the sterile PSS for the 14 day plot study.

In this study the plots were irrigated (3 L) only once with a “pure” *E.coli* strain in sterile PSS at cell concentrations of 10^3 and 10^4 cfu.mL⁻¹, respectively (Fig. 5).

Sampling of green beans

Green beans (300 g) were harvested daily from each sub-plot, 24 h after the previous day's treatment. The green beans harvested on the first day served as the control sample as no irrigation treatment had been applied. The beans were hygienically and randomly harvested from various plants in each plot and placed into sterile stomacher bags. The beans were weighed to ensure the correct mass, and then carefully hand-washed with 400 mL sterile PSS. To facilitate the analysis procedure the washwater was placed in sterile containers and then used for the microbial analysis.

Enumeration, isolation and identification

E.coli enumeration

The *E.coli* count of the river water, "pure" *E.coli* culture water and the green beans was determined according to the Standard Method APHA (2005). A dilution series was prepared in duplicate in PSS. Violet Red Bile Agar (VRBA) (Merck) was used for the enumeration. The VRBA plates were incubated for 24 h at 30°C (Merck Manual, 2005).

All the red colonies were counted to determine the numbers of *E.coli* present (Merck Manual, 2005). For further analysis representative colonies (red and pink) were statistically selected using the Harrison's disc method (Harrigan & McCance, 1974). The colonies selected were streaked out on VRBA before being transferred to Brilliance™ *E.coli*/Coliform selective agar (Oxoid). This was used to ensure purity and to isolate cultures. Colonies were streaked out a minimum of three times before a single pure colony was selected and cultured in MRS broth (Merck). Cultures were stored at -80°C in 40% glycerol.

Confirmation tests

Gram-staining - Gram staining and morphology was determined for each isolate microscopically (Leitz) (Gerhardt *et al.*, 1981).

MacConkey agar - Cultures were streaked out on MacConkey agar (Oxoid) and incubated at 37°C for 24 h (Gerhardt *et al.*, 1981). Growth on this medium was recorded as a positive result on the API 20E identification strip (Biomérieux, France).

Catalase and Oxidase tests - The catalase test was performed according to Gerhardt *et al.* (1981). Bubble formation was recorded as a positive result. The oxidase test was done according to Gerhardt *et al.* (1981) and a purple colour change was recorded as a positive result.

Oxidative-Fermentative (O-F) test - The O-F test was prepared according to Gerhardt *et al.* (1981). All the tubes were incubated for 48 h at 35°C. A yellow colouration in the aerobic and anaerobic incubated tubes was an indication of fermentative degradation. A yellow colouration in the aerobic incubated test tubes was an indication that the glucose were broken down by oxidation.

Motility - Motility was determined by using a hanging drop slide (Gerhardt *et al.*, 1981) and inspected microscopically (Leitz). Motility was recorded as a positive result.

API 20E - After the completion of the basic characterisation tests, all strains were subjected to the API 20E strip identification system (Biomerieux), according to the specific API instructions provided.

Numerical analysis

For the numerical clustering, the characteristics of the 162 strains composed of 72 isolates from the irrigation water (river water), 67 from the irrigated beans, 19 isolates from the “pure” culture studies (irrigation water and green beans) and four ATCC reference strains (ATCC 13135(=404), 10799(=158), 11775(=58) and 4350(=157) were included. Twenty-seven characters were included in the data set and analysed using the Sokal & Michener (SM) coefficient 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. Dendrogramme distances were calculated based on the phenotypic characteristics as calculation concept (Lockhart & Liston, 1970). (Lockhart & Liston, 1970)

Molecular methods

DNA template preparation

The DNA was extracted by first cultivating the pure bacterial strains on TSA (Oxoid) for 24 h at 37°C. A loopful of each culture was transferred to a 1.5 mL microcentrifuge tube containing 100 µL sterile nuclease-free water and boiled for 13 min. After being cooled on ice, it was centrifuged for 15 min at 14 000 x g after which the supernatant was transferred to a sterile 0.6 µL centrifuge tube and stored at -18°C (Altahi & Hassen, 2009).

uidA PCR

All the isolates from the river water and green beans that tested positive for *Escherichia coli* with API 20E were subjected to *uidA* gene PCR. The detection of the *uidA* gene, a highly conserved *E.coli* gene, which can therefore be used to identify *E.coli* strains on a molecular level. The PCR method is based on the method described by Heijnen & Medema (2006).

Each PCR reaction volume of 25 μL contained 0.4 μM of each primer UAL 1939b (5'-ATGGAATTTGCGCCGATTTTGC-3') and UAL2105b (5'ATTGTTTGCCTCCCTGCTGC-3') (Heijnen & Medema, 2006) to amplify the fragment of the *uidA* gene of 187 bp in size, 2.5 mM MgCl_2 , 1 x KAPATaq hotstart buffer, 0.2 mM of each dNTPs, 0.625 U KapaTaq Hotstart DNA Polymerase and 0.5 μL template DNA.

A positive control containing template DNA of a standard *E.coli* strain was added as well as a negative control that contained nuclease-free water and no template DNA. All the tubes were placed into the G-storm thermal cycler (Vacutec). Reaction conditions included: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 s; primer annealing at 59.7°C for 30 s; extension at 72°C for 30 s; with a final extension step at 72°C for 5 min followed by a 30 s cooling step at 4°C.

The PCR products were analysed using gel electrophoresis in a 1% agarose (SeeKem) gel containing 1 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide (Sigma). Gel electrophoresis was performed at 210 V for 20 min using the Baygene-BG-Power300 power supply. The PCR bands were visualised under a UV light.

Pathotype PCR

The pathotype PCR is a multiplex PCR (m-PCR) which was carried out on *uidA* PCR confirmed *E.coli* strains to determine whether any pathotypes are present. The m-PCR method was based on the method described by Omar & Barnard (2010). The m-PCR had a reaction volume of 12.5 μL , which consisted of 0.2 μM of each primer (Table 1), 1x Qiagen multiplex PCR mastermix, 4.74 μL RNase-free water and 0.25 μL of the template DNA.

A Standard Culture Mix (SCM) was prepared in advance by transferring 2 μL DNA of each of the EIEC, EPEC, EHEC, ETEC and EAEC cultures to a sterile 0.2 mL PCR tube and stored at -20°. A positive control, which contained 1.25 μL of SCM as DNA template, was then included with each batch of m-PCR samples tested. A negative control was also included, which contained RNase-free water and no template DNA. All the tubes were placed in the G-storm thermal cycler (Vacutec). Reaction conditions included: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 45 s; primer annealing at 55°C for 45 s; extension at 68°C for 2 min; with a final extension step at 72°C for 5 min followed by a 30 s cooling step at 4°C.

The PCR products were analysed using gel electrophoresis in a 1.25% agarose (SeeKem) gel containing 1 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide (Sigma). Gel electrophoresis was performed at 120 V for 90 min. The products were visualised under UV light.

Table 1 Primers sequences and expected sizes used for multiplex PCR to detect various *E.coli* pathotypes

| Pathotype | Primer* | Primer sequence (5' - 3') | Size (bp) | Reference |
|--------------------------------|--------------------|------------------------------|--------------|------------------------------------|
| Commensal (<i>mdh</i>) | Mdh01 | GGTATGGATCGTTCCGACCT | 300 | Tarr <i>et al.</i> , 2002 |
| | Mdh02 | GGCAGAATGGTAACACCAGAGT | | |
| EIEC (<i>ial</i>) | L- <i>ial</i> (F) | GGTATGATGATGATGAGTCCA | 650 | Lopez-Suacedo <i>et al.</i> , 2003 |
| | Ial(R) | GGAGGCCAACAATTATTTCC | | |
| EPEC/EHEC (<i>eaeA</i>) | L- <i>eaeA</i> (F) | GACCCGGCACAAGCATAAGC | 384 | Lopez-Suacedo <i>et al.</i> , 2003 |
| | L- <i>eaeA</i> (R) | CCACCTGCAGCAACAAGAGG | | |
| EHEC (<i>stx 1, stx2</i>) | Stx1(F) | ACACTGGATGATCTCAGTGG | 614 | Moses <i>et al.</i> , 2006 |
| | Stx1(R) | CTGAATCCCCCTCCATTATG | 779 | Moses <i>et al.</i> , 2006 |
| | Stx2(F) | CCATGACAACGGACAGCAGTT | | |
| | Stx2(R) | CCTGTCAACTGAGCACTTTG | | |
| ETEC (<i>LT, ST</i>) | LT(F) | GGCGACAGATTATACCGTGC | | |
| | LT(R) | CGGTCTCTATATCCCTGTT | 160 | Omar & Barnard, 2010 |
| | ST(F) | TTTCCCTCTTTTAGTCAGTCAACT | | |
| | ST(R) | GGCAGGATTACAACAAAGTTCACA | | |
| EAEC (<i>eagg</i>) | Eagg(F) | AGACTCTGGCGAAAG ACT GTATC | 194 | Pass <i>et al.</i> , 2000 |
| | Eagg(R) | ATGGCTGTCTGTAATAGATGAGAAC | | |

*F - Forward primer
R - Reverse primer

Triplex Polymerase Chain Reaction (t-PCR)

E.coli strains were selected for t-PCR, based on API 20E and PCR results, to determine what the phylo-group of the strains were. The PCR method was based on the method described by Clermont *et al.* (2000). The t-PCR had a reaction volume of 12.5 μ L, which consisted of 0.2 μ M of each primer (Table 2), 1x Qiagen multiplex PCR mastermix and 0.25 μ L of the template DNA.

Template DNA of a standard *E.coli* strain (ATCC 25922) was used as the marker (positive control). A negative control tube that contained nuclease-free water instead of template DNA was included. All the tubes were placed into the G-storm thermal cycler (Vacutec). PCR reaction conditions were: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 15 s;

primer annealing at 60°C for 30 s; extension at 72°C for 45 s; with a final extension step at 72°C for 5 min.

The PCR products were analysed using gel electrophoresis in a 2% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). Gel electrophoresis was performed 210 V for 30 min according to the instructional manual provided. PCR banding patterns were visualised under a UV light.

Table 2 Primers sequences and expected sizes used for triplex PCR to determine the phylogenetic groups of the *E.coli* isolates (Clermont *et al.*, 2000)

| Primer* | Primer sequence (5' - 3') | Size (bp) |
|----------------|------------------------------|--------------|
| chuA.1 (F) | GACGAACCAACGGTCAGGAT | 279 |
| chuA.2 (R) | TGCCGCCAGTACCAAAGACA | |
| yjaA.1 (F) | TGAAGTGTCAGGAGACGCTG | 211 |
| yjaA.2 (R) | ATGGAGAATGCGTTCCTCAAC | |
| TSPE4.C2.1 (F) | GAGTAATGTCGGGGCATTCA | 152 |
| TSPE4.C2.2 (R) | CGCGCCAACAAAGTATTACG | |

*F - Forward primer
R - Reverse primer

dnaJ sequencing

Strains were randomly selected for sequencing based on the API 20E data, specifically where strains from the irrigation river water and beans were found to be biochemically identical. The PCR method was based on the method described by Nhung *et al.* (2007). The PCR had a reaction volume of 50 µL which consisted of: 0.4 µM of each primer DN1-IF (5'-GATYTRCGHTAYAACATGGA-3') and DN1-2R (5'-TTCACRCCRTYDAAGAARC-3') (Nhung *et al.*, 2007); that amplify the *dnaJ* fragment of 758 bp in size; 2.5 mM MgCl₂; 1 x KAPATaq hotstart buffer; 0.2 mM of each dNTPs; 1 U KapaTaq Hotstart DNA Polymerase; and 1 µL template DNA.

All the tubes were centrifuged briefly before being placed in the G-storm thermal cycler (Vacutec). PCR reaction conditions: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 s; primer annealing at 50°C for 30 s; extension at 72°C for 1 min; with a final extension step of 72°C for 7 min.

The PCR products were analysed using gel electrophoresis in a 2% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). For gel electrophoresis the Baygene-BG-

Power300 power supply was programmed at 120 V for 30 min according to the instructional manual provided (Baygene catalogue). The products were visualised under UV light.

The PCR products were sequenced by the Central Analytical Facility of Stellenbosch University. The DNA sequencing analysis was done using the BigDye™ Terminator V3.1 sequencing kit (Applied Biosystems) based on the manufacturer's protocol with slight modifications. The electrophoresis of the sequences were performed on either the ABI3130xl or ABI3730xl (Applied Biosystems) using a 50 cm Capillary array and POP7 (Applied Biosystems).

oriC-locus sequencing

E.coli strains were randomly selected for sequencing based on the API 20E data. These were strains from the river water and were biochemically identical to the strains on the green beans a few days after. The PCR method was based on the method described by Roggenkamp (2007). The PCR had a reaction volume of 50 µL which consisted of: 0.5 µM of each primer EcOriC-73-f (5'-CACTGCCCTGTGGATAACAA-3') and EcOriC-292-r (5'-TATACAGATCGTGCGATCT-AC-3') (Roggenkamp, 2007); that amplify the *oriC* fragment of 217 bp in size; 2.5 mM MgCl₂; 1 x KAPATaq hotstart buffer; 0.2 mM of each dNTPs; 1 U KapaTaq Hotstart DNA Polymerase; and 1 µL template DNA.

All the tubes were placed in the G-storm thermal cycler (Vacutec). The PCR reaction conditions were: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s; primer annealing at 58°C for 30 s; extension at 72°C for 1:30 min; with a final extension step at 72°C for 15 min.

The PCR products were analysed using gel electrophoresis in a 1% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). Gel electrophoresis was performed at 120 V for 20 min. The products were visualised under UV light.

The PCR products were sequenced by the Central Analytical Facility of Stellenbosch University. The DNA sequencing analysis were done using the BigDye™ Terminator V3.1 sequencing kit (Applied Biosystems) based on the manufacturers protocol with slight modifications. The electrophoresis of the sequences were performed on either the ABI3130xl or ABI3730xl (Applied Biosystems) using a 50 cm Capillary array and POP7 (Applied Biosystems).

Sequencing analysis

The Basic Local Alignment Search Tool (BLAST) was used to identify the generated sequences to sequences that are available in the GenBank library. The phylogenetic tree analysis was done with phylogenetic analysis using parsimony (PAUP) software with 1 000 bootstraps and the neighbour-joining algorithm (Roggenkamp, 2007). The sequences of each gene (*dnaJ* and *oriC*) were used to construct phylograms.

RESULTS AND DISCUSSION

Carry-over of *E.coli* from irrigation water to beans under controlled conditions - 14 days (Study I - plots a and c)

The *E.coli* loads in the Plankenburg irrigation water as well as the effect of the irrigation water 24 h after the daily application onto the green beans is shown in Fig. 6. The *E.coli* counts in the river water varied between 3 900 - 118 500 cfu.mL⁻¹ during the study period. All the counts obtained in this study were above the WHO recommended guideline of 1 000 cfu.100 mL⁻¹ (WHO, 1989), making the river unsuitable for irrigation purposes.

Green beans from plots a and c (Fig. 5) were sampled on day 0, before irrigation with river water began, and served as the control. On day 1, the results showed no *E.coli* on the green beans 24 h after it had been irrigated for the first time (Fig. 6). Previous research reported that when a surface is uncolonised it is difficult for an organism to survive when compared to when it is colonised (Monier & Lindow, 2005). Ackermann (2010) also found that when green beans were exposed to an inoculum of 10² cfu.mL⁻¹, there were no *E.coli* survivors on the green beans, which indicated that a concentration of 10² cfu.mL⁻¹ could be considered as “safe” for *E.coli* in irrigation water.

The counts for days 2 to 4 show that the longer the green beans were exposed to the contaminated irrigation water, the more the *E.coli* counts increased on the green beans (130 - 210 cfu.mL⁻¹). One explanation for this can be that the hairy surface of green beans could help entrap the *E.coli* (Ackermann, 2010). The *E.coli* also has various attachment mechanisms it could have used to attach to the green bean surfaces and hairs (Doyle & Erickson, 2008).

The first major *E.coli* impact on the green beans was found on day 5, when the irrigation water on day 4 had a higher count (50 500 cfu.mL⁻¹) and subsequently the green beans showed an increase in load to 15 250 cfu.mL⁻¹. A small decrease in *E.coli* counts in the irrigation water on day 5 were followed by a similar decrease on the green beans. On day 6 the highest *E.coli* count was observed in the irrigation water (118 500 cfu.mL⁻¹) followed on day 7 to an increased count of 87 000 cfu.mL⁻¹ on the green beans. Overall, the subsequent profile of the *E.coli* numbers on the green beans (days 4 - 7) was similar to the pattern found in the irrigation water (days 3 - 6).

On days 8 and 9 it was however observed that the *E.coli* counts on the green beans were higher than the counts of the irrigation water on days 7 and 8. On day 8 the *E.coli* counts reached

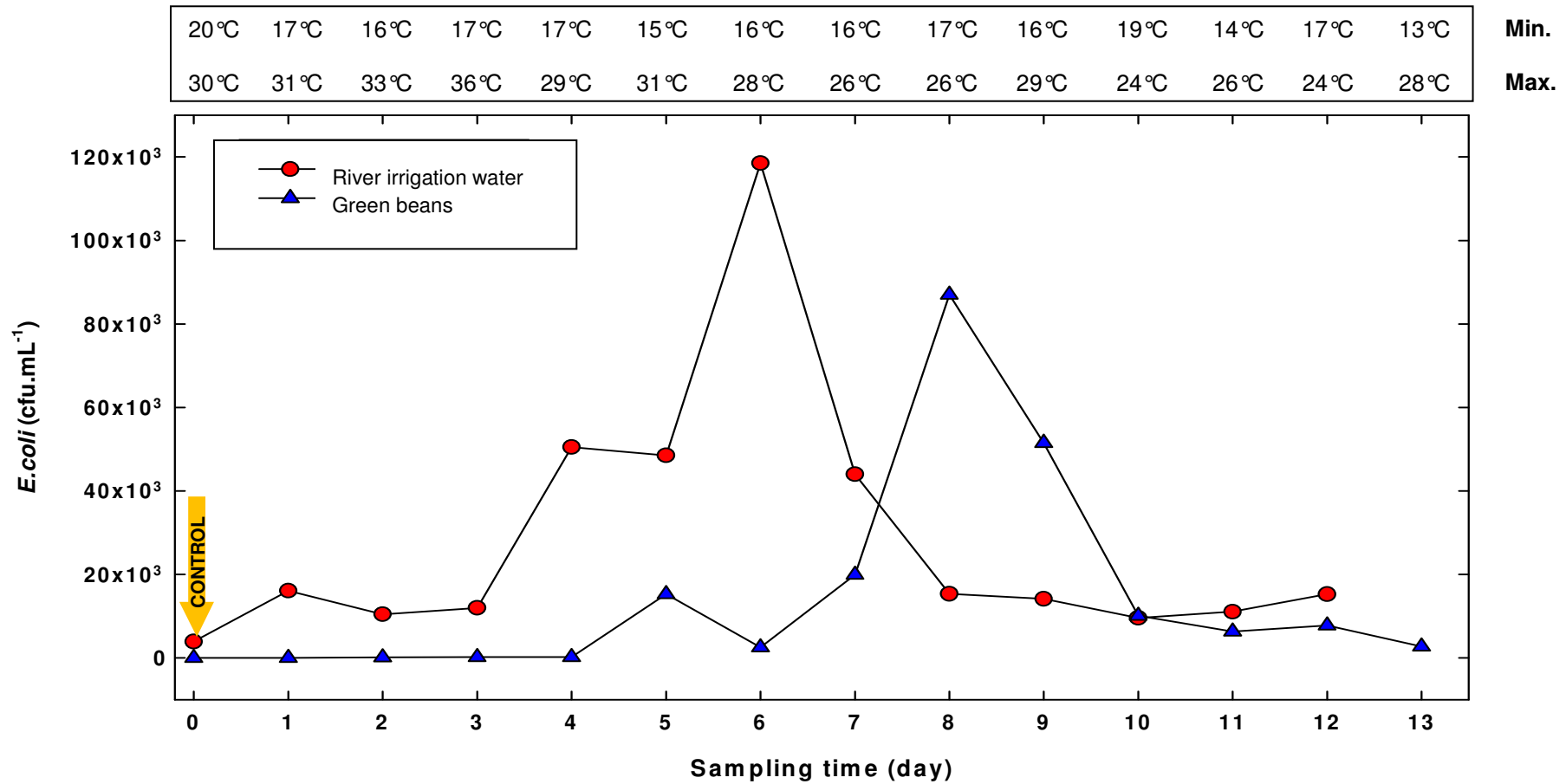


Figure 6 Study I - The 14 day plot study where green beans were irrigated daily for 13 days (days 0 - 12) with water from the Plankenburg River (Plank-1) and harvested 24 h after irrigation (days 1 - 13). Day 0 served as the control as the green beans had not been exposed to the river water before harvest. The *E. coli* values are averages of counts from the two sub-plots and each done in duplicate. The red circles represent the daily *E. coli* counts of the river water and the blue triangles the daily *E. coli* load on the beans 24 h after irrigation. The minimum and maximum environmental temperatures on the day of irrigation were also recorded.

87 000 cfu.mL⁻¹ on the green beans, compared to the irrigation water on day 7 which was only 44 000 cfu.mL⁻¹. The reason for this was the effect of the high count in the irrigation water on day 7. Another reason could be due to the formation of biomass clumps. Ackermann (2010) reported that *E.coli* aggregates to form clumps and even biofilms. Although hard to break apart, once a clump is broken apart it could contain millions of cells, which will lead to a large increase in the number of enumerated cells.

Although the counts on the green beans were still higher on day 9, the loads started to decline to around 10 000 cfu.mL⁻¹ by day 10. The lower and more stable counts of the irrigation water was probably the reason why the green bean counts decreased and then also stabilised.

Other reasons for this variation in numbers may include the impact of natural microbial inhabitants on the beans competing for nutrients and space. It is known that in the river water as well as on green beans there are many natural inhabitants present that can adapt well to the environment on the green beans, leading to the organisms growing better and being able to compete for the nutrients more successfully when compared to *E.coli*. Cooley *et al.* (2006) found that *Enterobacter asburiae*, a plant endophytic bacterium, can limit the growth and survival of *E.coli* on produce. In this study *E.asburiae* strains were isolated on days 4, 8, 9, 11, and 12 (see isolation section) from the green beans, which may be a reason why the *E.coli* counts on the green beans were low and started declining on those days. Another reason might also be due to quorum sensing, where an organism has the ability to regulate its own numbers if the population density is too high (Easton *et al.*, 2005).

Another factor that must be considered in plot studies is that in this study the outside temperature varied between 13° and 36°C over the 14 day study period (Fig. 6). Inside the tunnel the temperatures were found to vary 6° - 8°C higher than the outside temperature. This at times resulted in temperatures reaching more than 40°C inside the tunnel.

Temperature can also affect the bacterial attachment to produce and the type of attachment mechanism used (Ells & Hansen, 2006; Patel *et al.*, 2011). It has previously been found that moisture in combination with warmer and higher temperatures could result in bursts of growth on produce, as also was possible in this study (Doyle & Erickson, 2008). Higher temperatures usually increase the growth rates, resulting in the microbes being able to utilise more nutrients, which in turn results in larger growth increases (Doyle & Erickson, 2008). Although the temperatures were high (26°C) on day 8, it was not the highest temperature found during the 14 day study. The highest outside temperature of 36°C was observed on day 3, which in contrast had a low *E.coli* count of 200 cfu.mL⁻¹ on the green beans. This is an indication that temperature was not always the main reason for the large cell numbers or increases. UV-rays might also have had an influence on the counts, even though it was limited by the hobby tunnel. More research is therefore necessary to determine the impact of the UV-rays.

Carry-over from “pure” culture *E.coli* containing irrigation water to green beans under controlled conditions - 14 days (Study I - plots b and d)

In this study (Fig. 5 b and d) the green beans were irrigated with the “pure” *E.coli* culture at a concentration of 10^6 cfu.mL⁻¹ (Fig. 7) and harvested 24 h after irrigation. The aim of this plot study was to determine if *E.coli* is transferred to the green beans without any “natural” competitors present in the irrigation water.

The general daily profile of the “pure” *E.coli* loads on the green beans (Fig. 7) was similar to the profile of the green beans when river water was applied (Fig. 6). However in this case the inoculation concentration of 10^6 cfu.mL⁻¹ was kept constant. Overall, the population profile of the *E.coli* on the green beans again gradually increased to day 7 reaching 445 000 cfu.mL⁻¹. In this case it was speculated that the similar profiles found in both Figs. 6 and 7 could possibly be ascribed to the numbers reaching a maximum load on the beans. The *E.coli* loads in the irrigation water started declining from day 8 onwards, which could have resulted in the wash-off of the *E.coli* on the green beans with subsequent irrigation, as it did not properly attach to the surface.

The large increase in numbers by day 7 could also be due to *E.coli* clumps being broken during the washing of the green beans or even the impact of temperature. However, the maximum environmental temperature on day 7 was 26°C, which is lower when compared to the maximum temperature of 36°C on day 3. At the maximum temperature, the population reached a value of 33 000 cfu.mL⁻¹, which suggests that temperature is not the main reason for the large growth increase by day 7.

It has been reported that irrigation water can increase the leaching of nutrients to the plant surface and increase the solubility of the sugars leading to favourable growth circumstances (Brandl & Mandrell, 2002). Brandl & Mandrell (2002) also reported that *Salmonella enterica* in the cilantro phyllosphere had large growth increases in the first few days after inoculation, due to the organism being able to utilise all the simple sugars, whereas the numbers started to decline when the cells were unable to utilise the more complex sugars. This might explain why there was an overall increase from day 4 - 8 and a decline afterwards. This could be another explanation for the large growth increase by day 7

The data shows that when the applied irrigation water was at a constant cell concentration of 10^6 cfu.mL⁻¹, then 50% or less of the cells attached to the beans. Overall, it was found that when the *E.coli* concentration (10^6 cfu.mL⁻¹) in the irrigation water is high, it could be carried-over to the green beans and could result in a health hazard for the consumer.

Survival of a “pure” *E.coli* culture on green beans after “once-off” irrigation (Study II)

Green beans in Study II (Fig. 5 e,f,g,h) were irrigated with “pure” *E.coli* cultures at 10^3 and 10^4 cfu.mL⁻¹ cell concentrations, respectively. The aim of Study II was to determine what the lowest *E.coli* concentration is that would be transferred from irrigation water to produce and how long the

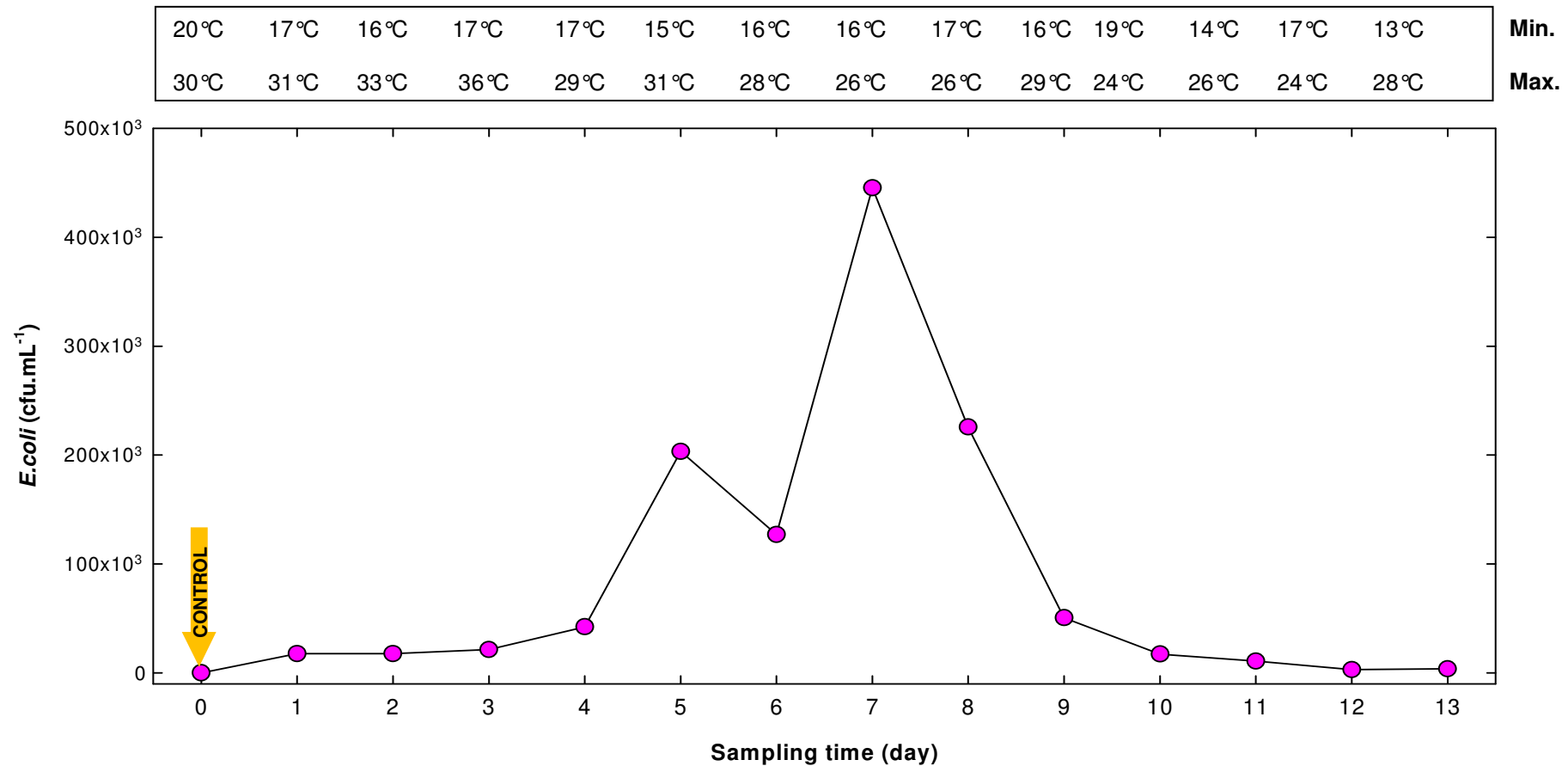


Figure 7 Study I - The 14 day plot study where green beans were irrigated daily for 13 days (days 0 - 12) with the “pure” *E.coli* culture at a concentration of 1×10^6 cfu.mL⁻¹ and harvested 24 h after irrigation (days 1 - 13). Day 0 served as the control as the green beans had not been exposed to the “pure” *E.coli* culture irrigation water before harvest. The *E.coli* values are averages of counts from the two sub-plots and each done in duplicate. The minimum and maximum environmental temperatures on the day of sampling were also recorded.

cells will survive on the green beans. The data obtained is shown in Table 3. No *E.coli* was found on the green beans on day 0 before irrigation (control sample).

In this study it was found that there was only 10 cfu.mL⁻¹ present on the green beans 24 h after having been irrigated with the 10³ cfu.mL⁻¹ *E.coli* concentration in the irrigation water. From day 2 onwards no survivors were detected on the irrigated green beans after being irrigated with the 10³ cfu.mL⁻¹ irrigation water. The data from this study supports the results reported by Ackermann (2010) who found under strict laboratory conditions that there were no survivors on green beans after exposure to a 10³ cfu.mL⁻¹ cell concentration.

Table 3 Survival of the “pure” *E.coli* culture inoculated into the irrigation water at 10³ and 10⁴ cfu.mL⁻¹ concentration on the green beans. Results are the average of the two plots, each sample done in duplicate

| Irrigation water concentration | Day 0 | Day 1 | Day 2 | Day 3 |
|--------------------------------|-------------------------|-------|-------|-------|
| | (cfu.mL ⁻¹) | | | |
| 10 ³ | 0 | 10 | 0 | 0 |
| 10 ⁴ | 0 | 70 | 20 | 0 |

When the concentration of *E.coli* in the irrigation water was increased to 10⁴ cfu.mL⁻¹, a similar survival profile on the green beans was found (Table 3). After the first irrigation session an *E.coli* cell count of 70 cfu.mL⁻¹ was found on day 1, which then decreased to 20 cfu.mL⁻¹ by day 2. From day 3 onwards no *E.coli* were detected on the green beans. Ackermann (2010) also found that 10⁴ cfu.mL⁻¹ is the lowest *E.coli* concentration that green beans could be exposed to for carry-over still to occur.

The reason why only a few *E.coli* survived on the green beans during this study could be that the population was too small to colonise and compete for nutrients with the organisms “naturally” present on the green beans. Low nutrient availability could also have been responsible as well as a lack of moisture since the green beans were only irrigated once and not on a daily basis. Moisture on produce surfaces is important for microbial growth, as it was previously reported that increasing moisture levels leads to increases in the leaching of nutrients to the produce surface resulting in microbial growth increases (Brandl & Mandrell, 2002). Low survival numbers could also have been

due to other bacteria that is naturally present on the green beans and well adapted to the environment (Cooley *et al.*, 2006), limiting growth and survival of the “pure” *E.coli* culture. Another reason could be ascribed to dehydration as the tunnel temperatures were fairly high and there was possibly not sufficient moisture on the green beans after only being irrigated once when compared with green beans that had been irrigated on a daily basis (Brandl & Mandrell, 2002). Daily irrigation provides a moist environment, which is more favourable for microbial growth.

Characterisation and identification of isolates from river water and irrigated beans

Irrigation water and green beans

During the 14 day irrigation periods (Fig. 6) a total of 72 organisms were isolated from the river water (Table 4). Of the 72 isolates, 30 (41.7%) were identified as *E.coli* with the API 20E system with the ID ranging between 97.4 - 99.9%. All the *E.coli* strains were Gram negative, rod shaped, motile, O-F positive, catalase positive, oxidase negative and grew on MacConkey agar (Buchanan & Doyle, 1997; Pu, 2009) (Table 4).

On Brilliance™ *E.coli*/Coliform selective agar (Oxoid), *E.coli* should produce purple colonies (Fig. 8) (Anon., 2011). However, some strains (10) identified as *E.coli* with the API 20E grew as pink colonies on this agar (Fig. 8). For the other characteristics, all these pink colony strains were Gram negative, rod shaped, motile, O-F positive, catalase positive, oxidase negative and grew on MacConkey agar as expected for *E.coli*. All coliforms are β -galactosidase positive (Anon., 2011), while *E.coli* is additionally also β -glucuronidase positive. When only β -galactosidase is present, only one of the chromogenic agents (Rose-Gal) is cleaved, resulting in the pink colour. When β -glucuronidase is present the other chromogenic agent (X-Glu) is also cleaved. The cleaving of both chromogenic agents results in a purple colony colour (Anon., 2011). However, according to Martins *et al.* (1993) depending on the physiological state of the *E.coli* strains, β -glucuronidase activity is not always expressed, even though the *uidA* encoding β -glucuronidase is present.

During the isolation phase some colonies selected with the Harrison's disc method (Harrigan & McCance, 1974) were found to be a mixture of at least two culture types. This was only observed once the colonies were streaked out on the Brilliance™ *E.coli*/Coliform selective agar (Fig. 9). These mixed cultures were then separated, purified and re-confirmed to a more acceptable ID level by the API 20E identification system. This happened twice for the river water, where a *Citrobacter freundii* strain (purple, ID = 96.7%) and a *Pantoea* spp. (pink, ID = 99.8%) were separated from the same original colony. Similarly, an *E.coli* strain (purple, ID = 98.5%) and a *Serratia fonticola* strain (pink, ID = 91.9%) were separated from the original colony (Table 4).

Table 4 The 72 microbial species isolated from the Plankenburg River water used for irrigation of green beans and some of their phenotypic characters

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram. +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|-----------|--------|----------|---------|----------|---------|-------------------------------|------|
| W1 | 1 | Pink | + | - | rods | + | +/+ | + | - | <i>E.aerogenes</i> | 96.7 |
| W2 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W3 | 1 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea spp 2</i> | 71.3 |
| W4 | 1 | Pink | + | - | rods | + | +/+ | + | - | <i>E.cloacae</i> | 95 |
| W5 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W6 | 1 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| W7 | 1 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera spp</i> | 53.4 |
| W8 | 2 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter aerogenes</i> | 91.2 |
| W9 | 2 | Pink | + | - | rods | + | +/+ | + | - | <i>Serratia odorifera 1</i> | 91.7 |
| W10 | 2 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W11 | 2 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| W12 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W13 | 3 | Pink | + | - | rods | + | +/+ | + | - | <i>E. cloacae</i> | 95 |
| W14 | 3 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 97.4 |
| W15 | 3 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 97.4 |
| W16 | 3 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 97.4 |
| W17 | 4 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W18 | 4 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W19 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>E.asburiae</i> | 99 |
| W26 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>E.asburiae</i> | 99 |
| W27 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 99.9 |
| W20 | 5 | Purple | + | - | rods | + | +/+ | + | - | <i>Citrobacter freundii</i> | 91.9 |
| W21 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Serratia odorifera 1</i> | 99.9 |
| W22 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli 1</i> | 98.7 |

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|-----------------------------------|------|
| W23 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 99.9 |
| W24 | 5 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 97.4 |
| W29 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>E.aerogenes</i> | 96.7 |
| W30 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>Citrobacter freundii</i> | 96.7 |
| W31 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 97.4 |
| W32 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.5 |
| W33A | 7 | Purple | + | - | rods | + | +/+ | + | - | <i>Citrobacter freundii</i> | 96.7 |
| W33B | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea</i> spp 1 | 99.8 |
| W34 | 7 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 97.4 |
| W35 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea</i> spp 1 | 94.6 |
| W36 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Citrobacter braakii</i> | 99.8 |
| W37 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Citrobacter koseri/farmeri</i> | 92.4 |
| W38 | 8 | Pink | + | - | rods | + | +/+ | + | - | <i>E.cloacae</i> | 95 |
| W39 | 8 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| W40 | 8 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 98.9 |
| W41 | 8 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.5 |
| W42 | 8 | Pink | + | - | rods | + | +/+ | + | - | <i>E.aerogenes</i> | 96.7 |
| W43 | 9 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.9 |
| W44 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 98.7 |
| W45 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 98.7 |
| W46 | 9 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.9 |
| W47 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 99.9 |
| W48 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 97.9 |
| W49 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.9 |
| W50 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>E.coli</i> 1 | 99.9 |
| W51 | 10 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 98.9 |

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|-----------------------------------|------|
| W52 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| W58 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99.4 |
| W59 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 83.4 |
| W60 | 11 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.8 |
| W61 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W62 | 12 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> | 97.4 |
| W63 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera spp</i> | 91.4 |
| W64 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Citrobacter koseri/farmeri</i> | 73.7 |
| W65 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W66 | 12 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| W67 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter aerogenes</i> | 96.7 |
| W68 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W69 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W70 | 13 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| W71 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99 |
| W72 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 99.4 |
| W73 | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| W74 | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W75A | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Serratia fonticola</i> | 91.9 |
| W75B | 14 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 98.5 |
| W76 | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| W77 | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Serratia liquefaciens</i> | 86.9 |

* Chrom. = Chromogenic agar (Brilliance™ E.coli/Coliform selective agar); McC = MacConkey agar; Gram = Gram staining; Morph. = Morphology; O-F = Oxidative-Fermentative; Cat. = Catalase and Ox. = Oxidase

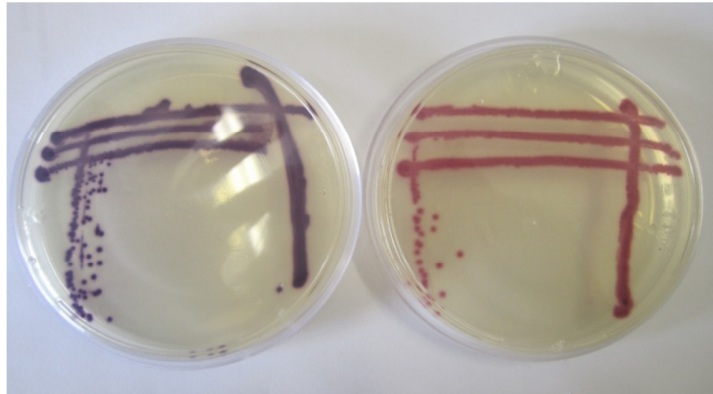


Figure 8 The isolated *E.coli* strains growing as purple or pink colonies on Brilliance™ *E.coli*/Coliform selective agar.

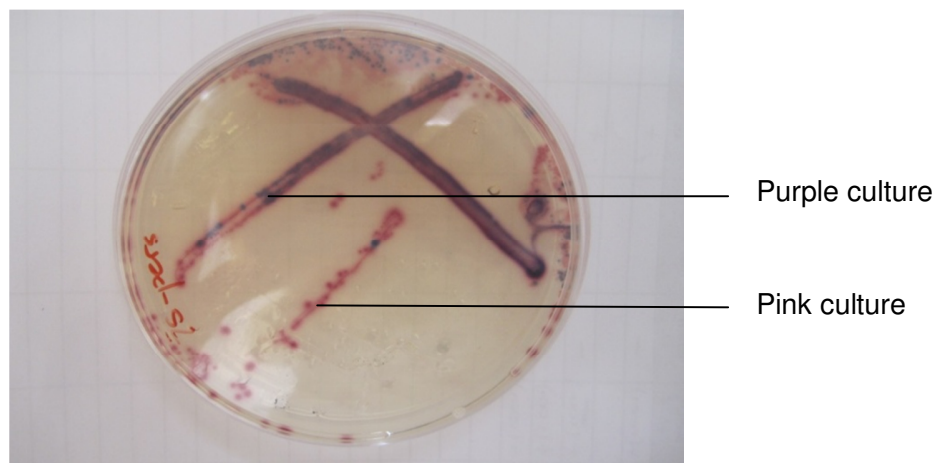


Figure 9 The mixture of two cultures is clearly visible on the Brilliance™ *E.coli*/Coliform selective agar.

In this study three species of *Citrobacter* were isolated from the river: *Citrobacter koseri/farmeri* (2 strains); *C.braakii* (1 strain); and *C.freundii* (3 strains). The *Citrobacter* strains were all Gram negative, rod shaped, motile, O-F positive, catalase positive, oxidase negative and MacConkey agar positive (Table 4). As expected the *C.koseri/farmeri* and *C.braakii* strains grew as pink colonies on the chromogenic agar, but what was surprising is that all three of the *C.freundii* isolates grew as purple colonies on Brilliance™ *E.coli*/Coliform selective agar. It is usually just *E.coli* that grows as purple colonies on Brilliance™ *E.coli*/Coliform selective agar (Anon., 2011). However, studies by Pérez *et al.* (1986) have reported that *C.freundii* could test positive for β -glucuronidase,

which would result in a purple colony colouring on the agar. In all these cases API 20E still gave a high identification ID of 96.7% for two of the isolates and 91.9% for the other *C. freundii* isolate.

Although *E. coli* was the most frequently isolated organism, there were other species also present and isolated more than once: *Klebsiella pneumoniae* (4 strains); *Enterobacter cloacae* (10 strains); *Enterobacter aerogenes* (5 strains); *Kluyvera* spp. (7 strains); *Serratia odorifera* 1 (2 strains); *Enterobacter asburiae* (3 strains); and *Pantoea* spp 1 (2 strains). *Pantoea* spp 2, *Serratia liquefaciens*, *Serratia fonticola* and *Citrobacter braakii* were only isolated once from the river water.

A total of 67 organisms were isolated from the green beans that were irrigated with the Plankenburg River (Table 5). No organisms were isolated from the green beans on day 0, as the green beans were not irrigated with the contaminated river water before harvest. The first organisms could only be isolated on day 3 as minimal carry-over was observed during the first two days.

In this study 20 (29.9%) of the 67 strains were identified as *E. coli* with the API 20E system. The API 20E gave good to excellent identifications with values ranging between 97.4 - 99.9% (Table 5). All the *E. coli* strains were Gram negative, rod shaped, motile, O-F positive, catalase positive, oxidase negative and grew on MacConkey agar, as expected for *E. coli* (Buchanan & Doyle, 1997; Pu, 2009).

One *E. coli* strain also grew as pink colonies on Brilliance™ *E. coli*/Coliform selective agar (chromogenic agar) as some of the *E. coli* isolates from the river water. Of the total *E. coli* strains (50) isolated from the irrigation water and green beans, 11 strains (22%) grew as pink colonies on the Brilliance™ *E. coli*/Coliform selective agar. It is interesting to note that during the green bean isolations, no mixed colonies were found with the green beans.

Other organisms that were isolated more than once from the green beans included *Klebsiella pneumoniae* (4 strains), *Enterobacter cloacae* (21 strains), *Enterobacter asburiae* (7 strains), *Enterobacter amnigenus* 2 (2 strains), *Klebsiella oxytoca* (2 strains), *Pantoea* spp 1 (2 strains), *Pantoea* spp 2 (2 strains), *Kluyvera* spp. (2 strains) and *Enterobacter aerogenes* (3 strains). Strains of *Pantoea* spp 3 and *Enterobacter sakazakii* were only isolated once from the green beans and not found in the river water (Table 5).

Klebsiella pneumoniae was found four times in the river as well as four times on the green beans. *E. cloacae* (18 vs. 10 isolates) and *E. asburiae* (7 vs. 3 isolates) were more frequently isolated from the green beans than the river water. This might be because they were selected more frequently with the Harrisons disc method or that these organisms could adapt and colonise better on the green bean environment. The *E. asburiae* is known to be naturally present on fresh produce, which could explain the higher count on the green beans (Cooley *et al.*, 2006).

Organisms that were isolated from the green beans, but not from the river water included: *Enterobacter amnigenus* 2; *Enterobacter sakazakii*; *Klebsiella oxytoca*; *Pantoea* spp 2; and *Pantoea*

Table 5 The 67 microbial species isolated from the green beans 24 h after being irrigated with the Plankenburg River water and some of their phenotypic characters

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram. +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|-----------|--------|----------|---------|----------|---------|------------------------------|------|
| B1 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B2 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B5 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B6 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B7 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B8 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B10 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B11 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B13 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B14 | 4 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B16 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B17 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B18 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Kluyvera</i> spp | 99.9 |
| B19 | 5 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea</i> spp 1 | 98.2 |
| B21 | 6 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| B22 | 6 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella oxytoca</i> | 97.4 |
| B23 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B24 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B25 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B26 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B27 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B28 | 6 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| B29 | 6 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 99.4 |
| B30 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 93.7 |
| B31 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 99.5 |

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mot. +/- | O/F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|-------------------------------|------|
| B32 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 93.7 |
| B33 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 93.7 |
| B34 | 7 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 93.7 |
| B35 | 8 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.5 |
| B36 | 8 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B37 | 8 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.5 |
| B38 | 8 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter sakazakii</i> | 91.2 |
| B39 | 8 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99.5 |
| B40 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter aerogenes</i> | 96.7 |
| B41 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B42 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter aerogenes</i> | 99.7 |
| B43 | 9 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| B44 | 9 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter aerogenes</i> | 96.7 |
| B45 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B46 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B47 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B48 | 10 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B49 | 10 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B50 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B51 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea spp 1</i> | 71.3 |
| B52 | 11 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| B53 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea spp 2</i> | 71.3 |
| B54 | 11 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B55 | 12 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B56 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Klebsiella oxycota</i> | 98 |

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mob. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|---------------------------------|------|
| B57 | 2 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea</i> spp 3 | 99.8 |
| B58 | 12 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B59 | 12 | Pink | + | - | rods | - | +/+ | + | - | <i>Klebsiella pneumoniae</i> | 97.7 |
| B60 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter asburiae</i> | 99 |
| B61 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter amnigenus 2</i> | 96.6 |
| B63 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Pantoea</i> spp 2 | 99.6 |
| B64 | 13 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter amnigenus 2</i> | 96.6 |
| B66 | 14 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 97.4 |
| B67 | 14 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B68 | 14 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 97.4 |
| B69 | 14 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 99.9 |
| B70 | 14 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli 1</i> | 97.4 |
| B71 | 15 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B72 | 15 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B73 | 15 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B74 | 15 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |
| B75 | 15 | Pink | + | - | rods | + | +/+ | + | - | <i>Enterobacter cloacae</i> | 95 |

* Chrom. = Chromogenic agar (Brilliance™ E.coli/Coliform selective agar); McC = MacConkey agar; Gram = Gram staining; Morph. = Morphology; O-F = Oxidative-Fermentative; Cat. = Catalase and Ox. = Oxidase

spp 3. Other organisms that were found in the river water, but not on the green beans include: *Serratia liquefaciens*; *Serratia fonticola*; *Serratia odorifera* 1; *Citrobacter koseri*/farmer; *Citrobacter braakii*; and *Citrobacter freundii*. Not one strain of *Serratia* or *Citrobacter* were found on the green beans, but three different *Serratia* and *Citrobacter* isolates were found in the river water. This might be an indication that *Serratia* and *Citrobacter* cannot grow on green beans. However, further investigation is necessary to prove if this is true.

All the species from the river water that were not present on the green beans (*Serratia* and *Citrobacter* isolates) were isolated at a low frequency from the river water (three isolates or less). This might suggest that these organisms were not present in high numbers in the river water, which could have resulted in the low number of isolates being isolated from the river water samples. This low concentration of these species in the irrigation water could have resulted none being carried-over to the green beans as the levels were too low to be carried over.

E.coli “pure” culture isolated from the inoculated irrigation water and from the green beans

The “pure” *E.coli* culture used to irrigate the green beans (Fig. 5 b and d) at a cell concentration of 10^6 cfu.mL⁻¹ was recovered from both the irrigation water and the green beans during this study (Table 6). Based on their phenotypical characters, the *E.coli* strains from the green beans were identical to the irrigated “pure” *E.coli* strain.

In the second study where the “pure” *E.coli* was inoculated into the irrigation water at concentrations of 10^3 cfu.mL⁻¹ and 10^4 cfu.mL⁻¹ identical phenotypical strains were recovered from both the irrigation water and the irrigated beans (Table 7).

Linking studies

Phase 1 linking: Phenotypical distribution of isolates based on species identification with API 20E

According to the API 20E identification data, eight species that were also present in the river water were isolated from the green beans (Table 8). *E.coli*, *E.cloacae* and *E.asburiae* were the most isolated strains. This was taken as an indication of first phase linking as these strains from the irrigation water were phenotypically identical to the strains on the green beans based on species identification with the API 20E system.

Table 6 The “pure” *E.coli* (10^6 cfu.mL⁻¹) culture recovered from the inoculated irrigation water and from the green beans 24 h after being irrigated

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|---------------------------|------|
| PCW1 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB1 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB2 | 3 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB3 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB4 | 6 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB5 | 9 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB6 | 9 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB7 | 12 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB8 | 12 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB9 | 15 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| PCB10 | 15 | Purple | + | - | Rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |

* Chrom. = Chromogenic agar (Brilliance™ *E.coli/Colifrom* selective agar); McC = MacConkey agar; Gram = Gram staining; Morph. = Morphology; O-F = Oxidative-Fermentative; Cat. = Catalase and Ox. = Oxidase

Table 7 The “pure” culture *E.coli* (10^3 and 10^4 cfu.mL⁻¹) isolated from the irrigation water and on the green beans based on phenotypical analysis

| Isolate nr. | Isolation day | Chrom. | McC. +/- | Gram +/- | Morph. | Mot. +/- | O-F +/- | Cat. +/- | Ox. +/- | API 20E species ID | ID % |
|-------------|---------------|--------|----------|----------|--------|----------|---------|----------|---------|---------------------------|------|
| 104PCB1 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB2 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB3 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB4 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB5 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB6 | 2 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 104PCB7 | 2 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |
| 103PCB1 | 1 | Purple | + | - | rods | + | +/+ | + | - | <i>Escherichia coli</i> 1 | 99.9 |

* Chrom. = Chromogenic agar (Brilliance™ *E.coli*/Coliform selective agar); McC = MacConkey agar; Gram = Gram staining; Morph. = Morphology; O-F = Oxidative-Fermentative; Cat. = Catalase and Ox. = Oxidase

Table 8 Species present in both irrigation water and on the green beans (indication of “carry-over”- phase 1 linking)

| Species | Number of isolates |
|-------------------------------|--------------------|
| <i>Escherichia coli</i> | 50 |
| <i>Enterobacter cloacae</i> | 31 |
| <i>Enterobacter asburiae</i> | 10 |
| <i>Kluyvera</i> spp. | 9 |
| <i>Klebsiella pneumoniae</i> | 8 |
| <i>Enterobacter aerogenes</i> | 8 |
| <i>Pantoea</i> spp. 1 | 4 |
| <i>Pantoea</i> spp. 2 | 3 |

Phase 2 linking: Dendrogramme clustering based on the API 20E characters

The data in Fig. 10 shows the dendrogramme based on the statistical clustering of the API 20E data using the simple matching coefficient. This coefficient is based on both positive and negative characteristics. The x-axis represents the dissimilarity index, where as the y-axis shows the strains isolated from both the river water and green beans, and the “pure” culture *E.coli* isolates. Seven clear clusters were found. Cluster A was taken as representative of the *K.pneumoniae* group; B = *E.aerogenes*; C, D, E = *E.coli*; F = *E.asburiae* and G = *E.cloacae*. To do the phase 2 linking, 13 strains were selected from the five largest clusters (A, C, D, E and G) to determine whether the strain in the river water could be linked to the strain from the green beans.

In these five clusters, strains that were phenotypically identical (Appendix I) were isolated from both the river water and a day or more after irrigation had taken place, from the green beans. In the first example (Cluster A), *K.pneumoniae* was isolated frequently throughout the 14 day period from both the irrigation water and green beans. Specifically, this species was also isolated on day 8 from the water and on day 9 from the green beans. Both these incidences were thus taken as an indication that *K.pneumoniae* was carried-over from the river water to the green beans. Thus the two strains (W839 and B943) isolated on day 8 and 9 were selected as these strains showed a carry-over 24 h after irrigation. Further analyses were necessary to determine whether these strains (from the irrigation water and the beans) were similar based on sequencing analysis.

The second largest cluster (G) consisted of 27 *E.cloacae* strains (Fig. 10). Some of the *E.cloacae* strains were isolated from the river water on days 3 and 13. Some of the *E.cloacae*

strains in this cluster were also isolated from the green beans on days 4, 7, 14 and 15. The fact that *E.collocaae* strains were isolated on days 7 and 15, suggests that they were probably the same strain that survived on the green beans for a period of at least a day or more.

The four *E.colli* clusters (C, D1, D2 and E) (Fig. 10) only differed in their ability to utilise three carbon sources (Table 9). Cluster E was the largest cluster and contained the reference cultures and the “pure” *E.colli* isolates. A phenotypic link can also be shown between the *E.colli* isolates in this cluster on days 4 and 10 isolated from the river water, and on days 6 and 12 from the green beans.

Cluster C was a smaller *E.colli* cluster. It did, however, confirm the carry-over of similar *E.colli* strains (based on phenotypic characters) isolated on day 6 from the river water to those isolated on day 8 from the green beans.

Cluster D (Fig. 10) was further grouped into two smaller sub-clusters (D1 and D2). These two sub-clusters differed as cluster D2 strains were able to utilise LDC (Table 9). However, no D2 strains were isolated from the green beans. Only cluster D1 showed a carry-over of *E.colli* with identical strains being isolated on days 3 and 7 from the river water and on day 14 from the green beans. This was taken as confirmation that the strain survived for longer periods on green beans or it might have been present in the river water throughout the sampling period.

Table 9 The phenotypic differences (LDC, ODC and SAC) between the four *E.colli* clusters

| | L-lysine (LDC) | L-ornithine (ODC) | D-sucrose (SAC) |
|------------|---------------------------|------------------------------|----------------------------|
| Cluster C | + | + | + |
| Cluster D1 | - | - | - |
| Cluster D2 | + | - | - |
| Cluster E | + | + | - |

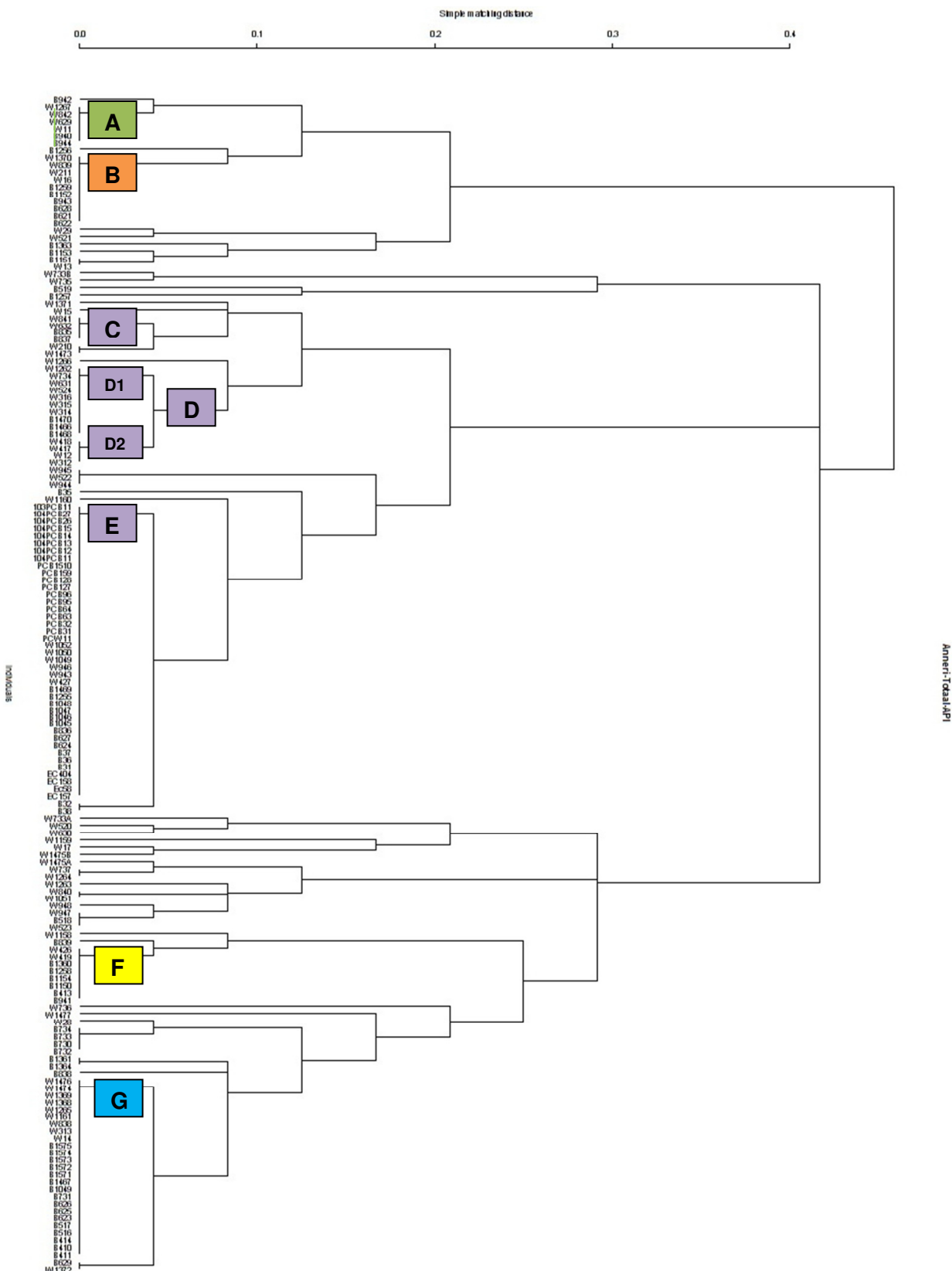


Figure 10 Dendrogramme illustrating the statistical clustering based on the API 20E data. Simple matching coefficient based on positive and negative characters was used to cluster similar isolates. Seven clusters were present (A – G). A = *K.pneumoniae*; B = *E.aerogenes*; C,D,E = *E.coli*; F = *E.asburiae* and G = *E.cloacae*.

From the data presented in this section and the clustering of similar isolates, the carry-over of several bacterial species especially *E.coli* has been shown to take place from the river water to the green beans. This was thus taken as proof of phase 2 linking. Some strains isolated from the green beans on different days show that the *E.coli* can survive on the green beans for 24 h or longer. In other cases, when a strain was present in the river water a few days before it was isolated from the green beans, it could be assumed that the strain was in the river water and after irrigation it was able to survive for longer periods on the green beans. It is also possible the strain was present in the river water during the whole sampling period, but the cell concentration was too low to be selected using the Harrison's disc method.

On a phenotypical level there were different clusters of *E.coli* and each could be used to confirm linking based on the days irrigated and the day when isolated from the green beans. These carry-over conclusions however, could not be completely established until each strain could be analysed on a molecular level.

Phase 3 linking: Identity confirmation based on *uidA* gene PCR

In this study the *uidA* gene which encodes from the enzyme β -glucuronidase (GUR) and which is associated with *E.coli* strains from the water and beans, was used for further identity confirmation (Martins *et al.*, 1993). Many studies have shown that *uidA* can be detected in 97 - 100% of *E.coli* strains (Rice *et al.*, 1990; Green *et al.*, 1991).

In this study, all 50 strains that were confirmed as *E.coli* with the API 20E data base were subsequently analysed with PCR. All (50 strains) tested positive for the *uidA* gene (Fig. 11). As these strains (from irrigation water to green beans) are confirmed to be *E.coli* on a molecular level, it supports linking. Even the API 20E confirmed, but pink (GUR-negative) colonies on Brilliance™ *E.coli*/Coliform selective agar *E.coli* strains tested positive for the gene (11 strains). It must be taken into consideration that in the literature it has been reported that the *uidA* gene could be present in *E.coli* strains, but that these strains will still be negative for β -glucuronidase activity (Bej *et al.*, 1991; Green *et al.*, 1991). The reason given for this is that their GUR activity is not always expressed, depending on the physiological state of the *E.coli* strain (Martins *et al.*, 1993).

The three strains that were purple on Brilliance™ *E.coli*/Coliform selective agar (chromogenic agar), but identified as *C.freundii* with the API 20E system were also studied. Of these three (W520, W733A and W630), two (W733A and W630) tested positive for the *uidA* gene (Fig. 11) and should thus be taken as *E.coli* strains. Previous studies that investigated the presence of the *uidA* gene in *C.freundii* reported none of the strains positive for the gene (Green *et al.*, 1991; Horakova *et al.*, 2008). It has also been reported that the API 20E system misidentified an *E.coli* strain as *C.freundii* strain in a previous study (Aldridge *et al.*, 1978). Bettelheim *et al.* (1993) similarly reported in another study that an *E.coli* pathogenic strain (*E.coli* O157) was

biochemically alike to *C.freundii*. All the above facts might explain why the API 20E system confirmed the two *E.coli* strains (W733A and W630) as *C.freundii*.

The other *C.freundii* strain (W520), did not test positive for the *uidA* gene, which indicated that according to PCR it is also not an *E.coli* strain (Fig.11). It had previously been found that *C.freundii* was not positive for the *uidA* gene (Horakova *et al.*, 2008).

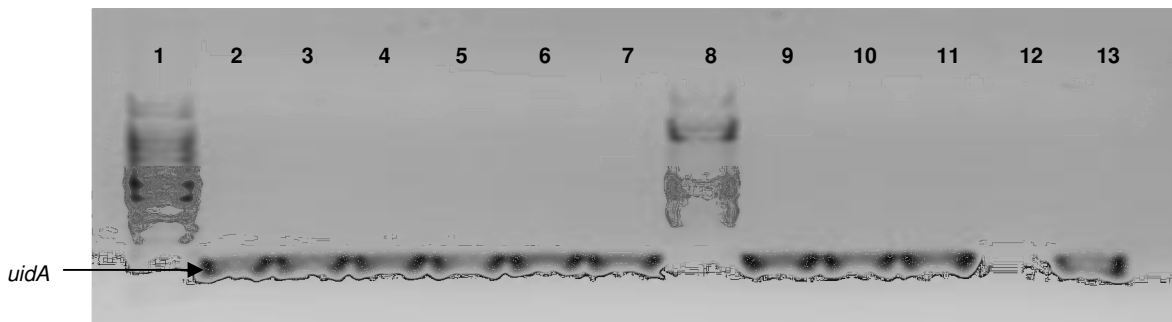


Figure 11 Agarose gel (1% agarose and 1 $\mu\text{g.L}^{-1}$ ethidium bromide) with PCR amplicons. Two (W733A and W630) of the three *C.freundii* (API confirmed) strains tested positive for the *uidA* gene (Lanes 2 - 4). The other strain (W520) did not test positive for *uidA* (Lane 8). Lanes 5 - 7, 9 - 11 = selected river and green bean *E.coli* strains that tested positive. Lane 1 = 100 bp marker; Lane 12 = negative control; Lane 13 = positive control.

However, further analysis is essential as it has been suggested by Martins *et al.* (1993) that the identification of *E.coli* should always be done in combination with the detection of the *uidA* gene as well as with other genotypical traits. In their study, Martins *et al.* (1993) reported that 2.3% of the *E.coli* strains examined did not test positive for the *uidA* gene. It was suggested that, depending on the physiological state of the *E.coli* strains, the *uidA* gene was not always expressed, even though it was present in the *E.coli* strains. Although the API 20E system confirmed that this strain was not an *E.coli*, further analysis is needed as it did test positive for GUR activity based on the purple colony colour on the chromogenic agar. A total of 52 (two *C.freundii* and 50 *E.coli* API 20E confirmed) strains from the river water and green beans tested positive for the *uidA* gene and were thus subjected to multiplex-PCR.

Phase 4 linking: Identity confirmation based on Multiplex-PCR

The 52 water and bean isolates that tested *uidA* positive as well as strain W520 that was not positive for *uidA*, were further subjected to multiplex PCR to determine if pathotypes were present. All the strains tested positive for the *mdh* gene, indicating all the strains were *E.coli* (Tarr *et al.*,

2002). The multiplex PCR data revealed that four strains (W630, W1371, W733A and W1052) from river water were EPEC types, as they were positive for the *mdh* and *eaeA* genes (Fig. 12).

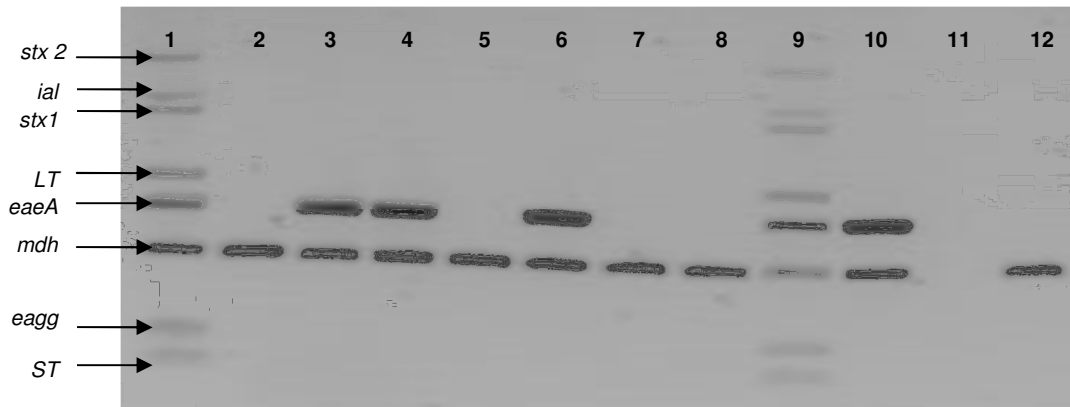


Figure 12 Agarose gel (1.25% agarose and 1 $\mu\text{g.L}^{-1}$ ethidium bromide) with multiplex PCR amplicons. Four river strains tested positive for the *eaeA* gene (EPEC). Lanes 1 and 9 = standard culture mix marker with virulence genes; lanes 2, 5, 7 and 8 = commensal *E.coli* strains; lanes 3 (W630), 4 (W1371), 6 (W733A) and 10 (W1052) = EPEC strains; lane 11 = negative control; lane 12 = positive control.

The *eaeA* gene, located on the “locus of the enterocyte effacement” (LEE) pathogenicity island, encodes the transmembrane protein, intimin, which plays an important role during cell adhesion to the epithelial cells (Afset *et al.*, 2003). EPEC can be taken as a dangerous *E.coli* pathotype and can cause infantile watery diarrhoea (Donnenberg & Kaper, 1992). In most countries, EPEC strains are frequently responsible for diarrhoeal diseases, especially among children (Matar *et al.*, 2002). The mechanism of the EPEC pathogenesis is an attaching and effacing (A/E) lesion, which is based on the adherence of the pathogen to the intestinal epithelium of the host (Vallance & Finlay, 2000).

It is interesting to note that two of the API 20E confirmed *C.freundii* strains that were PCR confirmed as *E.coli*, were the strains that classified as EPEC with the multiplex PCR. *C.freundii* can be the cause of a disease known as transmissible murine colonic hyperplasia. This is characteristic of mucosal proliferation and the A/E lesions, which are identical to the lesions of EPEC (Schauer *et al.*, 1995). It has previously been reported that *C.freundii* contained DNA that is similar to the *eaeA* gene that is responsible for A/E lesions caused by EPEC strains (Schauer & Falkow, 1993). The LEE is not identical between *E.coli* and *C.freundii*, but they are highly similar in terms of overall genetic organisation and gene function (Petty *et al.*, 2010). Horizontal gene transfer of the *eaeA* gene from EPEC to *C.freundii* might also be possible (Schauer & Falkow,

1993). The confirmation of the *uidA* gene (*E.coli* specific) is an indication that these *C.freundii* strains were really *E.coli*.

Sequencing analysis using the Basic Local Alignment Search Tool (BLAST) of the *dnaJ* and *oriC*-locus revealed that all four EPEC strains were identical to *E.coli* strains (99%). The four EPEC strains were isolated on days 6, 7, 10 and 13 and since the Plankenburg is a constant flowing river, their presence suggests there was a source continuously faecally polluting the river. More research is necessary to determine whether these EPEC strains were from human or animal origin.

The *C.freundii* strain (W520) that was not confirmed as *E.coli* by PCR (*uidA* negative) did, however, test positive for the *mdh* gene with the multiplex PCR. The *mdh* gene is a housekeeping gene that is not specifically conserved to *E.coli*. It could also occur in *Salmonella enterica* as well as in *C.freundii*. The level of identity of the *mdh* gene between *E.coli* and *C.freundii* was previously found to be 87.38% (Samuel *et al.*, 2004). Since *E.coli* and *C.freundii* are genetically very similar based on the *mdh* gene, it could be an explanation why the strain tested positive for the *mdh* gene. The strain W520 tested negative for all the other virulence factors with the multiplex-PCR.

Subsequent sequencing analysis was performed on the W520 strain and confirmed that it was definitely not an *E.coli* strain. The *dnaJ* was sequenced and the BLAST tool revealed the strain to be highly similar to *C.youngae* (97%) and *C.freundii* (92%). This suggests that the strain was most likely a member of the genus *Citrobacter* and not an *E.coli*.

Based on the multiplex PCR data no linking could be confirmed based on carry-over of pathotypes (EPEC). Even though EPEC strains were detected in the river water and not on the green beans, it could be that these strains were not selected and then enumerated. The detection of the *mdh* gene in the *E.coli* from the river water as well as those from the green beans is not considered enough evidence to confirm linking.

Phase 5 linking: Identity confirmation based on *E.coli* phylogenetic group determination (Triplex PCR)

E.coli can be divided into four main groups (A, B1, B2 and D) based on phylogenetic analysis (Clermont *et al.*, 2000) and, further sub-divided, depending on the different combinations of the three markers, into sub-groups A₀, A₁, B₁, B_{2,2}, B_{2,3}, D1 and D2 (Regua-Mangia *et al.*, 2010). This is shown in Table 10.

Table 10 *E.coli* phylogenetic groups based on the combinations of the genetic markers and DNA fragment (Regua-Mangia *et al.*, 2010)

| Phylogenetic group | <i>chuA</i> | <i>yjaA</i> | TSPE4.C2 |
|-----------------------------|-------------|-------------|----------|
| A ₀ | - | - | - |
| A ₁ | - | + | - |
| B ₁ | - | - | + |
| B ₂ ₂ | + | + | - |
| B ₂ ₃ | + | + | + |
| D ₁ | + | - | - |
| D ₂ | + | - | + |

From the API 20E clustering data (Fig. 10), 13 *E.coli* isolates were selected and further analysed using the triplex PCR method. The selected *E.coli* strains were chosen based on strain links between the river water and the green beans on a phenotypic level. The data obtained showed phylogenetically one *E.coli* isolate classified in group A₀ (*chuA*⁻, *yjaA*⁻, TSPE4.C2⁻), three in group B₁ (*chuA*⁻, *yjaA*⁻, TSPE4.C2⁺) and nine in B₂₃ (*chuA*⁺, *yjaA*⁺, TSPE4.C2⁺), respectively (Fig. 13). Of the selected strains none showed a link on a phylogenetic level, with the three main *E.coli* clusters having isolates from more than one phylogroup. This is an indication that the API 20E data cannot be used as a single method to prove direct linking. However, it is possible that the phylogenetic data can be used to identify the source of faecal pollution.

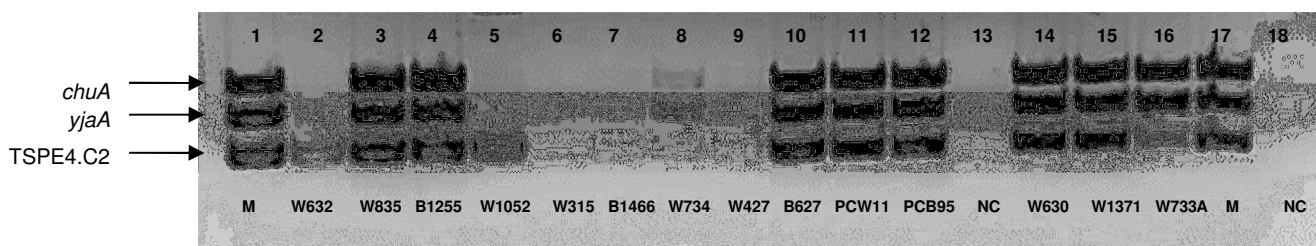


Figure 13 Agarose (2% agarose and 1 $\mu\text{g.L}^{-1}$ ethidium bromide) with triplex PCR amplicons. Lanes 1 and 17 is the marker (ATCC 25922). Lanes 2 - 10 = isolates from the river and green beans, including one EPEC strain in lane 5. Lane 11 = “pure” culture *E.coli* from river water. Lane 12 = “pure” culture *E.coli* from green beans on day 9. Lanes 14 - 16 = EPEC strains isolated from the river water. Lanes 13 and 18 = negative controls.

Phylo-group A - Although the samples size applied in this study was small ($n = 16$), only one isolate (W315) was classified in phylo-group A and more specifically phylo-group A_0 (Fig. 13). Phylo-group A was mostly associated with commensal strains, which are beneficial to the host and rarely cause disease (Iguchi *et al.*, 2009). Phylo-group A_1 was only positive for the *yjaA* gene, while A_0 was negative for the three genetic markers (Regua-Mangia *et al.*, 2010). The question whether A_0 was a good confirmation of *E.coli* has been discussed by Higgins *et al.* (2007), as there was no visible triplex amplicons in 8% of the 460 *E.coli* strains tested. Higgins *et al.* (2007) classified a strain from goose faeces under group A_0 , but with further 16S sequencing analysis the strain was identified as either an *Enterobacter hormaechei* or a *Pantoea* spp. This is also a clear indication that genotyping should be carefully used and in combination with other identification methods to categorise *E.coli* more accurately.

Gordon *et al.* (2008) also found strains that were grouped in phylo-group A_0 with the PCR triplex method that were rarely members of that group. In the study by Gordon *et al.* (2008), the genotype, A_0 , was either supposed to be classified under phylo-group B1 or where it was not possible to be classified in any of the phylogenetic groups, the strains were analysed by multi-locus sequence typing (MLST). The majority of the miss-assigned isolates were found in group A_0 . It was recommended that strains with the A_0 genotype should not be assigned to a phylo-group and that group A strains are not a clear phylogenetic group, but rather a clonal complex that may consist of many smaller clonal complexes.

The study by Gordon *et al.* (2008) further reported that strains grouped in phylo-group A_0 are rare. It was found in Australia that only 9% of isolates from non-human vertebrates, soil, water and sediment samples ($n = 888$) as well as 5% from human samples ($n = 619$) were genotyped under phylo-group A_0 . This is similar to the data found in this study. Due to the “miss”-classification reported in the literature, it might even be that the A_0 isolate (W315) should be in another phylo-group. Another study reported that MLST (85 - 90%) assigned more strains to the correct phylo-group than the triplex-PCR method (80 - 85%) (Gordon *et al.*, 2008). More research, such as analysis with MLST should be used to confirm whether this is true.

Phylo-group B1 - EPEC and EHEC strains could be found in phylo-group B1 as this group is usually associated with intestinal pathogens (Tramatu *et al.*, 2008; Carlos *et al.*, 2010). One EPEC strain (W1052), isolated from the river was classified under group B1 (Fig. 13). There were also three other isolates (W632, W427 and B1466) that were classified under group B1. The sequencing descriptors (BLAST) were different for the W1052 EPEC strain, compared to the other three.

Three isolates (W632, W427 and B1466) did not test positive for any of the intestinal pathotypes with the multiplex PCR (Fig. 12), which suggests that these three strains are more associated with commensal *E.coli*. Although commensal *E.coli* is not harmful, it could however

develop into a pathogenic strain at a later stage due to virulence gene acquisitions (Escobar-Páramo *et al.*, 2004a; Chapman *et al.*, 2006). Carlos *et al.* (2010) also reported *E.coli* strains that can persist in the environment belong mostly to group B1.

Phylo-group B2 - Three of the EPEC strains isolated from the river (W630, W733A and W1371), which also had similar BLAST sequencing descriptors (94 - 99% similar to *E.coli* O83:H7), were classified under group B₂₃. According to Tramatu *et al.* (2008) and Gómez-Duarte *et al.* (2010) most intestinal pathogens are usually classified under phylo-group A or B1, while B₂₃ is mostly associated with extraintestinal pathogens.

Gómez-Duarte *et al.* (2010) found no intestinal pathogen isolates in group B2 when they investigated stool samples of children's diarrhea. What they did find was that some STEC (VT and *eae*) and EAEC (*aggR*) isolates clustered in group D and not as expected in groups A or B1. They explained this as horizontal transfer of the *eae* and *aggR* genes from the A or B1 group. This is possible because the VT toxin genes are present in phages, the *eae* genes in pathogenicity islands and the *aggR* genes in virulence plasmids. Therefore, it might explain why the EPEC isolates, which are also associated with the *eae* genes, were classified in group B₂₃.

Many studies have reported EPEC strains belonging to phylo-group B2. Iguchi *et al.* (2009) found 98 genes that were specific to strains belonging to phylo-group B2 regardless of the pathotype. Virulence genes that are usually associated with specific pathotypes can, however, also be found all over the phylogeny, even in commensal strains (Escobar-Páramo *et al.*, 2004a). Genes such as *chuA* are not associated with specific pathotypes, but are specific to phylogenetic groups B2 and D (Escobar-Páramo *et al.*, 2004a). Studies have reported that EPEC *E.coli* is mostly classified in either phylo-group B1 or B2 depending on the type of *eae* gene. Phylo-group B1 is associated with the *eae* β 1 and B2 with *eae* α 1 or *eae* α 2 (Escobar-Páramo *et al.*, 2004a; Tramatu *et al.*, 2008).

Houser *et al.* (2008) investigated *E.coli* isolates shed by healthy lactating dairy cattle and also found EPEC strains belonging to group B2, but also to group A, B1 and D. ETEC strains were also found in group B2 and D, while extraintestinal pathogens were found in all four groups. It was concluded that in the intestinal gut, phenotypic and genotypic changes are continuously occurring due to gene transfer and genetic mutations which leads to the development of certain gene carrying bacterial species. Another study also placed EPEC strains isolated from rainwater tanks mostly in the phylo-group B2, while some of the EPEC strains were distributed across all four groups (Ahmed *et al.*, 2011).

When the phylogenetic grouping of atypical EPEC was determined by Afset *et al.* (2008) using the method of Clermont *et al.* (2000), 56 strains were found belonging to all four phylogenetic groups. The majority (24) of the strains were classified in the B2 group, followed by group B1 (16). Three major clusters were detected based on the virulence gene profile of the

EPEC strain. One of the clusters included group A, B1 and D, while the other two clusters had strains only belonging to group B2. The main separation criteria between the four groups were due to the differences in their virulence profiles. It was concluded that atypical EPEC was very diverse regarding its phylogenetic and virulence profile.

Other studies also found 14.3% of the EPEC isolates belonging to group B2 and more specifically 4.5% of these strains were classified in group B2₃. However most of the intestinal *E.coli* belonged to group A and D, while the extraintestinal bacteria belonged to group B2 (Regua-Mangia *et al.*, 2010).

Apart from the three EPEC strains being classified under group B2₃ in this study, six other strains isolated from the river and the green bean samples (not EPEC strains) were also classified under group B2₃. When commensal *E.coli* isolates were phylogenetically distributed by Duriez *et al.* (2001), it was found that A, B1 and D are the main phylo-groups present in the human gut and that these strains must acquire virulence factors to become pathogenic. The commensal strains allocated in all four the phylogroups, all had virulence factors, but fewer than the pathogenic strains in the corresponding group. Since the virulence pattern of commensal and pathogenic *E.coli* classified in the B2 phylo-group were the same, it appears that some commensal B2 strains could be virulent, even though it was rare.

The B2 phylo-group is usually associated with extraintestinal *E.coli* (ExPEC) that can cause meningitis (MENEK), urinary tract infections (UPEC) and septicemia (Escobar-Páramo *et al.*, 2006). According to Hamelin *et al.* (2007), phylo-group B2 has the most pathogenic strains, especially ExPEC strains (Hamelin *et al.*, 2007). Although ExPEC strains occur mostly in phylo-group B2 or D, they can also be found in phylo-group A and B1 while carrying a variety of ExPEC associated virulence genes (Ahmed *et al.*, 2011). These virulence genes of ExPEC strains are inherited vertically within evolutionary ancestry or by horizontal transfer between ancestry on pathogenicity islands or plasmids (Johnson *et al.*, 2001).

Since the six strains (B835, B1255, PCW11, PCB95, B627 and W734) in group B2₃ did not test positive as intestinal pathogens (EPEC, EHEC, ETEC, EIEC, EaggEC and DAEC) there might be a possibility that these strains belong to the ExPEC group and could pose a risk for extra-intestinal infections. Even commensal strains from phylo-group B2 harbour more virulence genes than the other commensal strains found in the other groups (Escobar-Páramo *et al.*, 2004a). In this study no PCR was performed to determine if ExPEC strains might be present, therefore future research is required to determine if these isolates belong to the ExPEC group.

Using phylogenetic classification to identify the source of the faecal pollution

Many studies have been done regarding the use of phylogenetic classification to determine the source of faecal pollution. Strains from animal and human origin were analysed by Goulet &

Picard (1986) and they showed that only 1.6% of the animal strains belong to group B2, compared to the 9% of strains from human origin.

More recently, Carlos *et al.* (2010) identified sources of faecal pollution by using phylogenetic classification based on genotyping. Strains that were classified as group B1, were from human and different animal hosts, especially the herbivores (cow, goat and sheep). Since *E.coli* are found in human hosts as well animals it is not a good indicator of herbivore faecal contamination. According to Carlos *et al.* (2010) group A were mostly associated with omnivorous mammals. Groups D₁ and D₂ are also not good indicators because human, cow, pig and sheep isolates were found among the D phylo-group. However, chicken and goat hosts were not classified in phylo-group D. This could indicate that faecal pollution by these animals pose a higher risk of extraintestinal *E.coli*. Humans were the only host that had isolates classified in all the groups, with the exception of group A₀. Strains that were classified in group B2₃ were only found in humans. Therefore, based on the results found, group B2, especially group B2₃ is a good indicator of human faecal pollution.

Escobar-Páramo *et al.* (2006) observed that bird isolates mostly belonged to group D and B1, non-human mammals to group A and B1, whereas humans were mostly associated with group A and B2. Group B1 was also the predominant group for zoo animals (Higgins *et al.*, 2007). Again, this shows the influence of domestication on the phylo-distribution of *E.coli*. Domesticated or farm animals are subjected to high antibiotic pressure and have more B1 strains, which are more antibiotic resistant than B2 strains. This could be the reason why these animal strains are mostly associated with phylo-group B1 (Escobar-Páramo *et al.*, 2006).

The phylogenetic distribution and virulence distribution of commensal *E.coli* strains isolated from 10 mammals living in the same zoo, revealed that *E.coli* from herbivores were mostly identified as phylo-group B1, with all the strains containing virulence factors also classified in this group (Baldy-Chudzik *et al.*, 2008). For the carnivores and omnivores more strains were classified in phylo-group A than the herbivores. The virulence gene positive strains were classified in phylo-group A. Phylo-group A virulence genes were mostly associated with ETEC pathotypes and phylo-group B1 with EHEC/EPEC pathotypes.

The phylo-distribution of *E.coli* isolates in surface waters were investigated by Hamelin *et al.* (2007). They found that the majority of the isolates with virulence factors were present where sewage effluents were being released into the river. Most pathogenic strains belonged to phylo-groups B2 and D, with ExPEC pathotypes being the most frequently found. Non-pathogenic strains belonged mainly to phylo-groups A and B1. Phylo-groups A and B1 were also mostly associated with sea-gulls, while phylo-groups B2 and D were mostly associated with agriculture, wildlife and human faecal pollution. The EPEC strains were again reported to be distributed among all four groups.

It has been reported that beside gut characteristics, the diet of the host plays an important role in the phylo-group distribution. Carlos *et al.* (2010) found that genetic markers were specifically distributed among omnivorous and herbivore mammals, with omnivorous mammals, such as humans and pigs, having the highest diversity of indexes. Similarly, Escobar-Páramo *et al.* (2006) reported that domestication (diet modification and high antibiotic pressure) was the main factor that shapes the genetic structure of *E.coli*. Domestication also lead to a lower diversity in microbiota, which probably explains why humans had a lower diversity compared to animals.

It has also been reported that the phylo ditribution among geographically different human populations were different for each population. This is probably because they are influenced by the geographic/climatic conditions which influence many other biologic processes, hygiene levels, dietary fators or the way food is processed by refrigeration chains, the use of antibiotics and host genetic factors. Some *E.coli* strains could be primarily adapted to the conditions of the human gut (Duriez *et al.*, 2001; Escobar-Páramo *et al.*, 2004b).

If the findings of the above reported studies are applied to the strains W630, W33A and W1371 in this study, the EPEC strains classified as B₂₃, might be considered of human origin. The other B₂₃ strains (B835, B1255, PCW11, PCB95, B627 and W734) could also be from human origin. If the faecal pollution was from human origin it might be that the source was upstream and from the informal settlement, Kayamandi. It has previously been reported that the faecal pollution of the Plankenburg River increased after it flowed past Kayamandi (Barnes & Taylor, 2004). The A₀ group might be from omnivorous origin, similar to the findings of Carlos *et al.* (2010) who found no strains of human host classified under group A₀. The B1 isolates could be from human or animal origin, but literature indicates that most B1 strains are from animal origin. Thus, further studies are needed to determine if this is in fact true. If one can identify the source, it might help to try and eliminate the problem in the future to ensure a less contaminated river.

Overall, the phylo-groups did not facilitate linking except for the “pure” culture *E.coli*. The “pure” culture *E.coli* from the irrigation water were in the same phylogroup (B₂₃) as the *E.coli* on from green beans.

Phase 6 linking: Linking based on *oriC*-locus and *dnaJ* sequence analysis

Strains, based on the API 20E clustering data, (Table 11) and that indicated a carry-over from the river water to the green beans were selected for further sequencing analysis. These strains were found to be identical at the phenotypical level, but sequencing analysis was needed to confirm the API 20E data. The selected strains included two of the EPEC strains that did not belong to any of the API 20E clusters. *E.coli* reference strains R58 (ATCC 11775) and R157 (ATCC 4350) were also included.

The chromosomal replication origin, *oriC*-locus and the housekeeping gene, *dnaJ* were chosen as sequencing base since they are highly conserved genes (Nhung *et al.*, 2007; Roggenkamp, 2007). It is known that the 16s rRNA cannot be used to distinguish between species that are closely related, since there are high similarities in this gene region between species (Spröer *et al.*, 1999; Roggenkamp, 2007).

Table 11 Species selected for sequencing analyses based on API 20E clustering data

| Strain | Source | Isolation day | Species | Phenotypic cluster |
|--------|----------------------------|---------------|---------------------|--------------------|
| W839 | River water | 8 | <i>K.pneumoniae</i> | A |
| B943 | Green beans | 9 | <i>K.pneumoniae</i> | A |
| W632 | River water | 6 | <i>E.coli</i> | C |
| B835 | Green beans | 8 | <i>E.coli</i> | C |
| W315 | River water | 3 | <i>E.coli</i> | D1 |
| W734 | River water | 7 | <i>E.coli</i> | D1 |
| B1466 | Green beans | 14 | <i>E.coli</i> | D1 |
| W427 | River water | 4 | <i>E.coli</i> | E |
| B627 | Green beans | 6 | <i>E.coli</i> | E |
| W1052 | River water | 10 | <i>E.coli</i> | E |
| B1255 | Green beans | 12 | <i>E.coli</i> | E |
| PCW11 | "Pure" culture water | 1 | <i>E.coli</i> | E |
| PCB95 | "Pure" culture green beans | 9 | <i>E.coli</i> | E |
| W313 | River water | 3 | <i>E.cloacae</i> | G |
| B411 | Green beans | 4 | <i>E.cloacae</i> | G |
| B731 | Green beans | 7 | <i>E.cloacae</i> | G |
| B1467 | Green beans | 14 | <i>E.cloacae</i> | G |
| B1573 | Green beans | 15 | <i>E.cloacae</i> | G |
| W630* | River water | 7 | <i>E.coli</i> | - |
| W733A* | River water | 7 | <i>E.coli</i> | - |

* Two EPEC strains included that were not in a clear API 20E cluster.

oriC-locus sequence analysis - The primers selected for the *oriC*-locus sequencing (EcOriC-73-f and EcOriC-292-r) were specific for *E.coli*, therefore only the *E.coli* strains (Table 11) were

sequenced and not the other coliforms. The neighbour-joining phylogenetic tree of the *E.coli* obtained from the *oriC*-locus sequences can be seen in Fig. 14. The *E.coli* sequences were highly similar and had no bootstrap support. This indicated that when based on the sequences of the *oriC*-locus, no significant differences could be found between the *E.coli* strains from the different API 20E clusters, even though the primers used were *E.coli* specific.

dnaJ sequence analysis - The *E.coli*, *K.pneumoniae* and *E.cloacae* strains included (Table 11) were also subjected to *dnaJ* sequence analysis. In Fig. 15 the neighbour-joining tree and bootstrap values of the *E.coli*, *K.pneumoniae* and *E.cloacae* *dnaJ* sequences are shown. The *dnaJ* sequences showed more differences than the *oriC* sequences of the *E.coli* strains from the different API 20E clusters.

It was, however, expected that the *oriC*-locus would give a greater divergence between the strains, compared to the *dnaJ* sequences. This is because the primers used for the *dnaJ* sequences were used for all *Enterobacteriaceae* species (Nhung *et al.*, 2007), whereas the *oriC*-locus primers were specifically used for *E.coli* (Roggenkamp, 2007). Roggenkamp (2007) found that strains from the same species will at least be 97% similar based on the *dnaJ* sequences. It might be that the *oriC*-locus fragment of 217 bp was too small to show any high divergence between the *E.coli* strains in comparison to the *dnaJ* fragment of 758 bp in this study.

Generally bootstrap values of >85% are considered significant and can be used to identify different clades of isolates (Reid *et al.*, 2000). In Fig. 15 the *E.coli*, *K.pneumoniae* and *E.cloacae* species could be clearly distinguished, with the *E.coli* strains dividing into two main clades with good bootstrap support (100% for *E.coli* clade 1 and 88% for *E.coli* clade 2).

The *K.pneumoniae* species had a significant bootstrap value of 100% (Fig. 15). These two *Klebsiella* strains included in this analysis did however show large *dnaJ* sequence differences. It is recommended that more *K.pneumoniae* strains as well as the type strain is included in future *dnaJ* analyses to investigate carry-over *Klebsiella* strains.

The *E.cloacae* species had a bootstrap value of 99% and differed significantly from the other two species. Within the *E.cloacae* species, the four bean isolates (B411, B731, B1467 and B1573) showed large differences in their *dnaJ* sequences (Fig. 15). These strains are therefore considered not to be identical, even though these strains grouped together in the same API 20E data cluster (G). This once again shows the importance of combining phenotypical data with molecular information to investigate carry-over. It cannot be confirmed whether these strains were not present in the river water, because initially not all the colonies were enumerated from the VRBA plates and only a few of the selected *E.cloacae* strains were sequenced. The only *E.cloacae* strain included in the *dnaJ* analysis that was isolated from the river water (W313) showed a degree of similarity with a strain isolated from the green beans (B1467) and grouped together with a bootstrap value of 100%. This might indicate carry-over, although this needs to be

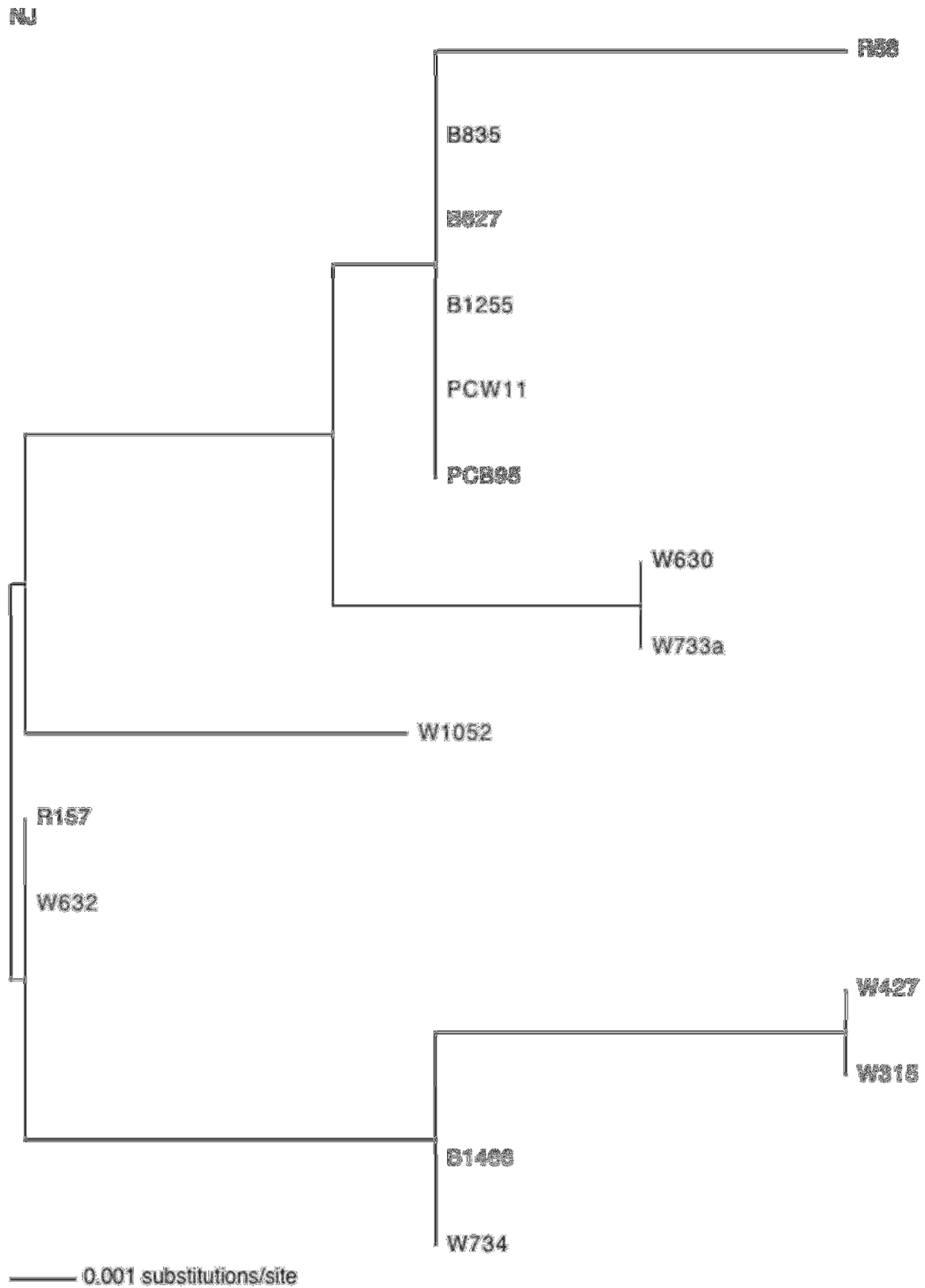


Figure 14 Neighbour-joining phylogenetic tree of *E. coli* strains based on the *oriC*-locus sequences.

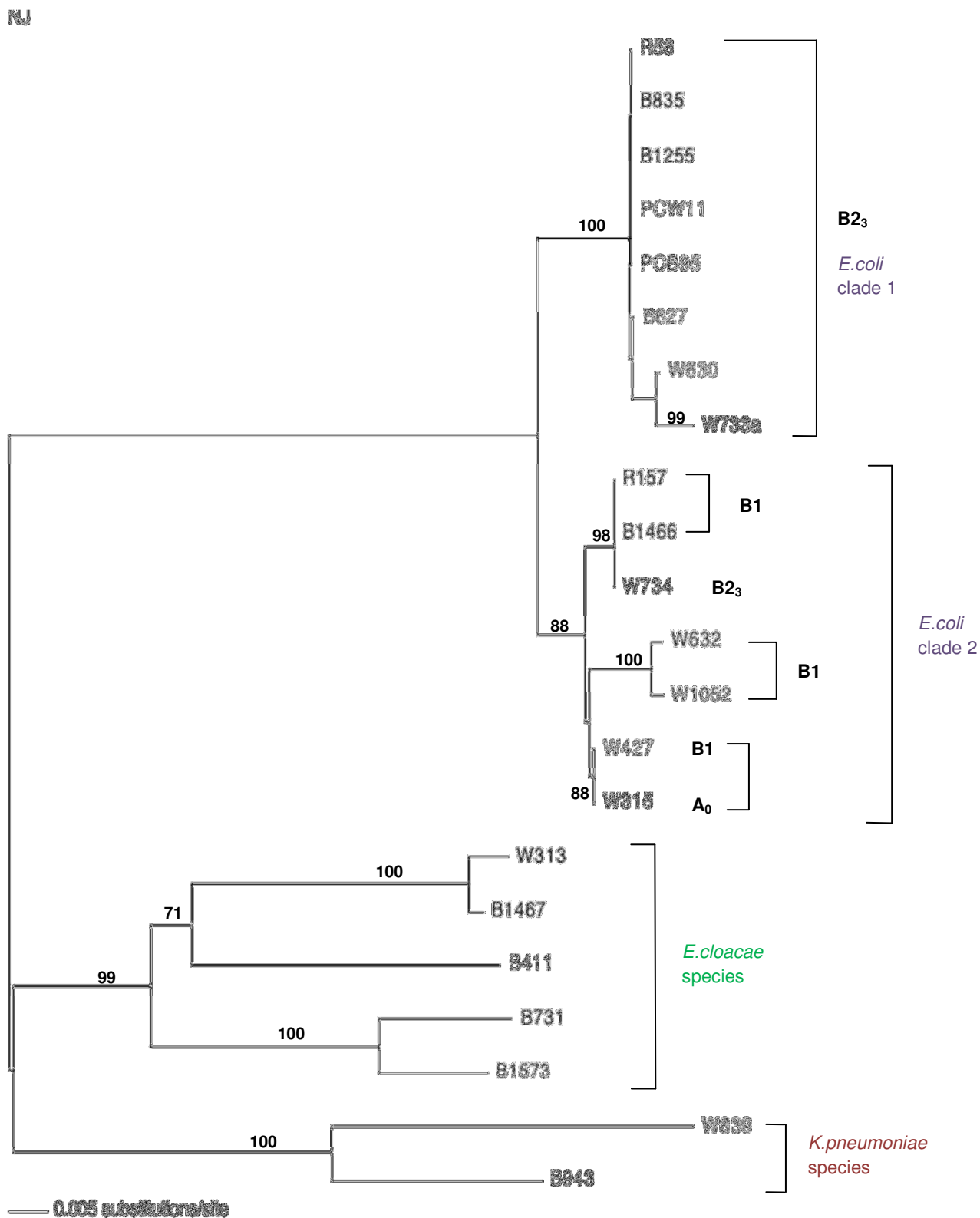


Figure 15 Phylogenetic tree of *E. coli*, *K. pneumoniae* and *E. cloacae* strains based on the *dnaJ* sequences with neighbour-joining and bootstrap values with included *E. coli* phylogenetic groups.

confirmed by other means such as phylogenetic analyses of less conserved genes of *E. cloacae*. Strain W313 was isolated from the river water on day 3 and strain B1467 was only isolated from the green beans on day 14. If B1467 are indeed a clone of W313, it might be that this specific strain was continuously present in the river water, but not always selected or it might be that this *E. cloacae* strain was able to survive for more than 10 days on the green beans.

The first *E. coli* clade contained the reference strain (R58), B835, B1255, PCW11, PCB95, B627, W630 and W733A, all of which had very similar *dnaJ* sequences. This clade had a bootstrap value of 100% and therefore differed significantly from the other *E. coli* clade. The clade contained two strains isolated from the river water and all were classified in the same phylo-group B2₃. All the strains were found in the API 20E cluster E, except for B835 which was in cluster C. Although linking could not be conclusively confirmed, carry-over of *E. coli* from the river water to green beans appear to be highly probable since both bean and water isolates (PCW11 and PCB95) grouped together in this clade. Due to the conserved nature of the *dnaJ* gene it was, however, not possible to distinguish between the reference strain (R58) and the environmental strains isolated from the beans and river water samples.

The first *E. coli* clade also contained two EPEC strains (W630 and W733A), but no EPEC genes were identified in any of the *E. coli* green bean strains chosen for phylogenetic analysis. It could be that EPEC strains do not survive very well on the surface of green beans or the EPEC numbers in the water were too low and neutralised during the dilution steps. More studies are however necessary to determine if this is true. As no EPEC strains were found on any of the green bean strains chosen for phylogenetic analysis and these two strains were not classified in any of the main API 20E clusters, it was expected that these two strains would group alone without any similar *E. coli* strains from the green beans.

Within the second *E. coli* clade three smaller groups emerged. The first group had a bootstrap value of 98%, and no differences in *dnaJ* sequences were observed for a strain isolated from the river water (W734), green beans (B1466) and the reference strain (R157). Both strains, W734 and B1466, were in the same API 20E cluster, D1. This could have been an indication of the carry-over of *E. coli* from the irrigation water to the green beans. In contrast each strain was classified under a different phylo-group. Strain W734 was classified in phylo-group B2₃ and B1466 along with the reference strain, in B1. Whether it is the same strain that persisted on the green beans for 7 days after being isolated from the river water or if a mutation occurred is unclear.

The next group in *E. coli* clade 2 had a bootstrap value of 100% and contained two strains, W632 and W1052 both isolated from the river water and both were classified in phylo-group B1. The phylogenetic tree analysis also showed little difference in *dnaJ* sequences. The strain W1052 was, however, identified as an EPEC strain in the previous analysis. W1052 was also placed in the API 20E data cluster E. Strain W632, on the other hand, was placed in a different API 20E data cluster (C). This is another example of where phenotypical analysis data provided more

discriminative information than phylogenetic analysis of *dnaJ* sequences, or genogrouping. The last group in *E.coli* clade 2 had a bootstrap value of 88% and also had two strains isolated from river water, W427 and W315. The two strains were however classified in two different phylo-groups, B1 and A₀ respectively. It could be that the W315 in group A₀, should actually be classified in B1 as it has been previously found that strains classified in group A₀ are B1 strains wrongly grouped or not *E.coli*. (Gordon *et al.*, 2008). The previous phenotypical analyses and molecular analysis (PCR, m-PCR and BLAST) identified the strain as *E.coli*; therefore it could be that the *E.coli* supposedly could belong to a different phylo-group as B1. Again both strains are from different API 20E clusters (E and D1), indicating the importance of using a variety of methods to prove carry-over at different levels. It could be that these strains were carried-over to the green beans, but were not enumerated or sequenced. If more strains were included in the phylogenetic analysis it might further confirm carry-over in each species.

CONCLUSIONS

During the 14 day plot study the *E.coli* counts in the irrigation water varied. Although the counts were always above the WHO irrigation guideline of a 1 000 faecal coliforms per 100 mL (WHO, 1989) and reached a maximum value of 118 500 cfu.mL⁻¹. This indicated that the Plankenburg River (Plank-1 site) is not suitable for irrigation. It was, however, found that minimal impact regarding the carry-over to green beans was observed when the irrigation water was in the 10³ cfu.mL⁻¹ range. It appears that *E.coli* does not survive for long on green beans when present at low concentrations (10³ and 10⁴ cfu.mL⁻¹) in the irrigation water. This could be an indication that it is possibly a “safe” concentration to irrigate fresh produce with and that no or a minimum number of *E.coli* will be carried over.

The counts on the green beans varied depending on the counts in the irrigation water. It was found that large *E.coli* increases on the green beans occurred even though the *E.coli* in the irrigation water was lower. This was also previously found by Ackermann (2010). The reason for this was possibly due to “clumping” as temperatures did not vary much across the 14 days. This indicates the importance that plate counts of the green beans could be lower than the actual count on the green beans due to the formation of clumps.

The data found in this study clearly indicated that there is indeed a carry-over of *E.coli* and other organisms from irrigation water to the green beans with daily irrigation. The effect of “once-off” irrigation at low concentrations lead to minimal impact and decreased survival of the *E.coli* on the green beans, possibly due to minimal moisture and nutrients.

The first phase of linking was proven using API 20E clustering where identical strains were found in the irrigation water and on the green beans a few days after being irrigated. Phase 2 linking statistically identified the linking of organisms based on the data (carbon source utilisation)

of the API 20E. *E.coli*, *E.asburiae* and *E.cloacae* were the species mostly isolated from the green beans as well as from the Plankenburg irrigation water. It was therefore concluded that these organisms are the most abundant organisms in the Plankenburg River.

The importance of combining phenotypical methods in combination with molecular methods was confirmed in this study, as the API 20E misidentified some *E.coli* as *C.freundii* isolates as well as *E.coli* pink colonies instead of purple colonies on Selective *E.coli*/Coliform chromogenic agar. Molecular methods such as PCR (phase 3 linking), m-PCR (phase 4 linking) and sequencing (phase 6 linking) were necessary for correct identification. For correct identification and linking all of these methods are necessary.

Multiplex-PCR identified four strains in the river water as EPEC strains. These strains were isolated over 14 days and showed that there was a continuous source faecally polluting the river water. Since the presence of EPEC strains have been shown, it is an indication that the Plankenburg River could possibly be a source of foodborne diseases making the river unsafe for irrigation and drinking purposes. Even though these strains were not found on the green beans, it might be that if more colonies were enumerated from the green beans EPEC strains would also have been found on the green beans.

Based on the genotyping data (phase 5 linking) the strains belonging to group A₀ and B₁ and the majority of selected strains belonging to phylo-group B_{2₃}, were identified. It has previously been found that strains belonging to phylo-group B_{2₃} are only found in humans (Carlos *et al.*, 2010). Therefore it could be that the faecal pollution in the Plankenburg River is from human origin. It can be speculated that the informal settlement, Kayamandi, might be the source as it has previously been found that the faecal pollution increased after the Plankenburg River flowed past Kayamandi (Barnes & Taylor, 2004). Further source-tracking methods would be of great importance to confirm whether Kayamandi is the only source contributing to the faecal pollution of the Plankenburg River.

Three of the EPEC strains were classified in phylogroup B_{2₃}, but it was found that ExPEC strains are mostly classified in phylo-group B_{2₃}. In this study the presence of ExPEC strains had not been determined, therefore there is a possibility that the Plankenburg River can contain many ExPEC strains as many strains that were not identified as an intestinal pathogens were classified in phylo-group B_{2₃}. It is recommended that a PCR analysis should be performed to determine if these strains are indeed present.

Overall, several diverse types of *E.coli* were found to be present in both the irrigation water and on the green beans indicating carry-over and linking. The API 20E data gave the first indication of different *E.coli* types as clusters with different phenotypical characters were found. Further confirmation of linking was confirmed with PCR methods and sequencing, which is necessary for accurate confirmation. The sequencing of the *oriC*-locus showed minimum differences between *E.coli* strains and had no bootstrap support, in comparison to the *dnaJ*

sequences which were able to yield different clades with significant bootstrap values (>85%). Therefore the *oriC*-locus was not a good method in this study to distinguish between different *E.coli* strains. Other DNA-fingerprinting methods such as REP-PCR or BOX-PCR could also be useful to determine if more differences can be observed between *E.coli* strains. The *dnaJ* sequences were able to distinguish clearly between the *E.coli*, *K.pneumoniae* and *E.cloacae* species. Linking of the environmental strains based on the *dnaJ* sequences could not be conclusively confirmed, however, carry-over of *E.coli* from the river water to the green beans appear to be highly probable since both bean and water isolates (PCW11 and PCB95) grouped together in the same clade.

The importance of molecular methods was showed as it is important for correct confirmation and identification. It was sometimes found that the phenotypic methods showed incorrect identifications. Therefore future studies should always include phenotypic and molecular methods for correct identification. The study still showed that there is a great probability of *E.coli* and other faecal coliforms being carried over from contaminated irrigation water to fresh produce. It is therefore concluded that highly contaminated irrigation water will result in high numbers of *E.coli* and other coliforms being carried over to fresh produce.

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APPENDIX 1

Phenotypic characters determined by API 20E

| Isolate | ONPG | ADH | LDC | ODC | CIT | H2S | URE | TDA | IND | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX | NO2 | N2 | MOB |
|---------|------|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|----|-----|
| Ec58 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| EC157 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| EC158 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| EC404 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B31 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B32 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B35 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B36 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B37 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B38 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B410 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B411 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B413 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B414 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B516 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B517 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B518 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B519 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B621 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B622 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B623 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B624 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B625 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B626 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B627 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B628 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B629 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B730 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B731 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B732 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B733 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B734 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B835 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B836 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B837 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B838 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B839 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B940 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B941 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B942 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B943 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B944 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |

| Isolate | ONPG | ADH | LDC | ODC | CIT | H2S | URE | TDA | IND | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX | NO2 | N2 | MOB |
|---------|------|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|----|-----|
| B1045 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1046 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1047 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1048 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1049 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1150 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1151 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1152 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B1153 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1154 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1255 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1256 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B1257 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1258 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1259 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B1360 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1361 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1363 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1364 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1466 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1467 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1468 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1469 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1470 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B1571 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1572 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1573 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1574 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B1575 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W11 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W12 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W13 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W14 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W15 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W16 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| W17 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W28 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W29 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W210 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| W211 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| W312 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W313 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W314 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W315 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W316 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |

| Isolate | ONPG | ADH | LDC | ODC | CIT | H2S | URE | TDA | IND | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX | NO2 | N2 | MOB |
|---------|------|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|----|-----|
| W417 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W418 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W419 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| W426 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| W427 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W520 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| W521 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W522 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W523 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W524 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W629 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W630 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W631 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W632 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W733A | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W733B | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| W734 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W735 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| W736 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W737 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W838 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W839 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| W840 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W841 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W842 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W943 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W944 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W945 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W946 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W947 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W948 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1049 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1050 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1051 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1052 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1158 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1159 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1160 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1161 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1262 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1263 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1264 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1265 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1266 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1267 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |

| Isolate | ONPG | ADH | LDC | ODC | CIT | H2S | URE | TDA | IND | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX | NO2 | N2 | MOB |
|----------|------|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|----|-----|
| W1368 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1369 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1370 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| W1371 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1372 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1473 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1474 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1475A | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1475B | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| W1476 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| W1477 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| PCW11 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB31 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB32 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB63 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB64 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB95 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB96 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB127 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB128 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB159 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| PCB1510 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB11 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB12 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB13 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB14 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB15 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB26 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 104PCB27 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 103PCB11 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Over the last few decades the consumption of fresh produce has increased, but it has also resulted in an increase in foodborne outbreaks. Irrigation water has been found to be a major source of faecal contamination and many outbreaks have been linked to contaminated irrigation water. Research has shown that many South African irrigation water sources have faecal coliform levels above the WHO and DWAF recommend guidelines of a 1 000 faecal coliforms per 100 mL. With these abnormally high faecal coliform levels there is an increase in the risk of carry-over to produce that is consumed raw or even after the application of minimal processing steps.

Once fresh produce gets contaminated, microbes can survive or even multiply on the produce but very little information is available on what impact varying environmental conditions will have on the survival and growth of potential pathogens. The ideal growth conditions for survival and growth of these pathogens are higher temperatures and moisture conditions, and nutrient rich environments. Since temperature and carbon/nutrient levels have major impacts on *E.coli* growth, it is important to know what the impact of different carbon concentrations, temperature and incubation time on *E.coli* growth in river water is. Thus, the overall objective of this study was to determine the impact of selected environmental factors on *E.coli* growth in river water that is used for irrigation purposes and to then to investigate the carry-over of *E.coli* from contaminated irrigation water to fresh produce under more controlled environmental conditions.

This first phase of the study focused on the impact of different environmental factors like carbon levels, temperature, incubation time and initial microbial load on the growth increases of *E.coli* and other "indigenous or viable aerobic" occurring microbes present in the Plankenburg river water. The same environmental factors were also monitored using a previously characterised environmental "pure" *E.coli* isolate to eliminate the impact of "competing indigenous" microorganisms.

The data obtained confirmed that an increase in temperature leads to an increase in the numbers of *E.coli* in non-sterile river water. It was also found that growth increases were limited at temperatures between 10° and 20°C, but that major increases occurred at temperatures of 25°C and higher. Therefore a general increase in river temperatures will impact the microbial load, especially when the river is highly polluted (high nutrient levels). One important aspect that was found was that regardless of the temperature, the *E.coli* in non-sterile river water died-off rapidly when the nutrient levels were low. It was therefore concluded that under specific conditions, the carbon (COD) level will be the major growth limiting factor in river water. This could be taken as an indication that when the river water is relatively unpolluted the faecal coliforms would not show large increases in numbers and not survive for long. Therefore a low carbon level in irrigation

water, could result in lower faecal coliform counts which in turn might result in less carry-over of *E.coli* from the water to fresh produce.

Based on the above it was essential to evaluate the impact of adjusted carbon levels of the river water on the growth of the *E.coli* present. At lower carbon levels (COD = <10 mg.L⁻¹) the *E.coli* was found to die-off faster at the higher temperatures, possibly due to nutrient limitations and nutrient competition by other non-faecal organisms. The growth profiles of *E.coli* at higher adjusted values (COD = >100 mg.L⁻¹) clearly showed major growth increases, with no die-off during a 24 h period, probably because of sufficient nutrient levels that were available. Overall it was clear that the carbon level of river water will have a major impact on microbial growth, survival and die-off and this will have an impact on pathogen carry-over and subsequent food safety when such water is used for irrigation of fresh produce.

Currently there are no guidelines or limitations regarding carbon (COD) levels as a method to try and control microbial growth in river water, therefore based on the data found in this study it would be of value to implement such a pollution limiting (especially the carbon (COD)) guideline. Such a guideline would be of value for river water intended for irrigation, in terms of carry-over loads and subsequent food safety.

As shown above many factors can affect the survival and growth of faecal coliforms in polluted irrigation water and their subsequent carry-over to fresh produce. Even though standard methods can be used to identify if irrigation water is faecally contaminated, it is difficult to follow the specific carry-over of microbes to produce using conventional techniques. It is thus important to be able to confirm that the identified organism on the fresh produce is from the faecally polluted irrigation water. Thus, the main objective of the next phase of the study was to specifically try and link the *E.coli* from the Plankenburg irrigation water to that found on the irrigated green beans.

The second study phase focused on: the effect of daily irrigation on carry-over; the effect of "once-off" irrigation on the survival of *E.coli* on the produce; identifying different types of *E.coli* in the irrigation water and those present on the irrigated fresh produce; linking (phenotypical and molecular methods) the different *E.coli* types in the irrigation water to those present on the irrigated produce; and using the information from the genotyping to possibly identifying the source (human or animal) responsible for the faecal pollution of the irrigation water.

The results from this phase indicated that members of the species *E.coli* are carried over from the water used to irrigate green beans, especially when the *E.coli* levels in the river water were high. These results showed that the Plankenburg River is heavy contaminated with faecal material (highest concentration - 118 500 cfu.mL⁻¹) and also exceeded the recommended WHO guideline of a 1 000 faecal coliforms per 100 mL (WHO, 1989). It was, however, found that the impact of carry-over that took place to green beans when the load in the irrigation water was in the 10³ - 10⁴ cfu.mL⁻¹ range, was minimal. This could be that the *E.coli* does not survive the irrigation process when present at concentrations lower than 10³ to 10⁴ cfu.mL⁻¹ in the irrigation water. This

could possibly be taken as an indication of a “safe” load for fresh produce irrigation and that no or a minimum number of *E.coli* will be carried over.

It must be noted that the lowest *E.coli* counts on the green beans were also found when the highest number of *E.asburiae* species (a natural bacterium), were present. This could be an indication that *E.asburiae* inhibits the growth of *E.coli* as reported in literature (Cooley *et al.*, 2006). It is thus possible that this observation could be used as a potential inoculation method to minimise *E.coli* carry-over to fresh produce.

It was also found in this study the *E.coli* levels on the green beans were higher than in the irrigation water on some days. This could have been due to accumulation or "clumping" as previously reported in literature (Ackermann, 2010).

After the enumeration steps to monitor faecal and *E.coli* levels, colonies from both the irrigation water and from the irrigated beans were selected, purified and characterised. A total of 67 microbes were isolated from the green beans and 72 from the irrigation water. Unique phenotypic (API) profiles were generated for each of the 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 a large degree of character variation between the different isolates, many were grouped together in several related sub-clusters. Based on the phenotypic groupings it was concluded that these isolates were related and originated from the same pollution source. It was thus concluded 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.

This data represented the first indication of linking, which was followed by PCR confirmation that all the strains phenotypically identified as *E.coli* were *uidA* positive. Interesting to note was that some miss-identification of *E.coli* strains as *C.freundii* strains did occur. Some *E.coli* strains were also found to be pink on Brilliance *E.coli*/Coliform selective agar. This suggests that phenotypical methods should be done in combination with molecular methods to positively confirm the identity of isolates.

All strains that tested *uidA* positive were subjected to multiplex PCR for further linking confirmation of carry-over. The multiplex PCR data showed that all the strains tested positive for the *mdh* gene, again indicating that all were members of the *E.coli* species. Among other linking data, this revealed the presence of EPEC strains in the irrigation water. This could make the Plankenburg River a potential source of disease, as EPEC is one of the six human intestinal pathogenic *E.coli* groups. The EPEC strains found in this study were isolated on different days over the course of the sampling period, indicating that it was not a "once-off" contamination source, but that there is a source constantly faecally contaminating the river water.

Based on the API clustering, the presence of the *uidA* gene and the multiplex PCR data, 13 of the *E.coli* isolates were selected and analysed using triplex PCR. All these strains showed

positive carry-over links between the irrigation water and the green beans. Of the triplex PCR data one, three and nine *E.coli* isolates were classified among phylogenetic groups A₀ (*chuA*⁻, *yjaA*⁻, TSPE4.C2⁻), B₁ (*chuA*⁻, *yjaA*⁻, TSPE4.C2⁺) and B₂₃ (*chuA*⁺, *yjaA*⁺, TSPE4.C2⁺), respectively. Of the strains none showed a direct phylogenetic link to the three main phenotypic *E.coli* clusters. Even though the triplex PCR method was not of value for linking purposes it is possible that the phylogenetic data can be used to identify the source of faecal pollution, as it is known that members of the group B₂₃ are only associated with human faecal pollution. The data obtained suggests that it could be argued that humans are the main source polluting the Plankenburg River upstream. It would also be of importance to confirm whether Kayamandi is the possible main source contributing to the faecal pollution of the Plankenburg River. Once the source of the faecal pollution is identified, it could be the first step to help minimise or possibly eliminate the constant pollution.

For further linking confirmation, DNA sequencing was done on selected strains, based on the API cluster analysis. The *oriC*-locus sequencing was unsuccessful in distinguishing between the different *E.coli* strains as it had no bootstrap support.

The *dnaJ* sequences did however show differences between the *E.coli*, *E.cloacae* and *K.pneumoniae* species. Overall it was concluded that it is highly probable for *E.coli* and other faecal coliforms to be carried-over from irrigation water to fresh produce. In the control study the "pure" *E.coli* culture was successfully linked with all the phenotypical and molecular methods. The importance of a "multi-method" approach to confirm carry-over was also indicated to prove carry-over at different levels.

For future studies it is recommended that confirmatory studies be done using larger numbers of *E.coli* isolates and subjecting them to both biochemical and molecular analyses. This could also be used to provide valuable information regarding possible linking in strains from irrigation water and fresh produce.

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