INVESTIGATION INTO THE LINK BETWEEN WATER QUALITY AND MICROBIOLOGICAL SAFETY OF SELECTED FRUIT AND VEGETABLES FROM FARMING TO PROCESSING STAGES (VOLUME 1)

Report to the
WATER RESEARCH COMMISSION
and
DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES

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EXECUTIVE SUMMARY

Background and motivation

An increase in the consumption of fresh and minimally processed fruits and vegetables globally (Ayers and Westcot, 1985; Beuchat, 2002) has resulted in one of the most important health challenges in terms of foodborne diseases. In the United States of America (USA) these dietary changes resulted in doubling of produce-associated disease outbreaks per year from 1973-1992 (USA Food and Drug Administration, 1998). However, over the past 10-15 years the number of foodborne disease outbreaks has declined. The number of CDC outbreak reports varied between 1243 to 1417 during 2000-2002 in comparison to only 800 to 850 outbreaks reported from 2009-2013 (http://www.foodsafetynews.com/2015/06/the-prevalence-of-foodborne-illness; accessed 12 July 2015). Specific examples include three outbreaks in 2004 in the USA and Canada due to Salmonella, affecting 561 people who consumed fresh tomatoes [Centres for Disease Control and Prevention (CDC), 2005]. In 2006 another outbreak was associated with the consumption of tomatoes, with 183 cases being reported in 21 states (CDC, 2006a). Subsequently, a *Salmonella* outbreak, occurring from April to August 2008 in the USA, was linked to the consumption of raw tomatoes, jalapeno and cilantro. This multi-state outbreak affected 1438 people with 282 people needing hospitalisation and possibly contributing to the deaths of two people (CDC 2008a). In 2008 a second outbreak was associated with cantaloupe. This affected 51 people in 16 states, with 16 people hospitalised (CDC 2008b).

Major disease outbreaks have also been associated with other pathogens such as *Escherichia coli* O157:H7, which affected 183 people in the USA in September 2006. Of these, 95 people were hospitalised, 29 people had haemolytic uremic syndrome and one person died (CDC 2006b). This outbreak was linked to the consumption of fresh bagged spinach. Additionally, a more recent multistate outbreak of *E. coli* O157:H7 in 2011 in 10 states of the United States caused 60 individuals to become ill (http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html; accessed 2015 June 25). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http:// www.bbc.co.uk/news/world-europe-13683270; accessed 2015 June 25). A Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated cantaloupes that left 146 people ill and caused 30 deaths in 28 states across the United States (http://www.foodsafetynews.com/2011/10/Cantaloupe-listeria-outbreak-84-sick-15-dead; accessed 2015 June 25).

According to the World Health Organisation about one third of 51 million deaths world-wide result from infectious and parasitic diseases. It has been estimated that worldwide 50 000 people die daily as a result of water-related diseases. Foodborne diseases due to food

contamination may occur at any point along the food chain, from the farm through food processing to the end user. However, the majority of foodborne outbreaks have been traced to contamination on the farm and the use of contaminated water (Westcot, 1997; Beuchat, 2002). Several studies have investigated or reviewed the impact of contaminated irrigation water on the spread of foodborne diseases (Ayers and Westcot, 1985; FDA, 1998; Venter, 2001).

Fresh produce is important from a national food supply and consumer health point of view while fresh fruit exports constitute a major part of the economy representing the third biggest earner of foreign exchange. South Africa has over the past 100 years built an international reputation as a major competitor in the export markets, delivering top quality and safe produce. South African farmers have further implemented measures to ensure compliance with new stringent good agricultural practices and food safety standards, allowing the country to remain a leading player in international food trade.

Unprecedented challenges facing the quality and quantity of our national water supplies have resulted in a new risk for farmers reliant on irrigation of cultivated crops (Gemmel and Schmidt, 2012; Chigor *et al*., 2013). Studies solicited by the Water Research Commission (WRC) entitled: "Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety" (WRC Report Number 1773/1-4/12) and "Health risk assessment in connection with the use of microbiologically contaminated source water for irrigation" (WRC Report Number 1226/1/04) have shown that the national water supplies are contaminated with pathogens and may pose a potential risk for the fresh fruit export industry and national safe food supplies. It was argued that a project that focuses on the risks in the supply chain will support and strengthen the findings in WRC Report Number 1773/1-4/12.

Aims:
The general and specific aims of this project were:

1. To investigate the links between water quality and microbiological safety in fresh minimally processed and frozen fruit and vegetables.
2. To review the literature study of WRC Report Number 1773/1-4/12 and expand the review to include a comprehensive literature study on processing water quality and its impact on the microbiological safety of food and potential effect on human health.
3. To establish co-operation with the team of researchers involved in WRC project K5/1773/4.
4. To select appropriate sites, with reference to i) different sources of untreated and treated water (excluding greywater), ii) different types of microbial contamination, iii) different types of farming – subsistence, emerging, commercial farming for local and export markets, iv) different crops and produce (specifically fruit and vegetables), v) different pathways of contamination: water for irrigation and water used for sprays, and iv) different pathways of contamination: water as transport and washing medium as part of packing and processing.
5. To propose and standardise the research methodology for the assessment of the impact

of contaminated irrigation water and processing water on microbial food safety.

6. To determine the extent (types and quantities) of contamination found in irrigation and processing water at the selected sites, with reference to i) indicators of water quality, ii) volume of water used for irrigation and processing, iii) microbial contaminants (viruses, bacteria, parasites), iv) physical parameters (pH, COD, EC, turbidity, temperature, etc.), and v) influence of personal on-site hygiene facilities vi) Influence of external quality control on food safety management at the farming and processing stages of production.
7. To investigate the impact of environmental parameters on the growth kinetics of contaminants, with reference to i) fate and survival, ii) decay rates, iii) surrogate organisms, and iv) different irrigation and processing technologies.
8. To study the extent of (types and quantities) contamination found on the irrigated raw produce (fruit and vegetables) up to harvest at selected sites, with reference to i) microbial contaminants (viruses, bacteria, parasites)m ii) microbial indicators/index of contamination/risk, iii) method of irrigation, agri-chemical spray water application, etc., and iv) investigate the potential link and/or association between contamination on raw produce and water applied (molecular epidemiology using a reference organism).
9. To study the extent (types and quantities) of contamination found on the raw produce (fruit and vegetables) after harvest, with reference to i) microbial contaminants (viruses, bacteria, parasites), ii) microbial indicators/index of contamination/risk, iii) additional contamination through handling, washing, storage, packaging, pre-freezing and minimal processing, etc., and iv) impact of biofilms in processing on product contamination.
10. To make interim and final recommendations for further research, with reference to: Further validation of results and Effective treatment options.

Research Findings

In this Volume the results for aims 2-7 (see page iii-iv) are reported on. The results for aims 8-10 and the extent to which the overall objective of this project was met will be reported on in Volume two of the report.

Aim 2: Literature review

After reviewing the literature study of WRC Report Number 1773/1-4/12, it was agreed to focus on aspects not covered in the first review. Topics covered in the previous literature study included i) the water situation in South Africa, ii) sources of water available, iii) quality of SA rivers, iv) water for agricultural uses, v) economic situation of the SA fruit and vegetable industry, vi) community importance of irrigated crops, vii) risk assessment of pathogens associated with vegetables and fruit, viii) pathogens associated with fresh produce including characteristics of bacterial pathogens (*Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, faecal streptococci and enterococci, *Shigella*, *Staphylococcus*, *Vibrio*, *Yersinia* and *Clostridium*), parasites (*Cryptosporidium* and *Giardia*) and viral pathogens (Norovirus and hepatitis A virus), ix) pre- and post-harvest sources of contamination, x) irrigation water and pathogen transfer, xi) infectious doses of the main water, soil and produce pathogens, as well as xii) treatment technologies for irrigation water.

Topics covered in this literature review therefore included i) heavy metal pollution and influence on safety of fresh produce, ii) agro-chemical spray water as a potential source of contamination of fruits and vegetables, iii) biofilms in water storage and distribution systems, iv) internalisation potential of human pathogenic bacteria in fresh produce, v) the incidence and control of foodborne pathogens on postharvest fruits and vegetables, vi) *L. monocytogenes* in vegetables and effect of minimal processing on its survival, vii) microbial source tracking using faecal indicator bacteria, and viii) microbial source tracking potential using phylogenetic analysis of noroviruses and hepatitis viruses.

Aim 3: Collaboration between researchers of WRC project 1773 and WRC project 1875

Co-operation within the team of researchers involved in WRC project 1773 and WRC project 1875 was established by organising four meetings and two methods workshops. Two of the research team members of the current project were also collaborators in the first project ensuring continuation, linking and consistency of sampling sites, crops and methodology employed. Additionally, one of the current research team members was appointed as a reference group member for the first project and visa versa.

Aim 4: Selection of appropriate sites and crop supply chains

A variety of fruit (tomatoes, peaches, pears and strawberries), vegetables (lettuce, spinach, cabbage, onions and broccoli), herbs (parsley, basil) and processed fresh produce (fruit salad and frozen vegetables) were sampled. For a description of the vegetable and fruit supply chains (up to, at and after harvest) included in this study, refer to Chapter 1, section 1.2 of this volume.

Water and produce samples were collected from selected sites in five provinces in South Africa, i.e. Gauteng (4 sites), KwaZulu-Natal (3 sites), Limpopo (2 sites), North West (3 sites), and the Western Cape (5 sites). Irrigation water sources included water from rivers, boreholes and dams as well as agricultural spray water. Irrigation methods included overhead irrigation (pivot and sprinklers), drip irrigation and micro irrigation systems. Different types of farming systems (commercial, small and large scale) were investigated and produce supplied for the local and international export market was assessed. Sites where water and produce samples were taken are described in detail in Chapter 1, section 1.3 of this volume.

Aim 5: Methodology

Standardised research methods for the assessment of the impact of contaminated irrigation and processing water on microbial food safety were selected. Methods selected included both standard ISO methods and published methods used by participants from both this and the previous WRC project. In order to transfer the methods and ensure consistency in test methods applied, two week-long training workshops were held, one at KwaZulu-Natal University, Department of Microbiology and one at the University of Pretoria, Departments of Food Science, Medical Virology and Microbiology and Plant Pathology. New methodology optimised and implemented included the Molecular detection system (3M, St. Paul, Minnesota, USA) for human pathogens (*E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp.) detection, Matrix Assisted Ionisation Time of Flight (MALDI-TOF) confirmation of the

presumptive *E. coli*, *Salmonella* and *Listeria* isolate identities, antibiotic resistance profiling, virulence gene profiling and Enterobacterial Repetitive Intergenic Consensus (ERIC) – PCR fingerprinting of *E. coli* isolates. Data analyses were based on results obtained with standard and new technologies. The standard ISO methods, published research methods and new methods are described in more detail in Chapter 1, section 1.4 of this volume.

Aim 6: Extent of microbial contamination found in the agriculture and agri-chemical spray water

Microbial analysis of irrigation water showed that the *E. coli* levels in irrigation water (river, dams and irrigation pipes) exceeded the DWAF (1996) guidelines of ≤ 1-1000 CFU/100 mℓ for crops to be eaten raw. However, although the *E. coli* counts of the primary irrigation source (rivers, dams) were unacceptably high, a reduction in numbers was observed for dam water and a further reduction was observed for the water from the micro-irrigation pipes, drip-irrigation and overhead irrigation pivot. *Salmonella* was isolated from up to 22% of the water samples from a selected site tested during the study. No *E. coli* O157:H7 and *Listeria* spp. were isolated from any of the irrigation water samples from any of the sites tested.

Hepatitis A viruses (HAVs) were detected in 76% of the selected South African (SA) surface water tested during this study. Genotyping of the HAV isolates showed the presence of HAV genotype IB in the water sources which confirmed human faecal contamination. Hence these faecally-contaminated water sources may be a potential transmission route of HAV infection and a potential source of contamination of irrigated fresh produce in SA. The isolation and genotypic characterization of disease causing Sapoviruses (SaV) (Genotype I.2 and GIV) from irrigation water sources during this study indicated the potential for fresh produce contamination during irrigation, especially since SaV GIV was isolated from the irrigation pivot point water sample on a commercial tomato/onion production farm. The high *E. coli* counts and the presence of human pathogenic viruses could constitute a potential health risk to the consumer.

During this study it was also shown that pesticide solutions used for agricultural spray purposes to control pests and insects can provide a suitable environment for the survival and growth of human pathogenic microbes, such as *L. monocytogenes*, *E. coli*, *Salmonella* and *Shigella* spp., whereas others are inhibitory to these microorganisms. Microbe growth was favoured mainly by the pesticides with organophosphates and carbamates as the active ingredients. Organophosphate pesticides contain diazinon and chlorpyrifos. Other active ingredients were abamectin (16-membered macrocyclic lactone derivative), phthalic acid diamide and poly-1-p-methene (insect growth regulator). Additionally, Sporekill was used in the wash water in the tomato packhouse on a commercial scale farm. The importance of using sanitizers and pesticides that can provide additional protection against waterborne pathogens should be further investigated. This should be taken into consideration in a risk assessment study for each crop and spray programme.

The mean turbidity levels in the irrigation water often exceeded the international turbidity of 0-1 NTU standard for water (DWAF, 1996). The high turbidity levels of the irrigation water

could be the result of soil erosion and runoff. There was a positive correlation between the biological oxygen demand (BOD), temperature and pH, and the turbidity levels in the water. The values for total dissolved solids (TDS) recorded were within the recommended range of 1000 mg/l for TDS in drinking water and 40 mg/ml for irrigation water as specified by the WHO (WHO, 1996). There was a positive correlation between the TDS and chemical oxygen demand (COD) levels in the irrigation water from two farms in KwaZulu-Natal. *E. coli* counts appeared to be influenced by TDS and COD in irrigation water from one of the farms. Microbial growth could have been supported by the presence of organic matter in the irrigation water.

The BOD levels of the irrigation water sources from selected farms were lower than the COD levels, which can be expected since BOD only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels. COD and TDS values were the only two parameters of water that seemed to have influenced the survival of the levels of total heterotrophic bacteria (THB) as well as *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms in the irrigation water. There was a significant positive correlation between the COD levels, the THB, *E. coli* and *Salmonella* counts in the water samples. South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by WHO of 10 mg/l.

Aim 7: Impact of environmental parameters on the growth kinetics of microbial contaminants

The studies evaluating the impact of environmental conditions on the growth kinetics of microorganisms were done on laboratory scale under simulated conditions. Results obtained for one of the studies showed that the effect of different irrigation technologies (drip, sprinkler and flood) on the survival of human pathogenic bacteria (*Salmonella* Typhimurium and *Listeria monocytogenes*) on fresh produce was crop dependant. Drip irrigation had the lowest risk of microbiological contamination for both tomatoes and lettuce. This is in agreement with previous reports in literature that irrigation methods that do not allow for direct contact of the contaminated water with the edible regions of the plant, facilitate a lower risk of contamination.

The human pathogenic bacteria *E. coli* O157:H7 and *S.* Typhimurium, *L. monocytogenes and Staphylococcus aureus* were unable to grow but were able to survive on tile surfaces at temperatures that simulate working environments (packhouses and minimal processing facilities) and could therefore potentially lead to the contamination of food products coming into contact with such surfaces. However, these pathogens could be considered low risk because *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* were not found to survive on fruit surfaces at high enough titres reported as the minimum infectious dose. In contrast *L. monocytogenes* was able to grow at low temperatures that occur in the supply chain and could therefore pose a potential health risk to the end consumer.

Environmental conditions such as exposure to high temperatures, UV and minimal processing steps (chlorine washing, hydrogen peroxide treatment, cooking, blanching, microwaving and freezing) contribute to a reduction in pathogen numbers. In WRC Report Number 1773/3/12 it was reported that minimal processing using chlorine washing was able to remove up to 3 log of the surface *L. innocua* (surrogate for *L. monocytogenes)* on spinach and tomatoes. In this study chlorine washing reduced the numbers of *L. innocua* spray-irrigated onto broccoli florets (rough, complex surface) by 1 log only. It was concluded that the effectivity of minimal processing steps to reduce microbiological surface contamination levels is crop dependant due to the surface characteristics, i.e. smooth versus hairy and/or rough. A combination of minimal processing methods such as chlorinated wash treatment, storage in modified atmosphere (MAP) (5% CO_2, 5% $O_{2)}$ and blanching, cooking and microwaving led to the greatest reduction in numbers. This was also observed for protozoa (*Cryptosporidium*) on fresh produce where a heat treatment step was necessary and the use of a combination of minimal processing methods caused the greatest reduction in pathogen numbers.

Biofilms have been reported to form rapidly in irrigation water storage facilities, irrigation pipes and wash-bath water in the packhouse or processing factory. Waterborne pathogens may be incorporated in these biofilms and shed over time during irrigation which constitutes a constant source of inoculum. This study highlighted the importance of implementing effective biofilm management systems in all water contact surfaces. The studies on *attachment* showed that *Salmonella* Typhimurium was able to attach to and colonize lemon fruit surfaces and also revealed an obvious direct proportion between cell densities and exposure time.

Internalisation studies confirmed the ability of pathogens to attach, survive, internalise and increase in plant tissue (Standing *et al*., 2013). It is now a scientific fact that waterborne pathogens can enter plants through growth cracks, roots and stomata and internalise in plants similar to plant pathogens. The ability of a pathogen to attach, colonise and survive on plant surfaces differ depending on the specific cultivar. However, it should be noted that in our and other studies pathogen concentrations higher than natural pathogen concentrations in the field were used. Although this situation in nature seldom applies, it shows the potential for contamination under extreme conditions such as flooding with highly contaminated water. Once internalised, the pathogens are protected against the effect of minimal processing steps employed to promote food safety in the supply chain.

General conclusions

Microbial analysis of irrigation water showed that the *E. coli* levels in the irrigation water (five rivers, ten dams) at the sites tested (fourteen in total) during this study exceeded the DWAF (1996) guidelines of $\leq$ 1-1000 CFU/100 mℓ for crops to be eaten raw. Although the *E. coli* counts of the primary irrigation water sources (rivers) were higher than the maximum allowable levels, a decrease in the *E. coli* numbers was observed for water sampled from storage dams, irrigation pivots and micro-irrigation pipes at several of the selected sites. The presence of *E. coli,* hepatitis A viruses (HAV), sapoviruses (SaV) and noroviruses (NoV) showed contamination from human origin from sewage works that contributed significantly to surface water contamination, posing a potential health risk to the consumer.

However, although the *E. coli* counts of the primary irrigation source (rivers) were unacceptably high, a reduction in numbers was observed for dam water and a further reduction was observed for the water from the micro-irrigation pipes, drip-irrigation and overhead irrigation pivot. *Salmonella* was isolated from up to 22% of the water samples from a selected site. No *E. coli* O157:H7 and *Listeria* spp. were isolated from any of the irrigation water samples from the sites tested.

Environmental conditions such as exposure to high temperatures, UV and minimal processing, genotyping of Hepatitis A virus (HAV) and Sapoviruses (SaV) strains isolated from irrigation water, showed that they were of human origin. This indicated that human faeces was the most likely source of the irrigated fresh produce contamination, posing a potential health risk to the consumer. The extent of potential crop contaminated by irrigation water should therefore be clarified which was the next step in this investigation and is reported on in volume 2 of this report.

Washing, hydrogen peroxide treatment, cooking, blanching, microwaving and freezing) contribute to a reduction in microorganism numbers including human pathogenic bacteria tested for. The effectiveness of minimal processing steps to reduce microbiological surface contamination levels is crop dependant due to the surface characteristics, i.e. smooth versus hairy and/or rough. Internalisation studies confirmed the ability of pathogens to attach, survive, internalise and increase in plant tissue. Once internalised the pathogens are protected against the effect of minimal processing steps employed to promote food safety in the supply chain. Agricultural chemicals can be used in the supply chain to reduce foodborne-pathogen-associated hazards, but the fact that some of the chemicals can support pathogen growth should be taken into consideration in a risk assessment strategy for each crop.

The effect of irrigation methods (drip, sprinkler and flood) on the surface contamination of fresh produce was found to be crop dependant and results showed that irrigation methods that do not allow for direct contact of the contaminated water with the edible regions lowered the contamination risk.

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CHAPTER 1

INTRODUCTION

1.1 Background, rationale and aims of research

An increase in the consumption of fresh and minimally processed fruits and vegetables globally (Ayers and Westcot, 1985; Beuchat, 2002) has resulted in one of the most important health challenges in terms of foodborne diseases. In the United States of America (USA) these dietary changes resulted in doubling of produce-associated disease outbreaks per year from 1973-1992 (USA Food and Drug Administration, 1998). However, over the past 10 to 15 years the number of foodborne disease outbreaks has declined. The number of CDC outbreak reports varied between 1243 to 1417 during 2000-2002 in comparison to only 800 to 850 outbreaks reported from 2009-2013 (http://www.foodsafetynews.com/2015/06/the-prevalence-of-foodborne-illness;accessed 12 July 2015). Specific examples include three outbreaks in 2004 in the USA and Canada due to Salmonella, affecting 561 people who consumed fresh tomatoes [Centres for Disease Control and Prevention (CDC), 2005]. In May 2004 another outbreak of *Salmonella enterica* serotype Enteritidis was reported, affecting 29 patients in the USA and Canada due to the consumption of fresh almonds (CDC, 2004). In 2006 another outbreak was associated with the consumption of tomatoes, with 183 cases being reported in 21 states (CDC, 2006a). Subsequently a *Salmonella* outbreak occurred from April to August 2008 in the USA with infections linked to the consumption of raw tomatoes, jalapeno and cilantro. This multi-state outbreak affected 1438 people, with 282 people needing hospitalisation and possibly contributing to the deaths of two people (CDC 2008a). In 2008 a second outbreak was associated with cantaloupe. This affected 51 people in 16 states, with 16 people hospitalised (CDC 2008b).

Major disease outbreaks have also been associated with other pathogens such as *Escherichia coli* O157:H7, which affected 183 people in the USA in September 2006. Of these, 95 people were hospitalised, 29 people had haemolytic uremic syndrome and one person died (CDC 2006b). This outbreak was linked to the consumption of fresh bagged spinach. Additionally, a more recent multistate outbreak of *E. coli* O157:H7 in 2011 in 10 states of the United States caused 60 individuals to become ill (http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html; accessed 2015 June 25). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http:// www.bbc.co.uk/news/world-europe-13683270; accessed 2015 June 25). A Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated cantaloupes that left 146 people ill and caused 30 deaths in 28 states across the United States

(http://www.foodsafetynews.com/2011/10/Cantaloupe-listeria-outbreak-84-sick15-dead; accessed 2015 June 25).

According to the World Health Organisation about one third of 51 million deaths world-wide result from infectious and parasitic diseases. It has further been estimated that worldwide 50 000 people die daily as a result of water-related diseases. Foodborne diseases due to food contamination may occur at any point along the food chain from the farm through food processing to the end user. However, the majority of foodborne outbreaks have been traced to contamination on the farm and the use of contaminated water (Westcot, 1997; Beuchat, 2002). Several studies have investigated or reviewed the impact of contaminated irrigation water on the spread of foodborne diseases (Ayers and Westcot, 1985; FDA, 1998; Venter, 2001).

Fresh produce is important from a national food supply and population health point of view while fresh fruit exports constitute a major part of the economy representing the third biggest earner of foreign exchange. South Africa has over the past 100 years built an international reputation as a major competitor in the export markets, delivering top quality and safe produce. South African farmers have implemented measures to comply with new stringent good agricultural practices and food safety requirements, allowing South Africa to remain a leading country in international food trade.

Unprecedented challenges facing the quality and quantity of our national water supply have resulted in a new risk for farmers reliant on irrigation of cultivated crops (Gemmel and Schmidt, 2012; Chigor *et al.*, 2013). Studies solicited by the Water Research Commission (WRC) entitled: "Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety" (WRC Report Number 1773/1-4/12) and "Health risk assessment in connection with the use of microbiologically contaminated source water for irrigation" (WRC Report Number 1226/1/04) have shown that the national water supply is contaminated with pathogens and may pose a potential risk for the fresh fruit export industry and national safe food supplies. It was argued that a project that focuses on the risks in the supply chain will support and strengthen the findings in WRC Report Number 1773/1-4/12.

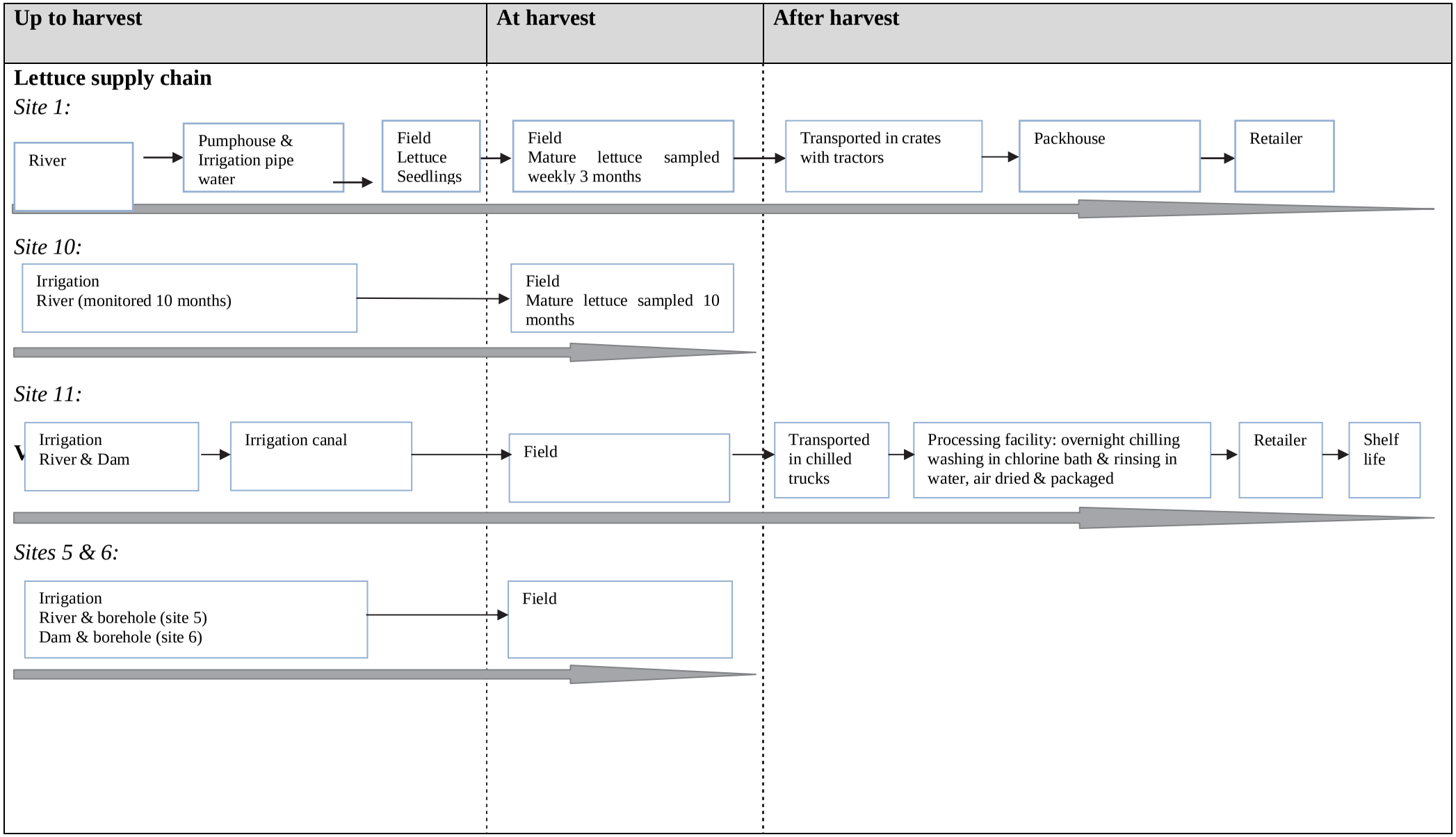
The current research team consisted of four groups from the Department of Microbiology and Plant Pathology, University of Pretoria (UP), Department of Food Science, UP, Department of Medical Virology, UP and Department of Microbiology, University of KwaZulu-Natal respectively. The research groups have extensive experience in water microbiology, water quality assessment, waterborne pathogens (including bacteria, viruses and protozoa), method optimization and development for enhanced pathogen detection, food safety, foodborne pathogens, risk assessment of fresh agricultural produce and minimally processed food. In order to do the research with the additional focus beyond the farm gate in the supply chain tasks were allocated according to expertise to achieve the aims as stipulated below.

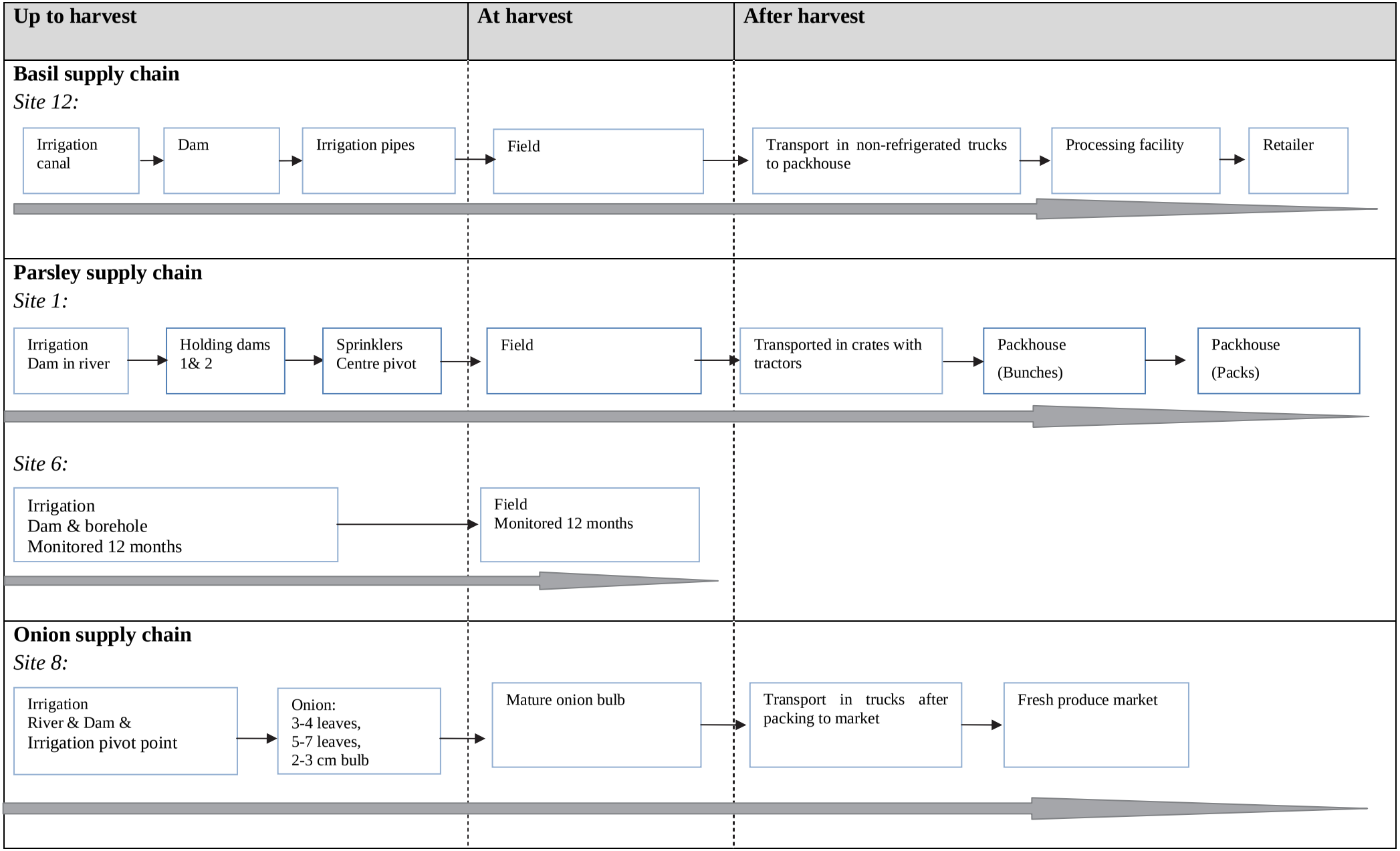
Aims:

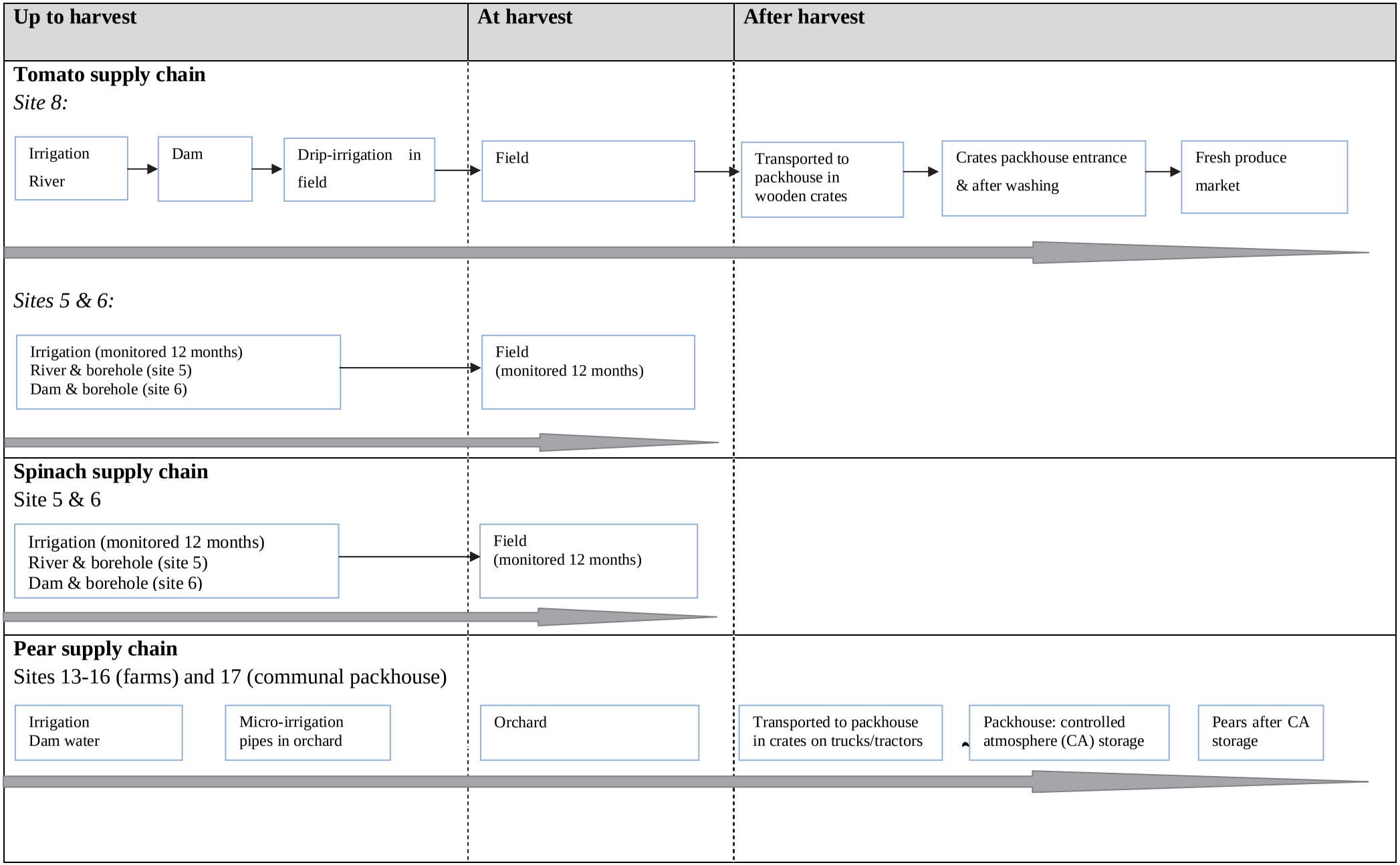
The general and specific aims of this project were:

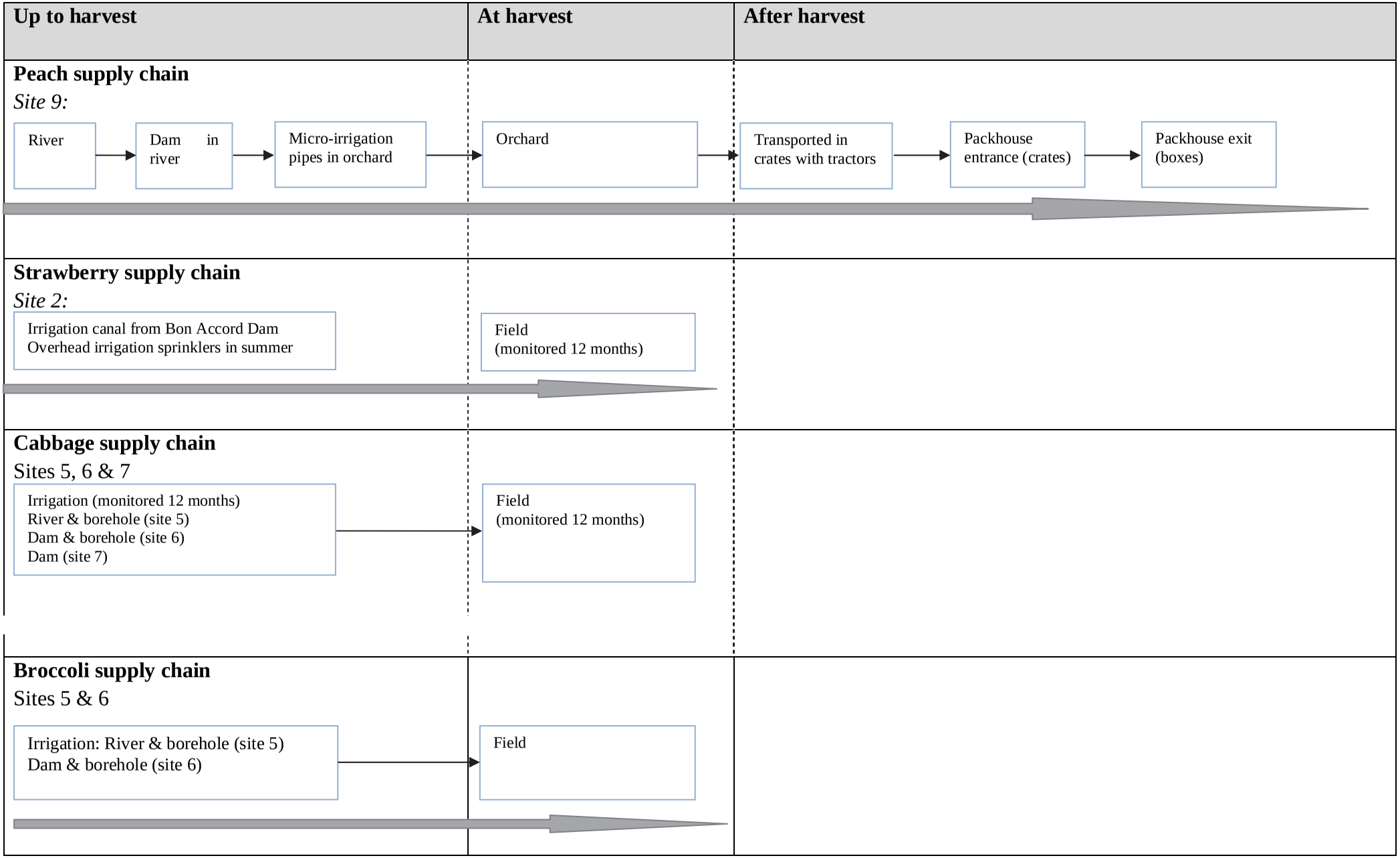
1. To investigate the links between water quality and microbiological safety in fresh minimally processed and frozen fruit and vegetables.
2. To review the literature study of WRC Report Number 1773/1-4/12 and expand the review to include a comprehensive literature study on processing water quality and its impact on the microbiological safety of food and potential effect on human health.
3. To establish co-operation with the team of researchers involved in WRC project K 5 / 1773/4.
4. To select appropriate sites, with reference to i) different sources of untreated and treated water (excluding greywater), ii) different types of microbial contamination, iii) different types of farming – subsistence, emerging, commercial farming for local and export markets, iv) different crops and produce (specifically fruit and vegetables), v) different pathways of contamination: water for irrigation and water used for sprays, and vi) different pathways of contamination: water as transport and washing medium as part of packing and processing.
5. To propose and standardise the research methodology for the assessment of the impact of contaminated irrigation water and processing water on microbial food safety.
6. To determine the extent (types and quantities) of contamination found in irrigation and processing water at the selected sites, with reference to i) indicators of water quality, ii) volume of water used for irrigation and processing, iii) microbial contaminants (viruses, bacteria, parasites), iv) physical parameters (pH, COD, EC, turbidity, temperature, etc.), and v) influence of personal on-site hygiene facilities vi) Influence of external quality control on food safety management at the farming and processing stages of production.
7. To investigate the impact of environmental parameters on the growth kinetics of contaminants, with reference to i) fate and survival, ii) decay rates, iii) surrogate organisms, and iv) different irrigation and processing technologies.
8. To study the extent of (types and quantities) contamination found on the irrigated raw produce (fruit and vegetables) up to harvest at selected sites, with reference to i) microbial contaminants (viruses, bacteria, parasites) – microbial indicators/index of contamination/risk, ii) method of irrigation, agri-chemical spray water application, etc., and iii) investigate the potential link and/or association between contamination on raw produce and water applied (molecular epidemiology using a reference organism).
9. To study the extent (types and quantities) of contamination found on the raw produce (fruit and vegetables) after harvest, with reference to i) microbial contaminants (viruses, bacteria, parasites), ii) microbial indicators/index of contamination/risk, iii) additional contamination through handling, washing, storage, packaging, pre-freezing and minimal processing, etc., and iv) impact of biofilms in processing on product contamination.
10. To make interim and final recommendations for further research, with reference to: Further validation of results and Effective treatment options.

1.2 Description of supply chains for selected fruit and vegetables




Up to harvest
At harvest
After harvest
Basil supply chain
Site 12:
Irrigation canal
Dam
Irrigation pipes
Field
Transport in non-refrigerated trucks to packhouse
Processing facility
Retailer
Parsley supply chain
Site 1:
Irrigation Dam in river
Holding dams 1& 2
Sprinklers Centre pivot
Field
Transported in crates with tractors
Packhouse (Bunches)
Packhouse (Packs)
Site 6:
Irrigation Dam & borehole Monitored 12 months
Field Monitored 12 months
Onion supply chain
Site 8:
Irrigation River & Dam & Irrigation pivot point
Onion: 3-4 leaves, 5-7 leaves, 2-3 cm bulb
Mature onion bulb
Transport in trucks after packing to market
Fresh produce market


Up to harvest
At harvest
After harvest
Tomato supply chain
Site 8:
Irrigation River
Dam
Drip-irrigation in field
Field
Transported to packhouse in wooden crates
Crates packhouse entrance & after washing
Fresh produce market
Sites 5 & 6:
Irrigation (monitored 12 months) River & borehole (site 5) Dam & borehole (site 6)
Field (monitored 12 months)
Spinach supply chain
Site 5 & 6
Irrigation (monitored 12 months) River & borehole (site 5) Dam & borehole (site 6)
Field (monitored 12 months)
Pear supply chain
Sites 13-16 (farms) and 17 (communal packhouse)
Irrigation Dam water
Micro-irrigation pipes in orchard
Orchard
Transported to packhouse in crates on trucks/tractors
Packhouse: controlled atmosphere (CA) storage
Pears after CA storage


Up to harvest
At harvest
After harvest
Peach supply chain
Site 9:
River
Dam in river
Micro-irrigation pipes in orchard
Orchard
Transported in crates with tractors
Packhouse entrance (crates)
Packhouse exit (boxes)
Strawberry supply chain
Site 2:
Irrigation canal from Bon Accord Dam
Overhead irrigation sprinklers in summer
Field
(monitored 12 months)
Cabbage supply chain
Sites 5, 6 & 7
Irrigation (monitored 12 months)
River & borehole (site 5)
Dam & borehole (site 6)
Dam (site 7)
Field
(monitored 12 months)
Broccoli supply chain
Sites 5 & 6
Irrigation: River & borehole (site 5)
Dam & borehole (site 6)
Field

Up to harvest	At harvest	After harvest
Two major frozen food brands in SA		Frozen cauliflower, sweet corn, green beans and carrots
Fruit salad from a processing facility of a major retailer in SA (Site 3) Fruit salad components: kiwi, mango, grape, strawberry, papaya, pine-apple. Products for local and export market.		*Site 3:* Fresh-cut fruit separate before mixing → Fruit salad after packaging → Packaged fruit salad – end of shelf life
Minimally processed fruit mangoes and melons from a processing facility of a major retailer in SA (Site 3)		*Site 3:* Whole fruit → Cut fruit → Packaged fruit (day 0) → Packaged fruit (day 5)

1.3 Description of selected sites and crops for different stages of production and processing

Water and produce samples were collected from selected sites in five provinces in South Africa, i.e. Gauteng (4 sites), KwaZulu-Natal (3 sites), Limpopo (2 sites), North West (3 sites), and the Western Cape (5 sites). Details of sites, irrigation water sources, type of irrigation, type of farming, produce, stages of sampling, and potential contamination sources are summarised below.

Selected Gauteng Province irrigation water and fresh produce sampling sites (1-4).

Site 1	A farm on the banks of the Crocodile River in the Crocodile (West) Marico Water Management Area, in Gauteng, was selected. River water on the farm is collected in a temporary dam, pumped to the farm through the pump house where the water is treated with ozone and distributed to different areas of the farm for crop irrigation
Important aspects for site selection	
Produce	Parsley and lettuce
Stages of sampling	**Parsley**: In the field, bunches in the packhouse and after packaging in the packhouse **Lettuce:** Seedlings 1 week before harvest, field lettuce for 12 weeks at harvest, in the pack house (after harvest) and at a retail store
Irrigation water sources	**Parsley irrigation:** Crocodile River, borehole, holding dam 1 (in the river) and 2 (on the farm), sprinklers, centre pivot and nursery water. **Lettuce irrigation:** Three different water sources on the chosen farm were tested for quality monitoring purposes: (1) the dam water, (2) the pump house water, and (3) water from the irrigation sprinklers on the plot where lettuce samples were taken. Testing the water quality at these three points therefore gave an indication of the water quality at the critical distribution points
Type of irrigation	Sprinkler, pivot
Type of farming	Commercial vegetable and herb farm
Potential contamination sources	Hands of packhouse workers, packhouse facility very dirty

Site 2	A small-scale strawberry farmer to the north of Pretoria, Pyramid district, Tshwane Metropolitan Area, Gauteng. During the hotter summer months overhead irrigation from the same water source is used to keep the fruit cool.
Important aspects for site selection	
Produce	Strawberries
Stages of sampling	In the field at harvest
Irrigation water sources	Irrigation canal from Bon Accord Dam
Type of irrigation	Overhead irrigation/ drip irrigation
Type of farming	Small scale strawberries
Potential contamination sources	Hands of packhouse workers

Site 3	**Fruit processing facility for major retailer in South Africa, Bapsfontein, Gauteng**
Important aspects for site selection	
Irrigation water sources	N/A
Type of contamination	N/A
Type of farming	N/A
Type of crops and produce	N/A
Irrigation technologies	N/A
Irrigation usage periods	N/A
Stages of harvesting	N/A
Potential pathways of contamination	Knives, cutting boards, hands of workers

Site 4	**Tshwane Fresh Produce Market** Pretoria, Gauteng
Important aspects for site selection	
Irrigation water sources	N/A
Type of contamination	N/A
Type of farming	N/A
Type of crops and produce	Tomatoes, onions
Irrigation technologies	N/A
Irrigation usage periods	N/A
Stages of harvesting	N/A
Potential pathways of contamination	N/A

Selected KwaZulu-Natal Province irrigation water and fresh produce sampling sites (5-7).

Site 5	Farm (130 hectares) located in Camperdown. Crops are not washed and packed on site
Important aspects for site selection	
Produce	Broccoli, spinach, jam tomatoes, Crisphead lettuce, cauliflower and cabbage.
Stages of harvesting	Throughout the year at harvest
Irrigation water sources	Borehole and river water
Irrigation technologies	Sprinkler
Type of farming	Commercial
Potential contamination sources	Animal grazing

Site 6	Farm (60 hectares) is located in Cato Ridge. Crops are washed and packed on site.
Important aspects for site selection	
Produce	Broccoli, lettuce, parsley, red cabbage, Chinese cabbage and coriander
Stages of harvesting	Throughout the year at harvest
Irrigation water sources	A mixture of dam and borehole
Irrigation technologies	Sprinkler
Type of farming	Commercial
Potential pathways of contamination	On-site contamination from workers' household waste

Site 7	Farm (360 hectares) located in Richmond. Crops are washed and packed on site.
Important aspects for site selection	
Produce	Tomatoes and cabbage
Stages of harvesting	Throughout the year at harvest
Irrigation water sources	Inanda Dam
Irrigation technologies	Sprinkler
Type of farming	Commercial
Potential pathways of contamination	Nearby contaminated river

Selected Limpopo Province irrigation water and fresh produce sampling sites (8-9)

Site 8	A fresh produce farm near Polokwane in the Limpopo Province, South Africa. Irrigation water is pumped from the Sand River which forms part of the Limpopo River basin.
Important aspects for site selection	
Produce	Tomatoes and onions
Stages of harvesting	**Tomatoes:** At harvest in the fields, after harvest from wooden crates at the packhouse entrance and after washing. Boxes were marked on the day of packing. Tomatoes from the marked boxes were sampled at the fresh produce market the following day. **Onions:** Sampled in the onion field at five different growth stages, i.e. 3-4 leaves, 5-7 leaves and 2-3 cm bulb size (before harvest), at harvest and after harvest (from the fresh produce market).
Irrigation water sources	Sand River, water storage dams
Irrigation technologies	Overhead irrigation pivot and drip irrigation
Type of farming	Commercial large scale
Potential pathways of contamination	Farm is downstream of two informal settlements, which contribute significantly to contamination of the Sand River. The river is also often contaminated with raw sewage from municipal sewage works near Polokwane.

Site 9	The study was conducted on a Global GAP (Good Agricultural Practices) certified commercial farm located in the Mookgophong (Naboomspruit) area in the Limpopo, South Africa.
Important aspects for site selection	
Produce	Peaches (Cultivar=Sunette)
Stages of harvesting	Orchard at harvest, and after harvest from crates at packhouse entrance after transport using tractors and from boxes after packing at the packhouse exit
Irrigation water sources	Sterk River, dam in the river, irrigation pipe water after filters in the orchard and micro irrigation pipes
Irrigation technologies	Micro irrigation and pivots
Type of farming	Commercial large scale.
Potential pathways of contamination	Crates in field, hands of pickers and people in the packhouse.

Selected North West irrigation water and fresh produce sampling sites (10-12).

Site 10	A vegetable-growing farm using water from a dam containing water diverted from the Skeerpoort River for irrigation purposes was chosen as the sampling site for fresh produce and water
Important aspects for site selection	
Produce	Lettuce
Stages of harvesting	Crisphead lettuce samples were collected monthly (at harvest) from a field irrigated with water from the Skeerpoort for 10 months.
Irrigation water sources	Sterk River
Irrigation technologies	Overhead irrigation
Type of farming	Commercial
Potential pathways of contamination	

Site 11	A Global GAP (Good Agricultural Practices) certified lettuce farm located in the Brits area in the North West Province. Process flow: The farm has a traceability system in place.
Important aspects for site selection	
Produce	Lettuce
Stages of harvesting	Crisphead lettuce samples were collected monthly (at harvest) from a field irrigated with water from the Skeerpoort for 10 months.
Irrigation water sources	Water from the Crocodile River and Hartbeespoort Dam feeds the irrigation canal channelled to the farm for irrigation purposes
Irrigation technologies	Irrigated by use of overhead sprinklers
Type of farming	Commercial large scale
Potential pathways of contamination	Crocodile River and Hartbeespoort Dam are susceptible to faecal contamination from informal settlements and wild animals

Site 12	A vegetable/herb production farm near Brits in the North West Province. Process flow: Basil samples are transported to a processing facility near Irene in Pretoria where the herbs are packed and distributed to major retailers in South Africa
Important aspects for site selection	
Produce	Basil
Stages of harvesting	At harvest from the field, after harvest following transport to a processing facility and packaging, as well as from a major retailer.
Irrigation water sources	Irrigation water is drawn from an irrigation channel and stored in a dam
Irrigation technologies	Overhead irrigation
Type of farming	Commercial
Potential pathways of contamination	Hands of packers in the processing facility

Selected Western Cape Province irrigation water and fresh produce sampling sites (13-16) and communal packhouse (17).

Process flow

After harvesting in the orchards from the three selected farms, the pears are transported in crates on trucks/tractors to a central packhouse. At the packhouse the pears are drenched once with water containing 75 ppm chlorine. After drenching the pears are stored in controlled atmosphere (CA) for 14 weeks, at oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5% and temperature set at -0.5°C (with variation at front and back of the room 0.2-minus 0.7°C), and subsequently exported.

Site 13 (V)	A commercial pear production farm near Somerset West, Western Cape.
Important aspects for site selection	
Produce	Pears (Cultivar=Packhams Triumph)
Stages of harvesting	In the orchard (at harvest), after harvest and after drenching upon arrival at the packhouse and from the packhouse after CA storage
Irrigation water sources	Dam on farm (Lourens River is the nearest known river)
Irrigation technologies	Micro irrigation
Type of farming	Commercial Conventional
Potential pathways of contamination	Wild ducks swimming in holding dam, Irrigation water (open holding dam) and on farm sources

Site 14 (L)	Commercial pear production farm near Grabouw, Western Cape
Important aspects for site selection	
Produce	Pears (Cultivar=Packhams Triumph)
Stages of harvesting	In orchard (at harvest), after drenching and from packhouse after CA storage
Irrigation water sources	Dam on farm (Vet River is the nearest known rive)
Irrigation technologies	Micro irrigation
Type of farming	Commercial Conventional
Potential pathways of contamination	Antelope drinking from / grazing around holding dam, Irrigation water (open holding dam) and on farm sources

Site 15 (G)	Commercial pear production farm near Grabouw, Western Cape
Important aspects for site selection	
Produce	Pears (Cultivar=Packhams Triumph)
Stages of harvesting	In orchard (at harvest), after drenching and from packhouse after CA storage
Irrigation water sources	Dam
Irrigation technologies	Micro irrigation
Type of farming	Commercial Conventional
Potential pathways of contamination	Irrigation water (open holding dam) and on farm sources

Site 16 (K)	Commercial pear production farm near Grabouw, Western Cape
Important aspects for site selection	
Produce	Pears (Cultivar=Packhams Triumph)
Stages of harvesting	In orchard (at harvest), after drenching and from packhouse after CA storage
Irrigation water sources	Dam
Irrigation technologies	Micro irrigation
Type of farming	Commercial Conventional
Potential pathways of contamination	Irrigation water (open holding dam) and on farm sources

1.4 Standardised research methods for assessment of the impact of irrigation water used during processing and microbial food safety

The aim was achieved by proposing standardised research methods for the assessment of the impact of contaminated irrigation and processing water on microbial food safety. It was decided to select all standard ISO methods, published methods used by group one (Prof Britz, Prof Buys and Prof Taylor) and existing methods currently used by the new participants of group two. In order to transfer the methods and ensure consistency in test methods applied, two week-long training workshops were held, one at KwaZulu-Natal University, Department of Microbiology and one at the University of Pretoria, Departments of Food Science, Virology and Microbiology and Plant Pathology. The methods used are summarised briefly below. Further details of the methods used for each of the studies can be viewed in the different sections in Chapters 3 and 4.

1.4.1 Water filtration

Three 1 litre water samples were collected in ethanol (70%) sterilized, air dried plastic bottles from each of the sites. Samples were transported in a cooler box and stored at 4 °C until analysis (within 24 h). All analyses were performed in triplicate. Each of the one litre water samples was filtered through cellulose nitrate filters (0.45 µm pore size) (Sartorius, Goettingen, Germany) for further microbiological analysis.

1.4.2 Fruit washings

Fruit was sonicated in Ringer's solution or 0.1% Peptone Buffered Water for an optimised period of time to release the microorganisms from the surface of the fruit. Fruit washings were then filtered as described above. Each 0.45 µM filter membrane was cut into small pieces, added to 9 mℓ TSB medium and serially diluted and coliform, *E. coli*, total viable bacterial and fungi/yeast counts determined after plating onto appropriate media.

1.4.3 Vegetable sampling and processing

Three twenty-five grams sub samples of each replicate were weighed into a sterile polyethylene bag containing 225 mℓ of 0.1% peptone buffered water (BPW) (Merck, Johannesburg, South Africa) and macerated for 5 min at 230 rev min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK).

1.4.4 Isolation of pathogens from irrigation water and vegetables

Escherichia coli, Listeria monocytogenes, Salmonella spp., *Enterococcus* spp., *Staphylococcus aureus*, aerobic and anaerobic spore formers were isolated from irrigation water and vegetables. Enumeration of bacterial pathogens was done according to the South African National Standards (SANS) (*E. coli*, SANS 688-2:1999; *L. monocytogenes*, SANS 11290-1; *Enterococcus*, SANS 7899-2:2004; *S. aureus*, SANS 688-1: 1999; *Salmonella* spp. SANS 6579:2003). Presumptive *E. coli*, *Salmonella* and *L. monocytogenes* were isolated and confirmed using API E20 and API *Listeria monocytogenes*, respectively. Presumptive *S. aureus* was confirmed with the Staphylase kit (Oxoid) after carrying out the catalase test.

1.4.5 Viable counts

After filtering the irrigation water, the membranes were aseptically cut into small pieces, added to 9 ml Tryptone Soy broth (TSB) (Biolab, Lawrenceville, Georgia, USA) medium and serially diluted. Similar to the water samples ad fruit washings, tenfold dilutions of each of the macerated fresh produce samples were prepared.

1.4.6 Multiplex PCR detection of Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica and Staphylococcus aureus

DNA was extracted from each of the enriched water and produce samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using the DNA as template using pathogen specific primers (Cebula *et al*., 1995; Standing *et al*., 2013).

1.4.7 Monitoring of water sources for the quantification of coliforms and Escherichia coli (Coli-lert-18® Method)

The total coliform numbers and presence of *E. coli* were determined by using a commercially available Colilert test kit according to the manufacturer's instructions (IDEXX Laboratory Inc., One IDEXX Drive, Westbrook, USA, Part no. 98-08877-00).

1.4.8 Scanning electron microscope examination

Material to be visualised was stored in fixing solution. Samples were subsequently rinsed 3 times in 0.075 M phosphate buffer. The remnants were then fixed in 0.5% aqueous osmium tetroxide for 1-2 hours followed by a rinsing with distilled water which was repeated 3 times. The samples were then dehydrated in a graded ethanol series of 30%, 50%, 70%, 90%, 100%, 100% and 100% for 10 minutes each. This was followed by critical point drying with liquid carbon dioxide and spluttering with gold before being viewed with a Jeol JSM-840 Scanning Electron Microscope.

1.4.9 VBNC microorganism detection

The presence of VBNC microorganisms in the irrigation water and on the fresh produce was detected via resuscitation of the artificially contaminated irrigation water and washings. Resuscitation of the samples was done using the Direct Viable Count (DVC) method of Kogure (1979). Resuscitation of the sample was done using the Direct Viable Count (DVC) method of Kogure (1979). A 97.3 ml sample was resuscitated using 0.2 ml nalidixic acid (0.002%) and 2.5 ml (0.025%) yeast extract. The samples were incubated in the dark on a shaker at 160 rpm at 25 °C for 6 h. Bacterial enumeration after resuscitation was obtained using the membrane filtration technique whereby 50 ml of the samples was filtered through nitrocellulose membranes. The membrane was plated on Coliforms Chromo Agar followed by incubation at 35 ± 2 °C for 24 h, for *Salmonella enterica* and *E. coli* O157:H7 respectively. Colourless and salmon-coloured colonies were enumerated and expressed as CFU/ml. The development of VBNC cells were confirmed by epifluorescent microscopy by staining the membranes after incubation with a 0.025% acridine orange solution for 5 min, air drying and mounting in non-fluorescent immersion oil. Only cells elongated at least twice, with respect to cell length, were counted as VBNC cells (Besnard *et al*., 2000).

1.4.10 Heavy metal impact on waterborne pathogens

The presence and concentration of toxic heavy metals, i.e. Arsenic (As), Lead (Pb), Mercury (Hg) and Cadmium (Cd) in the water samples in KZN were quantified using ICP-OES. This study was done to determine the effect of heavy metals on growth and presence of waterborne pathogens in water samples tested for VBNC. The rhizozone samples were first extracted using 0.02 M EDTA, buffered at pH 4.65 with 0.5 M acetate (soil: extractant ratio 1:10; 1 h; 20 °C), before determining the heavy metal concentrations in the filtered extract. Each sample solution was then made up to a final volume of 25 ml with distilled water and analyzed for the presence and concentration of heavy metals (Arora *et al*., 2008). Appropriate standards of the different heavy metals were prepared using varying concentrations of the heavy metals. The values obtained from ICP-OES of these standards were used for the construction of standard curves where the concentration of the heavy metals in the experimental samples was extrapolated.

1.4.11 Isolation of *Cryptosporidium* oocysts and *Giardia* cysts

Cryptosporidium oocysts and *Giardia* cysts were isolated from water using Filtration/IMS/FA by United States Environmental Protection Agency (EPA, 2005).

Filtration: Each 50 L raw water sample was filtered through an Envirocheck™ 1 µm HV filter.

Elution and Concentration: The Envirocheck™ filter capsule was filled with 125 mℓ prepared elution buffer (10 mℓ of Laureth-12 10% with 10 mℓ Tris solution, 2 mℓ EDTA solution and 150µℓ silicone antifoam agent filled to 1 ℓ distilled water) and placed on a shaker at 300 rpm for 15 min, followed by centrifugation at 1100 x g for 15 min. Another 125 mℓ elution buffer was then added and then treated as described above.

Separation: Equal volumes of Buffer A and B as well as *Cryptosporidium* and *Giardia* Dynabeads™ from the ImmunoMagnetic Separation (IMS) kit (Invitrogen Dynabeads GC Combo, Dehteq, South Africa) were added to the centrifuged samples and the oocysts isolated according to the manufacturer's instructions.

Staining of sample concentrate: The samples were then stained using *Cryptosporidium* Fluorescein-iso-thiocyanate (FITC) and *Giardia* FITC and examined using a fluorescent microscope according to the manufacturer's instructions.

PCR analysis: Identification of *Cryptosporidium* oocysts was done with real-time PCR according to the methodology of Ramirez and Sreevatsan (2006). Following staining of the oocysts on a microscope slide DNA was extracted through freeze thaw and a DNA extraction kit. To differentiate *Cryptosporidium* species a 272bp fragment of the small-subunit rRNA (18s rRNA) was amplified using published *Cryptosporidium*-specific sense primer (Limor *et al.*, 2002). The PCR products were identified by the melting curve analysis (Ramirez and Sreevatsan, 2006).

Positive control: ColorSeed™ (BTF Pty Limited, United Kingdom) capsules containing an exact amount of Cryptosporidium oocysts given were used as positive control as well as to

determine the recovery rate of the method. *Cryptosporidium* oocysts recovery rate was calculated using the formula: *Cryptosporidium* recovery (%) = (*Cryptosporidium* detected/Number of *Cryptosporidium* in ColorSeed™ as per Certificate of Analysis) x 100.
Negative control: Negative control is performed to ensure that the equipment and reagents are free of *Cryptosporidium* oocysts or *Giardia* cysts or other materials that could be misidentified as oocysts or cysts.

1.4.12 Thin layer agar method for enumerating cold injured bacteria in frozen food samples

The thin layer agar method of Wu *et al.* (2001) was used which entails pouring a thin layer of non-selective medium, trypticase soy agar, over a pre-poured and solidified selective medium for the isolation of specific microorganisms. Colonies were enumerated after incubation at 37°C for 24 hours and expressed as colony forming units (CFU) per mℓ.

New methods optimised and implemented to facilitate pathogen detection and for microbial source tracking purposes were as follows:

1.4.13 Confirmation of presumptive pathogen identities isolated from selective media

DNA was extracted from single colonies of presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates using the Quick-gDNA miniprep kit (Zymo Research, California, USA). The universal primers F-27 and R-1492 were used to amplify the 16s rDNA genes of the bacterial isolates (Weisburg *et al*., 1991). The PCR products were sequenced and a search of nucleotide databases conducted to confirm the microorganism identities.

1.4.14 Matrix Assisted Ionisation Time of Flight (MALDI-TOF) confirmation of the presumptive Escherichia coli, Salmonella and Listeria isolate identities

Purified bacterial cultures isolated from the selective media were transferred in duplicate directly to a MALDI-TOF steel polished target plate and subsequently analysed using Bruker MicroFlex LT MALDI-TOF in conjunction with Bruker Biotyper Automation Software and library and analysed according to the manufacturer's instructions (Bruker, Bremen, Germany).

1.4.15 Molecular Detection System (MDS) analysis

Twenty-four hour 3M Peptone buffered water (PBW) and 3M *Listeria* specific enrichment broths were used to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* 0157, including H7, (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012) (3M, St. Paul, Minnesota, USA).

1.4.16 Antibiotic resistance of E. coli isolates

After confirmation of the *E. coli* identities, forty-five isolates from the irrigation water (river dam, irrigation pivot point) and onion samples were subjected to antibiotic susceptibility testing using the Kirby Bauer disk diffusion technique. The *E. coli* isolates were cultured in twenty-five millilitres Brain Heart Infusion Broth each, incubated for 24 hours at 37 °C and a

hundred microlitres of each of the bacterial suspensions plated onto Mueller-Hinton agar plates (Merck, Darmstadt, Germany). Antibiotic resistance or susceptibility of *E. coli* isolates were scored according to criteria developed by the Clinical Laboratory Standards (CLSI, 2007).

1.4.17 Virulence genes in *E. coli* isolates

E. coli isolates were tested for the presence of virulence genes [shigatoxin 1 (*stx* 1), shigatoxin 2 (*stx* 2) and intimin (*eae*)] and the house-keeping *mdh* gene (internal PCR reaction control) as described Omar and Barnard (2010).

1.4.18 Enterobacterial Repetitive Intergenic Consensus (ERIC) – PCR fingerprinting of E. coli isolates

ERIC – PCR fingerprints were generated for *E. coli* isolates from water and produce. The polymerase chain reaction (PCR) mixtures contained 160 ng of template DNA, 200 µM of each primer in a total volume of 50µℓ. The forward and reverse primer sequences used to generate the DNA fingerprints were 5' ATGTAAGCTCCTGGGGATTCAC -3' and 5'-AAGTAAGTGACTGGGGTGAGCG -3' respectively (Mohapatra *et al* 2001). The PCR cycling conditions involved an initial denaturation step of 95 °C for 4 min, 30 cycles of denaturation (94 °C for 30 s), annealing (40 °C for 1 min) and extension (72 °C for 8 min) and a final elongation step at 72 °C for 15 min.

1.4.19 Statistical analysis

Data were analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC). A one-way analysis of variance was used to determine the difference in microbial counts. Means were analysed using the least significant difference (using the Fisher test) at a 5% level of significance.

Analysis of variance and correlation coefficients were calculated to determine associations between the measured traits using XLSTAT version 2014. To group the populations based on antibiotic phenotype similarities, a cluster analysis was conducted on the Euclidean distance matrix with the unweighted pair group method based on arithmetic averages (UPGMA) using XLSTAT 2014.

The ERIC – PCR DNA fingerprints of *E. coli* isolates were compared and analysed with the GelCompar II v. 6.5 (Applied Maths, Saint-Marten-Latem Belgium). Percentage similarities of digitised bands were calculated using the Pearson correlation coefficient and the unweighted pair group arithmetic mean (UPGMA) and complete linkage algorithms were used to derive a dendrogram. Relationships between the DNA fingerprints of each of the isolates were expressed as dendrograms.

4.1.20 Data storage

a) Records are stored in such a manner that they are readily retrievable and to prevent damage, deterioration, loss and access by unauthorised personnel.

b) Records are retained for 5 years, unless otherwise specified.

c) Electronic Records.

d) Electronic records are backed up regularly onto external hard drives and the cloud for secure storage.

e) Only authorised personnel have access to electronic records and changes are only made by appointed persons.

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CHAPTER 2

SYNTHESIS OF UPDATED AND EXTENDED LITERATURE REVIEW

2.1 INTRODUCTION

The increased demand for fresh produce and convenience foods world-wide and the associated highly publicised foodborne disease outbreaks, emphasised the importance of food safety assurance in the supply chain (Jacxsens *et al.,* 2009). Foodborne pathogens linked with foodborne disease outbreaks after fresh produce consumption included amongst others *Escherichia coli* O157:H7, *E. coli* O104:H4, *Salmonella* spp. and *Listeria monocytogenes*, noroviruses (NoV I and II), hepatitis A virus (HAV) and protozoa such as Giardia and Cryptosporidium. Fresh produce contamination with human pathogens can happen from pre- to post-harvest stages of processing up to marketing and prior to consumption. Potential sources of contamination at the pre-harvest level are soil, faeces, irrigation water, reconstituted fungicides and insecticides, dust, insects, manure (Althaus *et al.,* 2012). At the post-harvest level the produce could be contaminated by contact with asymptomatic human carriers, harvesting equipment, transport containers, surfaces in processing factories and polluted process water (Althaus *et al.,* 2012).

South African rivers have increasingly been reported to be of an unacceptable microbiological quality and that they pose a risk of transferring enteric bacterial pathogens onto irrigated produce (Gemmell and Schmidt, 2012; Ijabadeniy *et al.,* 2011; Chigor *et al.,* 2013; CSIR, 2010; Teklehaimanot *et al.,* 2014). Urban developments, presence of informal settlements with limited access to potable water, inadequate sanitation, and inefficient municipal sewage works are factors contributing to the deteriorating water quality (Odjadjare and Okoh, 2010).

This increases the potential risk of foodborne illnesses that could negatively impact on the fresh produce industries in South Africa. An example of the economic impact that foodborne pathogens can have on a country is well-known and best illustrated by the *E. coli* O104:H4 outbreak in the European Union in May 2011. The outbreak was initially associated with cucumbers and tomatoes from Spain and later linked to a German sprout producer. The resultant €225 million losses per week for the Spanish vegetable industry and other related sectors highlight the economic importance of such outbreaks as well as the importance of rapid accurate diagnostic test methods (http://www.bbc.co.uk/news/world-europe-13683270, accessed 2015 June 25). Therefore ensuring microbiological safety of local, imported and exported fresh produce is important to safeguard public health and retain a vibrant industry.

To investigate the extent of food safety concerns in the South African fresh produce industry, a solicited research project entitled “Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety” (WRC Report Number 1773/1-4/12), was initiated and funded by the Water Research Commission (WRC) and co-funded by the Department of Agriculture, Forestry and Fisheries (DAFF). In this study it was reported that the total coliform and faecal counts for irrigation water were generally much higher than standards set

by the Department of Water Affairs (DWA) and the World Health Organisation (WHO). Linking studies performed by genotypic and phenotypic characterisation of identified isolates from irrigation water and fresh produce in the same study reported at least 30 percent correlations between contamination sources and pathogen presence on the product. The conclusion from these studies was that microorganisms on fresh produce surfaces were present as a result of transfer from contaminated irrigation water. Subsequently, a follow up Water Research Commission (WRC) solicited project co-funded by DAFF: "*An investigation into the link between water quality and microbiological safety of fruit and vegetables from the farming to the processing stages of production and marketing*" (WRC Project No K5/1875/4, Water Research Commission Knowledge review 2010) was initiated.

Topics covered in the literature review of WRC Report Number 1773/1-4/12 include the following: 1) the water situation in South Africa; 2) sources of water available; 3) quality of SA rivers; 4) water for agricultural uses; 5) economic situation of the SA fruit and vegetable industry; 6) community importance of irrigated crops; 7) risk assessment of pathogens associated with vegetables and fruit; 8) pathogens associated with fresh produce including characteristics of bacterial pathogens (*E. coli*, *Salmonella*, *Listeria monocytogenes*, faecal streptococci and enterococci, *Shigella, Staphylococcus*, *Vibrio*, *Yersinia* and *Clostridium*), parasites (*Cryptosporidium* and *Giardia*) and viral pathogens (Norovirus and hepatitis A virus); 9) pre- and post-harvest sources of contamination; 10) irrigation water and pathogen transfer; 11) infectious doses of the main water, soil and produce pathogens as well as 12) treatment technologies for irrigation water. For the purpose of this literature review it was agreed to focus on aspects not covered in the WRC project K5/1773/4 literature review. Topics covered in this literature review include heavy metal pollution and influence on safety of fresh produce (section 2), agro-chemical spray water as a potential source of contamination of fruits and vegetables (section 3), biofilms in water storage and distribution systems (section 4), internalisation potential of human pathogenic bacteria in fresh production (section 5), the incidence and control of foodborne pathogens on postharvest fruits and vegetables (section 6), *Listeria monocytogenes* and vegetables and effect of minimal processing on survival of the pathogen (section 7), microbial source tracking using faecal indicator bacteria (section 8), microbial source tracking potential using phylogenetic analysis of noroviruses and hepatitis viruses (section 9).

2.2 HEAVY METAL POLLUTION AND INFLUENCE ON THE SAFETY OF FRESH PRODUCE

Besides pathogenic microorganisms, chemical contaminants are also of concern to public health safety (Qadir *et al*., 2008). Chemicals have the ability to cause serious health risks to consumers if they are able to contaminate fresh produce at significant concentrations. Contamination of fresh produce may occur by way of either naturally occurring substances or by synthetic chemicals (Table 1) which may be added or which are present during production or processing (UN, 2007).

Micronutrient elements are known to be essential for plant growth and human nutrition. However, some of these elements, such as copper (Cu), chromium, fluorine, molybdenum,

nickel, selenium (Se) or zinc (Zn) may also be toxic to both animals and humans at higher concentrations. Other trace elements, such as arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) may also be present in fresh produce (McLaughlin *et al*., 1999). Heavy metals are extremely harmful because of their non-biodegradable nature, long biological half-lives and their potential to accumulate in different body parts. Even low concentrations of heavy metals have damaging effects on man and animals because there is no good mechanism for their elimination from the body. These metals are even more toxic as they are soluble in water (Arora *et al*., 2008). Cadmium is a non-essential metal element that is known to cause damage even at very low concentrations. This metal can be easily taken up from soil by vegetable plants and can accumulate at high levels (Yang *et al*., 2009).

Heavy metals are also easily accumulated in the edible parts of leafy vegetables (Arora *et al*., 2008). However, the absorption and accumulation of heavy metals in fresh produce may depend upon various factors, such as temperature, moisture, organic matter, pH and nutrient availability (Sharma *et al*., 2007). Consuming heavy metal contaminated fresh produce is therefore of serious health concern, as it can dangerously deplete some essential nutrients in the body causing a decline in immunological defences, intrauterine growth retardation, impaired psycho-social behaviour, disabilities associated with malnutrition and a high prevalence of upper gastrointestinal cancer (Sharma *et al*., 2007; McLaughlin *et al*., 1999). It is therefore very important to identify chemical hazards that are applied to fresh produce.

Table 1: Naturally and added chemical hazards and the potential risks they impose on humans

Naturally occurring chemical hazards	**Potential health risks for humans**
Allergens (e.g. weeds, peanuts)	Allergenic reactions
Fungal toxins (mycotoxins; e.g. aflatoxin)	Multiple poisonings (acute or chronic)
Phytohaemagglutinin	
Alkaloids	Multiple poisonings (acute or chronic)
Added chemical hazards	**Potential health risks for humans**
Agrochemicals (pesticides and fertilizers)	Multiple poisonings (acute or chronic)
Toxic elements and compounds (e.g. lead, zinc, cadmium, mercury, arsenic, cyanide)	Multiple poisonings (acute or chronic)
Processing contaminants (e.g. lubricants, cleaning agents, sanitizers, coatings, paints, refrigerants and cooling agents, water / stream treatment chemicals, pest control chemicals)	Multiple poisonings (acute or chronic)
Persistent organic pollutants (POPs) are compounds that accumulate in the environment and the human body. Known examples are dioxins and PCBs (polychlorinated biphenyls).	Exposure to POPs may result in a wide variety of adverse effects in humans.
Agents from packaging material (e.g. plasticizers, vinyl chloride, adhesives, lead, tin)	Multiple poisonings (acute or chronic)

Source: (UN, 2007).

Toxic substances, such as pesticides, are used in pest control by protecting developing crops from harmful insects or competitive weeds or to remove potential vectors of disease. Pesticides can be very harmful to both humans and the environment (UN, 2007). Besides the fact that pesticides may be a chemical hazard, some of these pesticides can also support the growth of some bacterial species, such as *Pseudomonas*, *Salmonella*, *Escherichia coli*, *Acinetobacter*, *Aeromonas* and various coliforms (Ng *et al*., 2005).

It has been shown that metal concentrations in plant tissues, generally, increase with concentrations of metals in irrigation water. The concentrations of these heavy metals in roots are typically higher compared to the metal concentrations in leaves (Qadir *et al*., 2008). Recently, Arora *et al*. (2008) assessed the levels of different heavy metals such as iron (Fe), manganese (Mn), copper and zinc, in vegetables that were irrigated with water from different sources. It was found that continuous irrigation of vegetables with wastewater resulted in accumulation of heavy metals in the edible parts of food crops, ranging between: 116-378, 12-69, 5.2-16.8 and 22-46 mg/kg for Fe, Mn, Cu and Zn, respectively (Arora *et al*., 2008).

With the intention of reducing the concentration of toxic heavy metal contaminants (e.g. As, Cd, Pb) in irrigation water, Ayers and Westcot (1985) established standards for these metals in irrigation water (Table 2).

Table 2: Recommended maximum concentrations of trace elements in irrigation water

Element	Recommended Maximum Concentration (mg/l)	Remarks
Al (aluminium)	5.0	Can cause non-productivity in acid soils (pH < 5.5), but more alkaline soils at pH > 7.0 will precipitate the ion and eliminate any toxicity.
As (arsenic)	0.10	Toxicity to plants varies widely, ranging from 12 mg/l for Sudan grass to less than 0.05 mg/l for rice.
Be (beryllium)	0.10	Toxicity to plants varies widely, ranging from 5 mg/l for kale to 0.5 mg/l for bush beans.
Cd (cadmium)	0.01	Toxic to beans, beets and turnips at concentrations as low as 0.1 mg/l in nutrient solutions. Conservative limits recommended due to its potential for accumulation in plants and soils to concentrations that may be harmful to humans.
Co (cobalt)	0.05	Toxic to tomato plants at 0.1 mg/l in nutrient solution. Tends to be inactivated by neutral and alkaline soils.
Cr (chromium)	0.10	Not generally recognized as an essential growth element. Conservative limits recommended due to lack of knowledge on its toxicity to plants.

Element	Recommended Maximum Concentration (mg/l)	Remarks
Cu (copper)	0.20	Toxic to a number of plants at 0.1 to 1.0 mg/l in nutrient solutions.
F (fluoride)	1.0	Inactivated by neutral and alkaline soils.
Fe (iron)	5.0	Not toxic to plants in aerated soils, but can contribute to soil acidification and loss of availability of essential phosphorus and molybdenum. Overhead sprinkling may result in unsightly deposits on plants, equipment and buildings.
Li (lithium)	2.5	Tolerated by most crops up to 5 mg/l; mobile in soil. Toxic to citrus at low concentrations (<0.075 mg/l). Acts similarly to boron.
Mn (manganese)	0.20	Toxic to a number of crops at a few-tenth to a few mg/l, but usually only in acid soils.
Mo (molybdenum)	0.01	Not toxic to plants at normal concentrations in soil and water. Can be toxic to livestock if forage is grown in soils with high concentrations of available molybdenum.
Ni (nickel)	0.20	Toxic to a number of plants at 0.5 mg/l to 1.0 mg/l; reduced toxicity at neutral or alkaline pH.
Pb (lead)	5.0	Can inhibit plant cell growth at very high concentrations.
Se (selenium)	0.02	Toxic to plants at concentrations as low as 0.025 mg/l and toxic to livestock if forage is grown in soils with relatively high levels of added selenium. An essential element to animals but in very low concentrations.
Sn (tin)		
Ti (titanium)	----	Effectively excluded by plants; specific tolerance unknown.
W (tungsten)		
V (vanadium)	0.10	Toxic to many plants at relatively low concentrations.
Zn (zinc)	2.0	Toxic to many plants at widely varying concentrations; reduced toxicity at pH > 6.0 and in fine textured or organic soils.

Source: Ayers and Westcott, 1985.

2.3 AGRO-CHEMICAL SPRAY WATER AS A POTENTIAL SOURCE OF CONTAMINATION OF FRUITS AND VEGETABLES

Agricultural pesticides are extensively used to protect and improve the quality and quantity of food commodities and to combat certain diseases transmitted by insect and fungal vectors to fruits and vegetables (Benimeli *et al.*, 2003). Pesticides consist of a specific active ingredient which controls a specific group of pests as well other additives, or adjuvants which contribute to efficacy and stability of the product (Ng *et al*., 2005). They are classified according to their different chemical nature as well as their different functionality, such as organonitrogen, organosulphide, carbamate, organophosphorus, pyrethroid and organochloride pesticides (Donaldson *et al*., 2002). A more detailed understanding of whether these pesticides are inhibitory or stimulatory to the growth of the aquatic microbial community is one of the major challenges in environmental studies due to their different chemical formulations and the difference in the tolerance level of different microbial species to these pesticides (Widenfalk *et al*., 2004). Agricultural pesticides are transformed by physical, chemical and biological processes into one or more transformation products. As compared to the parent pesticide, these transformation products may pose a similar or increased ecotoxicological risk and could be stimulatory to growth and biological activity in the aquatic ecosystem (Belfroid *et al*., 1998).

Pesticides and the aquatic environment

Very little information has been presented concerning the subsistence of pesticides in water and especially their relationship with aquatic microorganisms (De Lorenzo *et al*., 2001)). Because of their highly toxic nature, pesticides represent one of the more important classes of micro pollutants (Schwartz, 1987). The majority of endocrine disrupting pesticides (EDPs) enter the water environment from agricultural sources, through leaching from the soil, surface runoff, spray drift, soil erosion and volatilisation. Pesticides that are not adsorbed to soil particles are highly water soluble and are relatively stable having great ability to leach through the soil. Pesticides are transported to surface water through leaching and during transportation transformation and degradation may also occur. Degraded pesticides are simplified to products, which can be either harmful or beneficial to aquatic organisms (De Lorenzo *et al*., 2001).

The microbial degradation pathways of EDPs include anaerobic and aerobic degradation. With anaerobic degradation being the major degradation pathway in the aquatic environment (De Lorenzo *et al*., 2001). For different microorganisms, their biochemical pathways to attack pesticides and the toxicity and mobility of their metabolites are different. Generally, the toxicity of the metabolites is higher, as their mobility is stronger than that of the parent compounds, and some metabolites may be accumulated in the natural environment. For example, DDE, a metabolic residue of DDT, may persist in the environment for a long time, and it may even influence the degradation level of its parent DDT. Because pesticides possess different molecular structures and physico-chemical properties, their sensitivities are different. The number and the position of chlorine atoms in benzene may influence biodegradation. The biodegradability of pesticides in the aquatic environment does give an indication of the

survival ability of the microorganism in agrochemical spray solutions (De Lorenzo *et al*., 2001).

Prior to pesticide application, agricultural water is used to mix the chemical to obtain the desired final concentration (Guan *et al*., 2001; Ng *et al*., 2005). The main sources of water used on farms are rivers, dams, lakes, boreholes, and streams (Nystrom *et al*., 1999; Koutsotoli *et al*., 2005). Faeces, soil and other inputs such as sewage overflow and introduced foodborne pathogenic bacteria in water sources can affect quality and safety of agricultural water (Kirby *et al*., 2003). Microbial status of farm water sources used for preparation of plant protection remedies should be considered prior to application of pesticides (Ng *et al*., 2005). Studies by Guan *et al*. (2001) and Coghlan (2000) showed that dilution of various commercial plant protective products with contaminated water may present suitable conditions for the survival and growth of pathogenic bacteria such as *L. monocytogenes*, *Salmonella* spp., *Shigella* spp. and *E. coli* O157:H7. Other studies confirmed that pesticide solutions prepared using contaminated water could result in the spread of foodborne pathogens to crops cultivated in the field, especially if the pesticide solutions support microbial growth (Guan *et al*., 2005). Furthermore, delay in using pesticide spray mixes should be avoided and is critical as it could allow the number of bacterial cells already in the water to increase (Guan *et al*., 2001; Ng *et al*., 2005). Guan *et al*. (2001) indicated that pesticide preparations are normally reconstituted and diluted in non-potable farm or agricultural water. Bacterial contaminants, including pathogens, from this source might then grow in the preparations prior to application to the produce.

Both Sulphur and Kumules with sulphur as active ingredient killed *S. aureus* and *L. monocytogenes*, but allowed survival of *E. coli* O157:H7 and *S*. Typhimurium (WRC Project K5/1875/4). It was reported by Ng *et al*. (2005) that Kumules DF with the same active ingredient as Kumules and Sulphur destroyed of *L. monocytogenes* and permitted the survival of *E. coli* O157:H7 and encouraged the growth of *S*. Typhimurium. Previous studies also demonstrated that Penncozeb 750 DF with the same active ingredients as Dithane, and Champ Dry Prill having the same active ingredient as Copper hydroxide, were all able to inhibit the survival of *E. coli* O157:H7, *L. monocytogenes* and *S.* Typhimurium after different time intervals (Ng *et al*., 2005). Furthermore, Guan *et al.* (2001) demonstrated that Dithane M45 (Mancozeb) and Lorsban 4E (Chloropirifos) killed *E. coli*, *L. monocytogenes* and *Salmonella* after one hour of contact and this correlates with the results observed in this study.

However, Guan *et al.* (2001) demonstrated that the product Ambush (permethrin) allowed survival and growth of *E. coli* O157:H7, *L. monocytogenes* and *S.* Typhimurium and Ng *et al*. (2005) indicated that the same product with the same active ingredient inhibited the same bacteria. Variation in results reported in these studies may be attributed to the fact that the pesticide products were obtained from two different manufacturers. It was further reported that after inoculation, pesticide solutions were incubated at different temperatures. Therefore the ability of Ambush to kill and support foodborne pathogen was attributed to possible differences in additives and/or adjuvants present in the different pesticide formulations. The exact composition of pesticides, which includes adjuvants and inerts, is usually not disclosed on the product label (Ng. *et al.,* 2005).

Verhaelin *et al.* (2013) reported that the human norovirus persisted in reconstituted pesticides including Rovral, Aliette, Teldor, Signum, Karate Z, Primor and Calypso. The pesticide solutions could therefore be a possible source of viruses in the fresh produce chain (Verhaelin *et al.*, 2013).

Depending on the product, the use of highly contaminated agricultural water used to mix pesticides may lead to an increase and inhibition of microbes in the pesticide solution. It is therefore important for growers to regularly monitor water quality for compliance to regulatory requirements. Potable water should be used to prepare pesticide sprays. Application of product solutions immediately after preparation and/or using chemical spray products that inhibit pathogen growth is encouraged. Mixing two or more pesticide products in one spray tank is also ideal as their effects on foodborne pathogens differ.

Fate and microbial biodegradation of pesticides in the environment

The understanding of the toxicity data involving microorganisms and pesticides are limited because most studies have focused mainly on microbial degradation of pesticides rather than the impacts of pesticides on natural microbial populations (Marrie *et al*., 2000). In addition, studies of pesticide effects on soil microbes are far more common than studies of those in aquatic environments, with further studies done in aquatic environments focusing mostly on he whole population of aquatic microbes (Marrie *et al*., 2000), thus making data specific to foodborne pathogens very scarce. Presently, there are about 900 pesticide products and about 600 active pesticidal ingredients on the market (Hall *et al.*, 2001). Millions of tons of pesticides are applied annually. However, less than 5% of these products are estimated to reach the target organism, with the remainder being deposited on the soil and non-target organisms, as well as moving into the atmosphere and water (Pimental and Levitan, 1986). When pesticides are sprayed on vegetables and on soil they consequently remain in the environment for a very long time and accumulate into food chains decades after their application to soil (Kannan et *al.*, 1994). Pesticides such as the organophosphates, which are more readily biodegradable, are therefore now being used in preference to the more persistent chlorinated pesticides. The fate of pesticides in the environment is determined by both biotic and abiotic factors since they are degraded in the environment principally by the action of microorganisms (Kookana and Aylmore, 1994). To predict the fate of pesticides in soil it is important to have an understanding of those microbes able to degrade pesticides, their activity and the factors that limit their activity in *situ* (Aislabie and Lloyd-Jones, 1995). There are several factors that affect biodegradation of pesticides. 1. In the environment these include: presence and number of appropriate microorganisms, contact between the microbe and the substrate, pH, temperature, salinity, available water, oxygen tension and redox potential, nutrient availability, presence of alternative carbon substrates, light quality and intensity, binding to surfaces, and alternative electron acceptors 2. Factors regarding the molecule consist of: chemical structure, molecular weight, functional groups, e.g. -CI, -CH_3, -COOH, -OH, concentration and toxicity and solubility in water (Robertson and Alexander, 1994).

When a biodegradable pesticide moves into the soil environment, a number of competing reactions may alter the availability of the molecule in the solution phase. These competing

reactions tend to ultimately influence the concentration and consequently the microbial adaptation and metabolism processes (Kearney and Kellogg, 1985). The rate of microbial breakdown of organic herbicides in soil water environments has been shown to depend on its concentration and its availability to the microbial community. The differences in molecular structure as well as in chemical and physical properties render the susceptibility of pesticides to biological degradation extremely variable (de Bertoldi *et al*., 1983). Adsorption of pesticide residues to inorganic matter has also been proposed as one of the factors affecting the availability of the pesticides to the microbial community. Several mechanisms have been proposed for adsorption of pesticides to soil organic matter. These are: Van der Waals forces, hydrophobic bonding, hydrogen bonding, ion exchange, and ligand exchange. Many of the biodegradable herbicides are acidic and adsorption depends on the pH of the system. The magnitude of adsorption of acidic pesticides by soil organic matter, however, is much lower than that of cationic or basic pesticides (Finstein and Morris, 1995). More important is the organic matter—clay complexes in most mineral soils. These complexes must be considered in evaluating pesticide adsorption because as a general rule, in a soil with organic matter content up to about 6%, both mineral and organic surfaces are involved in adsorption, but at higher organic matter contents most adsorption will occur on organic surfaces (for example at very low concentrations, little mineralization of 2,4—D occurred when this herbicide was present in aqueous systems at initial concentrations of 2 to 3 ng/ml or less, while 60% or more of the chemical initially present at higher concentrations was converted to CO_2 in 6 days). Apparently microorganisms did not assimilate the carbon derived from metabolism of 2,4—D at low concentrations, but high concentrations of pesticides may also be inhibitory to the resident microbial community (Boethling and Alexander, 1979). Fogerty and Tuovinen (1991) examined the degradation of [carboxy-14C] 2,4-D at different initial concentrations in prairie soil samples. After 56 days, about 90% of the initial 500 ng/ml of the substrate was detected, compared with 8% when 10 ng/ml of 2,4-D was initially applied. In contrast, Parker and Doxtader (1982) reported a direct correlation between the initial 2,4-D concentration (range, 1.3 to 134 ng/ml) and the length of the lag phase preceding active degradation in fine sandy loam. Measurement of the formation of $14CO_2$ from [U-14C]2,4-D showed that high concentrations (5,000 and 20,000 ng/ml) of 2,4-D inhibit the degradation of the parent compound in samples of various soil types, implicating that elevated levels of pesticides, higher than normally applied in the environment, retard the degradation by native soil microorganisms. It is a common trend that mixed cultures degrade pesticides more rapidly than pure cultures. Alternatively, not all microbial communities possess the ability to degrade a pesticide before the first application of that pesticide. In some instances biodegradation will require a new trait to develop within the community; for example, the evolution of catabolic genes, by either a relaxation of substrate specificity or inducer specificity of existing enzymes, or by the acquisition of specific enzymes by genetic exchange (Sahu *et al*., 1995). Eventually in such populations it is anticipated that there will be sufficient advantageous changes to existing genes, and genetic exchange between strains, either located on plasmids or transposable elements, to allow a cell to utilize the newly introduced compound (Pesticide).

When growth conditions are favourable, molecules of biogenic origin are readily degradable by microorganisms (Fogerty and Tuovinen, 1991). Pesticides are synthetic molecules which often contain functional groups such as chlorine substituents and novel arrangements rarely

found in nature of compounds such as diphenylmethane organophosphates and other biogenic molecules which increase recalcitrance, leading to greater persistence. Molecules containing a high amount of chlorine substitutions have proven to be more recalcitrant, thus the location and number of chlorine substituents on a pesticide molecule will affect recalcitrance; for example, 2,4,5-T [2,4,5-trich1orophenoxy acetic acid] is more recalcitrant than 2,4-D [(2,4-dichlorophenoxy) acetic acid] (Aronstein *et al*., 1991).

Pesticide metabolism

The mechanism of pesticide action on microbial species may not be the same as for the target organisms (insects, fungi and weed), as pesticides have been shown to interfere with respiration, photosynthesis, and biosynthetic reactions as well as cell growth, division, and molecular composition of microorganisms (De Lorenzo *et al*., 2001). However, Aislabie and Lloyd-Jones (1995) reported that some pesticides, such as 2, 4 dichlorophenoxy acetic acid (2, 4-D) and carbofuran, can serve as a carbon source to some microorganisms for growth. In the presence of alternative readily degradable carbon sources, bacteria are less likely to degrade the pesticide. At the same time, in the absence of essential nutrients such as carbon, nitrogen, phosphorus and oxygen, where there is adequate concentration of the pesticide to induce appropriate degradative enzymes and uptake mechanisms, the organism can degrade the pesticide and utilize it as a carbon source for growth (Nadeau *et al*., 1994). The pesticide could either be stimulatory or inhibitory to microbial growth, resulting in death, survival or growth of the microorganisms after inoculation, depending on the species and the chemical composition of the pesticide (Peter *et al*., 2005). Understanding pesticide metabolism in plants and microorganisms is necessary for pesticide development, for safe and efficient use, as well as for developing pesticide bioremediation strategies for contaminated soil and water.

2.4 BIOFILMS IN WATER STORAGE AND DISTRIBUTION SYSTEMS

Biofilms are important biological structures that form vital components in all ecosystems and can form in virtually every possible location (Manuzon and Wang, 2007; Reid *et al.,* 2007). The structures of biofilms inherently provide a protected mode of growth that allows the cells contained within them to survive in hostile environments (Costerton *et al.,* 1999). Hostile environments are created by variations in the bulk liquid phase such as: nutrient limitation, fluid flow, desiccation, toxic chemical gradients, UV radiation, and pH and temperature fluctuations (Costerton *et al.,* 1999). It is therefore no surprise that biofilm formation by microorganisms is the preferred way of life, with each planktonic cell being represented by up to a thousand cells attached to the surface (Costerton *et al.,* 1995; Momba *et al.,* 2000; Reid *et al.,* 2007; Watnick and Kotler, 2000). Donlan and Costerton (2002) showed that 99.9% of microorganisms, such as bacteria, found in natural aquatic ecosystems, incorporate into these structures. Huq *et al.* (2008) showed that they can contain up to 1.0 x 10^9 cells per clump although the size of the biofilm is greatly influenced by the chemical and physical conditions of the environment.

Surfaces in natural environments have been shown to be major sites of microbial activity (Van Loosdrecht *et al.,* 1990), with the majority of biofilms forming predominantly in aquatic

habitats (Costerton *et al.*, 1995; Reid *et al.*, 2007). The incorporation of pathogenic microorganisms is of particular concern as the biofilm structure protects them and therefore encourages their persistence in that environment. As parts of the biofilm structure are able to detach, they can be responsible for the majority of the planktonic cells found in the aqueous environment (Chang *et al.*, 2003; Percival *et al.*, 1998; Van der Wende *et al.*, 1989) and may alter the quality of the water (Chang *et al.*, 2003). Detached pieces of biofilm in the liquid phase may find their way onto crops. If a foodborne pathogen was incorporated into the biofilm structure that detached, it could cause subsequent infections and disease, thus having a profound effect on the agricultural sector as well as human health (Manuzon and Wang, 2007; Momba *et al.*, 2000).

Definition of a biofilm

The definition of a biofilm varies greatly between researchers but generally includes: clusters of cells that are adhered to a surface; cells that are suspended in fluid as floccules and cells that have aggregated and exist as pellicles at air-liquid interfaces (Costerton *et al.*, 1995; Hall-Stoodley and Stoodley, 2005). Despite the numerous descriptions of biofilms, they can be defined as complex biologically active heterogeneous communities of self-regulating sessile cells (Garrett *et al.*, 2008; Hall-Stoodley and Stoodley, 2005; Hood and Zottola, 1995; Stoodley *et al.*, 1999) co-adhered to each other and irreversibly attached to an inert or living surface via extra-cellular polymeric substances (EPS) secreted by the cells themselves (Costerton *et al.*, 1999; Garrett *et al.*, 2008; Huq *et al.*, 2008; Donlan, 2002). The EPS of a biofilm is responsible for keeping the cells of the biofilm immobilised and providing a primitive homeostasis, which aids in protecting the cells from variations in the bulk liquid phase (Costerton *et al.*, 1995; Donlan, 2002; Flemming *et al.*, 1999; Hall-Stoodley and Stoodley, 2005; Skillman *et al.*, 1999).

Biofilms are complex structures that can contain numerous microorganisms such as moulds, yeasts, algae, protozoa, archaea and viruses, in addition to bacteria (Huq *et al.*, 2008; Lindsay and von Holy, 2006; Manuzon and Wang, 2007). As a result of the range of microorganisms that can incorporate into these structures, multispecies biofilms are commonly encountered in nature. The microbial content of biofilms differs and can also vary over time, with different organisms becoming more dominant than others (Manuzon and Wang, 2007; Skillman *et al.*, 1999).

Biofilm Formation

Due to biofilm formation being the preferred lifestyle, these structures are encountered in almost all habitats and they develop as a result of a successful attempt at attachment to a surface by a microorganism, followed by subsequent formation of micro-colonies (Hermansson, 1999; Momba *et al.*, 2000). These micro-colonies develop into complex surface communities if conditions are favourable for continued growth (Hermansson, 1999). True biofilms can form in as little time as a few hours or days after bacterial cells have attached to a surface (Hood and Zottola, 1995).

The process of biofilm formation is dictated by various variables, namely: the species of the microorganisms involved, the conditioning layer, environmental factors, and essential gene products (Dunne, 2002). Cells of different species differ in their attachment strategies (O' Toole, 2003) which are governed by initial cell concentration, surface conditions, motility and the lifecycle of the respective microorganisms (Takhistov and George, 2004). Despite differences in attachment strategies between species, the stages of biofilm development appear to be conserved amongst them. Biofilm formation can essentially be broken down into the following steps: conditioning of the surface; transport of microbial cells to the surface; initial adhesion; attachment and colonization. Detachment is also an important point to consider in the life cycle of biofilms since pathogens may be released into the processing systems and contaminate food products and as such may be a cause for concern in the food processing industry (Characklis, 1990; Hood and Zottola, 1995)

Conditioning layer formation

In order for a biofilm of any kind to develop, a conditioning layer is first required, as it forms the foundation on which the biofilm structure develops (Garret *et al.,* 2008). The conditioning layer can develop almost immediately after a surface (either abiotic or biotic) is exposed to an aqueous environment (Schmid *et al.,* 2004). Subsequently, the chemical and physical properties of the surface are altered to a state quite different compared to the native surface (Donlan, 2002; Dunne, 2002). The conditioning layer contents correlate with the composition of the bulk fluid and are the result of particles sedimenting out of the bulk liquid due to gravitational force present (Characklis, 1990; Garret *et al.,* 2008; Hood and Zottola, 1995). The sediment is composed of various particles ranging from organic to inorganic molecules that provide anchorage and nutrients for the biofilm community (Garret *et al.,* 2008). Interactions between the substrates of the conditioning film and the surface often results in the altering of the pH, nutrient levels, ionic strength, temperature and surface tension to conditions more favourable for microorganism attachment, thus affecting the rate and extent of microbial colonization of that surface (Donlan, 2002; Garret *et al.,* 2008). The presence of microorganisms on the surface may also be involved in conditioning of the surface to facilitate the attachment of other microorganisms that were unable to bind initially (Hood and Zottola, 1995). This can be achieved either by the production of waste products in the existing biofilm, which can be utilised by other microorganisms or by providing a surface that they can attach to through EPS production (Patil and Anil, 2005).

Transport to a surface

Before any attachment can occur, the microorganisms have to be transported to a proximity close enough to the surface (usually <1 nm) (Characklis, 1990; Dunne, 2002). This can occur either randomly (via diffusive- or convective transport) or directly (via chemotaxis and active movement) (Costerton, 1999; Dunne, 2002; van Loosdrecht *et al.,* 1990). Random transport of cells to a surface involves transport of the cells to a well-conditioned surface by chance, whereas with directed movement, transport can be by chance or through motile appendages during a chemotactical response to a concentration gradient at the interfacial region (Garrett *et al.,* 2008; van Loosdrecht *et al.,* 1990). Active movement of a cell towards a surface generally occurs at a faster rate as it is not reliant on convection currents or on diffusion gradients (van Loosdrecht *et al.,* 1990). The properties of the surface also greatly influence the attachment

rate. With smoother surfaces longer initial attachment phases are often experienced when compared to rougher surfaces. This effect is however, only initially experienced and doesn't affect the amount of microorganisms that can potentially attach.

Initial adhesion

Factors such as surface functionality, temperature, pressure conditions, bacterial orientation, available energy and local environmental variables determine whether cells that arrive on a conditioned surface will adhere or not, and whether the adherence will be reversible or irreversible (Characklis, 1990; Garrett *et al.*, 2008). The final determination of whether or not the cells will adhere comes down to the net sum of attractive or repulsive forces generated between the attachment surface and the cell surface, and whether or not the cell has the capability to overcome the repulsive energy barriers that it will experience at the surface (Dunne 2002; Skillman *et al.*, 1999). If the repulsive forces present are greater than the attractive forces, cells will cease association with the surface and attachment will not occur and vice versa (Garret *et al.*, 2008).

Reversible attachment

If the attractive forces are greater, and the cells have reached a close enough proximity to the surface, they are able to associate with that surface and undergo initial adhesion (Garret *et al.*, 2008; van Loosdrecht *et al.*, 1990). During initial adhesion, the cells up regulate specific genes that are required for attachment, such as genes for cell surface proteins and excretion products, and down regulate or inhibit genes that are no longer required, such as surface appendages (Costerton, 1999; Jefferson, 2004; Garret *et al.*, 2008). This signal cascade, that is triggered once the cells have overcome all the negative energy barriers associated with the solid surface to which they are attached, can be associated with the cells ability to sense the environment in which they find themselves (Jefferson, 2004). This profound change in phenotype ensures that the cells progress from reversible- to irreversible attachment thus giving biofilm-associated phases of the bacterial life cycle significantly different phenotypes compared to their planktonic counterparts (Costerton, 1999; Costerton *et al.*, 1999; Manuzon and Wang *et al.*, 2007; Watnick and Kotler, 2000). Initial adhesion can occur within minutes (Costerton, 1999) or seconds as shown by Takhistov and George (2004). The authors showed that *Listeria monocytogenes* cells in pure culture were able to undergo random reversible attachment within 3-5s of contact with a surface. Although the adhesion was sparse at first, attachment did become more concentrated over time.

Irreversible attachment

The transition from reversible- to irreversible attachment starts when the adhered cells start secreting adhesive polymers. The adhesive polymers increase the cells ability to attach to the surface but also adjust the microenvironment surrounding the cells (Hood and Zottola, 1995; Takhistov and George, 2004). In the absence of chemical- or physical intervention, the cells are able to firmly attach to the surface and start colonising the area (Dunne, 2002). Maturation of the biofilm can only occur once the cells have irreversibly attached to the surface and involves an increase in the density and complexity of the biofilm through cell division of cells trapped in the biofilm or the attachment of subsequent cells (Dunne, 2002; Jenkinson and Lappi-Scott, 2001; O' Toole, 2003; Takhistov and George, 2004; Webb *et al.*, 2003). This

increase in cell biomass first results in the formation of micro-colonies before a fully mature biofilm structure is achieved through the formation of inter-colony bridges (Lindsay and von Holy, 2006; Takhistov and George, 2004).

Detachment

The ability of attached cells to proliferate is highly dependent on the availability of nutrients as well as the cell growth kinetics (Takhistov and George, 2004). As the biofilm grows in density and size, the ability of nutrients to diffuse to cells within the biofilm decreases and most often, the nutrient levels in the immediate environment surrounding the biofilm also start to decrease. At this point, the biofilm is said to have reached a critical mass and the equilibrium of the structure shifts from the incorporation of cells, to the dissociation of cells from the biofilm (Dunne, 2002; Garret *et al.,* 2008). Once the biofilm cells return to the planktonic state of growth, the biofilm developmental life cycle is said to be complete (Stoodley *et al.,* 2002).

The dissociation of cells can occur through the generation of planktonic daughter cells from actively growing cells at the periphery of the biofilm structure (Donlan, 2002; Dunne, 2002) or the highly regulated process of quorum sensing, Quorum sensing has been shown to be the primary cause of cell release (Lindsay and von Holy, 2006). Under limiting nutrient conditions, cells contained within the biofilm use quorum sensing to instruct other cells to release enzymes, in order to break down the EPS which holds them together to release valuable polysaccharides (Garret *et al.,* 2008; Takhistov and George, 2004). The degradation and utilisation of polysaccharides in the EPS not only supports the on-going survival of the cells but also weakens the biofilm structure, thus allowing the release of cells into the bulk liquid phase for colonisation on virgin areas (Costerton and Lewanolowski, 1995; Costerton *et al.,* 1999; Donlan, 2002; Dunne, 2002; Garret *et al.,* 2008; Lindsay and von Holy, 2006; Shirtliff *et al.,* 2002; Takhistov and George, 2004).

Not all removal of cells from the biofilm structure is co-ordinated from within the biofilm itself. Cells can also be removed through high shear forces whereby clumps of cells are released into the bulk fluid from the extremities of the structure (Characklis, 1990; Donlan, 2002). The mechanism in which cells are released from the biofilm affects the phenotype displayed by those cells directly after release. Cells that are forcibly removed by shearing often demonstrate biofilm characteristics whereas cells that underwent planned release convert back to the planktonic phenotype relatively quickly (Donlan, 2002). The return to the planktonic phenotype involves the up regulation of suppressed genes such as the production of motile appendages and the down regulation of genes that facilitated adhesion to a surface such as cell surface proteins and excretion products (Costerton, 1999; Garret *et al.,* 2008). Numerous studies have demonstrated that the release of planktonic cells from a biofilm structure serves as a persistent source of contamination in the bulk fluid (Costerton *et al.,* 1995; Hanna and Wang, 2007; Shirtliff *et al.,* 2002; van der Wende *et al.,* 1989).

Biofilm architecture

The structures of biofilms are not uniform or mono-layered but rather heterogeneous, and often appear as patches on a surface (Donlan and Costerton, 2002; Momba *et al.,* 2000). The thickness also varies amongst biofilms, with some only a few layers thick whilst others consist of complex compositions of different microorganisms (Lewandowski and Beyenal, 2003; Lindsay and von Holy, 2006; Shirtliff *et al.,* 2002; Stoodley *et al.,* 2002). Despite this variance in content and structure, all biofilms have a similar design.

The biofilm structure is made up of numerous micro-colonies which are said to be the basic structural units of the biofilm architecture. Micro-colonies take on the appearance of mushroom shaped projections that are anchored to a point on the surface and are linked to each other through inter-colony bridges (Costerton *et al.,* 1999; Donlan and Costerton, 2002; Dunne, 2002; Garret *et al.,* 2008). Most of the microorganisms present within the micro-colonies incorporate into the 'crown' of the mushroom shaped appendages with very few being found in the 'stalk' (Costerton, 1999).

All of the micro-colonies are surrounded by water channels that allow the convective flow of water through the biofilm structure (Costerton *et al.,* 1995). These channels can reach widths of 0.3µm (Costerton *et al.,* 1995) and are not only responsible for hydrating the biofilm but also allowing the passage of nutrients into the structure and the removal of waste from deep within (Costerton *et al.,* 1999; Garret *et al.,* 2008; Lindsay and von Holy, 2006; Shirtliff *et al.,* 2002). The availability of nutrients and the removal of waste to and from the biofilm respectively, and the microorganisms present within the biofilm, all contribute to micro-environmental changes as the biofilm grows and the matrix thickens (Momba *et al.,* 2000). Local variations are often encountered as different regions vary in their pH and nutrient- and oxygen availabilities (Jefferson, 2004). These local variations provide various habitats within the structure that facilitate the incorporation of multiple species with different growth requirements (Flemming *et al.,* 1999). The ability of the primitive circulatory system formed by the water channels to supply cells with nutrients and remove waste, ultimately limits the maximum growth potential of the biofilm (Dunne, 2002).

Factors that affect physical properties and architecture

The structure of a biofilm is greatly influenced by the genetic factors of the microorganisms that incorporate into the biofilm. However, physical factors imposed on the biofilm from the environment can also affect the physical properties and architecture of the structure (Hall-Stoodley and Stoodley, 2002). Factors such as the flow regimen of the bulk fluid, temperature, surface properties, nutrient availability and the EPS composition can all have effects of varying degrees.

The EPS, which is not only involved in attachment of cells to a surface but is also a determining factor in the strength of the attachment, makes up 75-90% of the biofilm structure, whilst only 10-25% of the content consists of actual microbial cells (Costerton *et al.,* 1999; Donlan and Costerton, 2002). The EPS varies in chemical and physical properties based on the microorganisms that produce them and the surrounding environmental factors, with even individual micro-colonies within a biofilm varying greatly in their immediate

surroundings due to differences in EPS composition (Costerton *et al.,* 1999). The majority of literature refers to the EPS as a substance that 'cements' the cells of a biofilm to each other and a surface, much like a calcified exoskeleton; when in fact, the EPS is actually a highly hydrated gelatinous slime layer (Donlan, 2002; Dunne, 2002; Momba *et al.,* 2000). The extracellular polymeric substances of the biofilm are generally made up of polysaccharides and proteins but may also contain lipids and nucleic acids, all of which influence the strength of the matrix (Goodwin and Forster, 1985; Urbain *et al.,* 1993). Other particles such as mineral crystals, corrosion molecules and clay particles can also become entrapped in the polysaccharide matrix (Donlan, 2002). Extracellular polymeric substances that are predominantly made up of polysaccharides, such as those produced by diatoms, tend to be stronger and therefore make it increasingly difficult for the biofilm to be removed from a surface (Becker, 1996).

In addition to the EPS, the flow regimen of the bulk fluid can also greatly influence the strength of the structure against environmental factors and it can also have an effect on the morphology of the biofilm structure (Stoodley *et al.,* 1999). The structure tends to display more filamentous properties with increased flow velocity and shows a distinctive orientation in relation to the flow of the bulk fluid (Stoodley *et al.,* 1999). Biofilms that develop in high-shear environments tend to have stronger structures that are more resistant to mechanical shearing, compared to biofilms that develop in low-shear environments which have a much lower tensile strength and therefore break more easily (Costerton *et al.,* 1995; Donlan and Costerton, 2002). The stronger structures can also be attributed to the density of cells contained within the biofilm. In high shear environments with higher fluid velocities, higher mass transfer conditions are experienced, thereby supplying the cells with more nutrients and allowing increases in cell densities (Simões *et al.,* 2006; Stoodley *et al.,* 1999). A study by Simões *et al.* (2006) found that biofilms under high shear conditions, with increased nutrient concentrations, contained higher numbers of culturable cells due to the increased mass transfer occurring compared to biofilms under less turbulent conditions, which had less active inner cells. Nutrient availability on its own can also influence the physical properties of the biofilm (Simões *et al.,* 2006). Stoodley *et al.* (1999) found that increasing the carbon and nitrogen concentration in the bulk liquid phase caused an immediate change in the morphology of the biofilms in terms of surface coverage and thickness.

Physical factors such as changes in temperature have a profound effect on the formation of biofilms as well as their physical properties once the structures have developed. Numerous studies have considered the effects of temperature on biofilm formation in a variety of environments and have found that temperature changes had effects on the microbial community composition (Anderson-Glenna *et al.,* 2008; Lindström *et al.,* 2005), cells entering a viable but not cultural state (Whitesides and Oliver, 1997), and increased/ decreased gene regulation (White-Ziegler *et al.,* 2008). Lower temperatures have been found to promote biofilm formation by bacteria due to the up regulation of genes required for attachment (White-Ziegler *et al.,* 2008) and the increased stability of the extracellular polymeric substances required by the cells to attach to a surface (Else *et al.,* 2003; Garret *et al.,* 2008). A study by Villain-Simonnet *et al.* (2000) showed that the polysaccharides of the *Rhizobium Leguminasorum* EPS increased in strength with the increase in temperature.

However, this was only achieved up to a critical point where the gelatinous EPS turned into a solution. This change from a gel-like structure to a solution can influence the adherent ability of the microorganisms and as a result have an effect on the strength and structure of the biofilm. Changes in temperature also influence nutrient uptake by affecting enzyme activity. Deviations from the optimum enzyme temperature decrease the enzymes activity and as a result the nutrient uptake, irrespective of the availability of nutrients present (Garret *et al.,* 2008). In a study done by Ndiongue *et al.,* (2005) it was found that there was a marked difference in the amount of time required for a biofilm growing at 6°C to reach steady state biofilm heterotrophic plate count (HPC) numbers, compared to the biofilm that developed at 18°C, which reached steady state much faster.

Benefits of communal living

Life within a biofilm is the preferred way of life for microorganisms. This is evident in most environments, with the number of cells attached to a surface far exceeding the number of cells found in the planktonic state. Biofilm formation is favoured, as the biofilm matrix can afford a certain degree of protection to the cells that it contains, by forming a primitive homeostasis (Costerton and Lewanolowski, 1995; Costerton *et al.,* 1995; Donlan, 2002; Flemming *et al.,* 1999; Hall-Stoodley and Stoodley, 2005; Skillman *et al.,* 1999). This primitive homeostasis can protect the cells contained within the biofilm structure from adverse environmental factors such as chemical or biological antimicrobial agents (Costerton, 1995; Costerton and Lewanolowski, 1995; Hood and Zottola, 1995; Huq *et al.,* 2008) which include: predation by phagocytic amoebae, bacterivorous microorganisms, bacteriophages and protozoa (Costerton *et al.,* 1995; Donlan and Costerton, 2002; Huq *et al.,* 2008; Webb *et al.,* 2003, as well as UV radiation, pH and temperature fluctuations (Hall-Stoodley and Stoodley, 2005). Environmental stresses such as nutrient limitation, fluid flow, oxidative stress, desiccation and toxic chemical gradients can also trigger biofilm formation (Donlan and Costerton, 2002; Huq *et al.,* 2008). It is therefore no surprise that biofilm formation usually occurs under adverse conditions as an attempt to survive in unfavourable environments.

One of the largest benefits of community living is that it enables resistance to a number of removal strategies, such as antimicrobial and antifouling agents and shear stress (Shirtliff *et al.,* 2002). Biofilm cells have profoundly increased tolerance towards antimicrobial agents such as antibiotics, disinfectants or germicides (Webb *et al.,* 2003). This increased tolerance towards such agents can be observed as an early phenotype in cells that have incorporated into biofilms, with biofilm cells generally displaying higher tolerance than their planktonic counterparts (Aruscavage *et al.,* 2006). Mechanisms such as delayed penetration due to the biofilm matrix, altered growth rate of biofilm cells, greater cell density, physiological changes within the cells themselves and micro-environmental changes that affect the activity of the antimicrobial agents can all be responsible for increased resistance (Costerton *et al.,* 1999; Donlan and Costerton, 2002; Dunne, 2002; Manuzon and Wang, 2007). Increased resistance to antimicrobial agents can also be attributed to transfer of genes on plasmids between cells of the biofilm (Donlan and Costerton, 2002; Jefferson, 2004). The biofilm structure facilitates the rapid transfer of transmissible genetic elements such as plasmids and phages between cells due to the close proximity of cells, and promotes the spread of multiple bacterial resistance genes (Donlan, 2002; Jefferson, 2004; Watnick and Kotler, 2000). Horizontal gene transfer

between cells of a biofilm can also increase the structure's resistance to anti-microbial agents (Jefferson, 2004).

Another advantage to the biofilm mode of growth is the survival of cells under starvation conditions where the surface associations provide selective growth advantages to the cells contained within the biofilm. The biofilm structure allows cells to use nutrients that are often found in greater abundance in the conditioned layer on the surface, especially during starvation conditions when nutrients are found at greater concentrations at surfaces than in the bulk fluid (Hood and Zottola, 1995; Lindsay and von Holy, 2006). The biofilm matrix can also serve as a source of nutrients when the surrounding environment's nutrients are limited. The cells are able to utilise the polysaccharides within the matrix not only to sustain their growth, but also to provide the opportunity for detachment (Costerton and Lewanolowski, 1995; Costerton *et al.,* 1999; Donlan, 2002; Dunne, 2002; Garret *et al.,* 2008; Lindsay and von Holy, 2006; Shirtliff *et al.,* 2002; Takhistov and George, 2004). Nutrient availability is a major determining factor in microorganism life strategy and is also an integral part of the survival of the biofilm population (Takhistov and George, 2004).

Techniques used to measure and observe biofilm accumulation and identify microorganisms that have been incorporated

Information on surface colonisation by biofilms in numerous environments is increasing as techniques used to study the population density, structure and function of biofilm communities improves (Araya *et al.,* 2003). Several techniques can be employed to study biofilm formation, although many of them are labour intensive, destructive to the biofilm structure, have low sensitivity (Bakke *et al.,* 2001) or don't take into consideration viable but not culturable cells (VBNC) (Araya *et al.,* 2003).

Quantification of biomass

Quantification of the biofilm biomass can be carried out via in-situ studies without cell removal or by removing the cells from their attachment surface in order to determine the number of cells present. Both mechanisms of biofilm study have advantages and disadvantages and the choice of method is dependent on the researcher, the type of research to be carried out and the equipment available (Butterfield *et al.,* 2002). It is necessary to standardise techniques as they vary greatly amongst and as a result, prevent comparison between studies.

In indirect methods, the biofilm biomass is determined non-invasively and often involves methods that quantify cellular components to determine microbial numbers (Hood and Zottola, 1995). Such components include: Adenosine triphosphate (ATP), proteins or exopolysaccarides (EPS) (Hood and Zottola, 1995). Bakke *et al.* (2001) made use of the indirect method of optical density measurements to determine biofilm cell mass and biofilm total mass. They found that the amount of light absorption through the biofilm could be used *in situ* and in real time, whilst still being sensitive enough to determine the cell biomass.

In direct methods, dislodging of cells is followed by direct counting via plate counts, microscopic techniques or radiolabelling (Hood and Zottola, 1995). Techniques that require

the removal of cells from a surface to determine the biomass, have been the preferred method for biofilm study for many years. Such methods usually initially involve the dislodging and separating of all the cells associated with the biofilm. Dislodging of cells can be done by scrapping off of the cells with a sterile blade (Banning *et al.,* 2003), removing attached cells with swabs (Hallam *et al.,* 2001), stomaching of biofilm samples (Gagnon and Slawson, 1999), removal with beads (Lehtola *et al.,* 2006) or through vortexing. Due to inconsistencies in cell harvesting protocols, there are always discrepancies in the number of cells that are removed. Direct methods of quantification have the added disadvantage that clumps of cells are often not dispersed and are therefore quantified as a single cell rather than a mass of cells. Cells that are forcibly removed often also experience a certain degree of damage; although these cells are still viable, they may be non-culturable and this may give incorrect results during enumeration (Lindsay and von Holy, 2006).

Methods for visualising biofilms

Microscopy continues to be the most widely used tool in the analysis of biofilms (Huq *et al.,* 2008). Microscopic techniques that are commonly used include: Confocal Scanning laser microscopy, Scanning Electron Microscopy (SEM) and Atomic force microscopy. Atomic force microscopy is of particular interest as it doesn't involve metal coating or staining of specimens before non-invasive imaging under near physiological conditions is performed (Beech *et al.,* 2002). It is also advantageous as it allows real time in-vivo visualisations of biofilms on different surfaces in their native states (Beech *et al.,* 2002). Despite its advantages over SEM, SEM is still most commonly used as it conveniently allows for the examination of larger areas, thus allowing specific areas to be located and zoomed in on (Beech *et al.,* 2002). However, the image obtained doesn't necessarily show the true morphology of the biofilm as the reagents and methods used for sample preparation dehydrates the biofilms and gives the EPS a fibrous appearance versus the thick gelatinous substance that would be visible before sample preparation (Donlan and Costerton, 2002). The use of fluorescent markers or probes is also commonly used to detect and visualise specific microorganisms within the biofilm structure (Manz *et al.,* 1993). This technique is extremely useful as it allows the spatial organisation of the biofilm to be determined (Manz *et al.,* 1993).

Identification of the genetic diversity

The microbial composition of a biofilm can be determined through identification of the genetic diversity found within the microbial community. In order to accurately assess the genetic diversity, molecular methods are preferred as not all microbes can be recovered through standard cultivation methods (Manuzon and Wang, 2007). The genetic diversity can be determined by extracting the DNA from the cells and sequencing highly conserved regions such as the 16S region in bacteria and the ITS region in fungi. The 16S rRNA gene is ideal for identification as it is highly conserved, forms part of the stable genome and can be compared to the 16S rRNA gene of all other bacteria, the 16S rRNA gene of archeobacteria and the 18S rRNA gene of eukaryotes (Clarridge, 2004); thus allowing identification of all species present.

Identification can be carried out with genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998). Denaturing gradient gel

electrophoresis has been successfully applied to numerous studies to compare the bacteria present in a particular environment despite many of them not even being described yet (Araya *et al.,* 2003; Cody *et al.,* 2000). Another popular technique employed to identify microorganisms is matrix assisted laser desorption ionisation time of flight (MALDI TOF) analysis. MALDI TOF is a mass spectrometry ionisation technique that allows the identification of microorganisms through their protein profile. The protein profile of each microorganism is unique and can be compared to a protein profile database to identify the microorganism (Albrethsen, 2007). The only disadvantage of this technique is that it requires the cultures to be culturable.

Biofilms in aquatic environments

The microbiology of rivers, streams and other natural water bodies has been neglected worldwide in the past with more interest being paid to potable water systems (Neu and Lawrence, 1997). Various microbial and chemical guidelines have been set in place to monitor and regulate the quality of the countries irrigation water (DWAF, 1996). However, in order to enforce monitoring and compliance, a strong institutional framework is required (Steele and Odumeru, 2004). Guidelines governing the microbial quality of irrigation water worldwide vary significantly between countries and different water sources, thereby demonstrating the widespread uncertainty about the risks associated with the poor microbial quality of irrigation water.

Irrigated agriculture accesses water supplies from large reservoirs, dams, rivers, groundwater, municipal supplies and industrial effluent. The quality of these surface- and groundwater supplies varies greatly and is dependent on the source (DWAF, 1996; Steele and Odumeru, 2004). The presence of pathogens and indicator organisms in irrigation water can be influenced by various factors, such as the intrinsic physical and chemical characteristics of the catchment area, and the magnitude and range of human activities and animal sources that act as point sources for pathogen release into the environment (WHO, 2008). Numerous studies carried out on different natural water bodies within South Africa have shown that the water is below drinking water standards due to faecal contamination (Biyela *et al.,* 2004; Lin *et al.,* 2004; Obi *et al.,* 2002) and it also often shows levels of contamination that deem it unfit for irrigated agricultural purposes according to the South African water quality guidelines (DWAF, 1996). As a result, monitoring of ground and surface water microbial quality in South Africa has come to the forefront as many potential faecal pollution sites within the hydrological cycle exist due to the formation of numerous densely populated informal settlements that lack basic services such as sanitation and a safe potable water supply.

Water storage tanks

Collection and storage of untreated natural water sources, when they are available, is common practice (Momba and Notshe, 2003) in countries such as South Africa, where a great proportion of the rural communities lack access to clean potable water facilities (DAFF, 2010; Momba and Notshe, 2003). The collection and storage of water in large storage tanks can be problematic as it often leads to deterioration of water quality and an increased risk of contamination due to stagnation of the water and long storage periods (Graham and VanDerslice, 2007; Le Chevailler *et al.,* 1996).

The conditions under which the water is stored often intensifies the poor quality of the water, as stored water is more susceptible to environmental influences and contamination, than in its natural habitat (Jagals *et al.,* 2003). However, the water contained within water storage containers can be tainted prior to storage via storm runoff, faulty septic systems, contaminated soil, runoff from manure storages, or livestock/ wildlife faeces (Beuchat, 2002; Cessford and Burke, 2005). The collection and storage of untreated water supplies such as roof catchments (rainwater harvesting), surface water and groundwater, which are contaminated with pathogens, is concerning as it can provide an ideal environment for pathogen proliferation.

Numerous studies have been done to monitor the microbial quality of water that is transported and stored in small household containers (Jagals *et al.,* 2003; Momba and Kaleni, 2003; Momba and Mnqumevu, 2000; Momba and Notshe, 2003). Many of these studies have shown that the transport and storage of water after collection from the source, results in microbial deterioration of the water which often leads to levels of heterotrophic bacteria that are unsuitable for human consumption. The quality of the water that is collected can drastically affect the rate of deterioration of the stored water (Ahmed *et al.,* 2008; Ahmed *et al.,* 2010; Kahinda *et al.,* 2007; Zhu *et al.,* 2004). Biofilm development on the interior surface of water storage containers also contributes to the deterioration of the water quality. Numerous studies have demonstrated that this environment is ideal for biofilm formation (Jagals *et al.,* 2003; Momba and Kaleni, 2002; Momba and Notshe, 2003). As biofilms in water storage devices, much like distribution system biofilms, are able to shed, they can be responsible for the majority of the planktonic cells found in the aqueous environment (Chang *et al.,* 2003; Jagals *et al.,* 2003; Le Chevailler *et al.,* 1996; Percival *et al.,* 1998; Van der Wende *et al.,* 1989).

The type of material an abiotic surface is composed of has been shown to influence biofilm formation. Water storage containers made of plastic based materials, such as polyethylene, have been observed to support more bacterial incorporation into biofilms on their interior surfaces than those made of metal based materials (Momba and Kaleni, 2002; Momba and Notshe, 2003). In addition, studies have shown that plastic based water storage containers have a greater affinity to support the incorporation of faecal coliforms into biofilm structures (Momba and Kaleni, 2002; Momba and Notshe, 2003). This is concerning as these biofilms can act as reservoirs for coliforms and pathogenic microorganisms, that can, through growth and detachment, be responsible for the majority of the planktonic cells found in the aqueous environment (Chang *et al.,* 2003; Percival *et al.,* 1998; Van der Wende *et al.,* 1989).

Biofilms formation in water distribution systems

Biofilms have been found to develop under many conditions and locations. However, the interior surfaces of water distribution systems are particularly ideal: the water is under laminar flow conditions, there are large surface areas, there is a continuous supply of nutrients and the surface chemistry is fitting (Garrett *et al.,* 2008). The extent to which microbes become associated with a surface can significantly enhance their survival and growth potential within the distribution lines of water distribution systems (Ridgway and Olson, 1981).

Routine water samples taken from water distribution systems and natural habitats are generally used as indicators of the quality of the water. However, the majority (99.9%) of the

microbial ecology of such water systems is attached to surfaces that the water is exposed to (Donlan and Costerton, 2002; Huq *et al.,* 2008; Juhna *et al.,* 2007). This gross underestimation of the amount of microorganisms present in these systems is partially due to the difficulty involved in determining the extent of biofilm formation due to the nature of sampling (Boe-Hansen *et al.,* 2003). As a result, biofilms in these environments have been rarely examined in the past with many researchers opting to use models to represent what could happen under different environmental (e.g. temperature, organic matter) and operating parameters (e.g. flow rate, chlorine residual) (Berry *et al.,* 2006; Camper *et al.,* 1999; Gagnon and Slawson, 1999). However, biofilm monitoring within water distribution systems has improved with the introduction of new sampling procedures that involve installing biofilm samplers into the walls of industrial pipes and vessels, thus allowing monitoring to occur efficiently without damage to the system (Juhna *et al.,* 2007).

Biofilm formation in irrigation water distribution systems

The quality of water used in irrigated agriculture is of utmost importance as plants require water to grow and this exposes them to an array of contaminants if unsafe water is used (Aruscavage, 2006). It has been shown that illnesses associated with the consumption of fresh produce, can be traced back to crop contamination as a result of contaminated irrigation water (Heaton and Jones, 2008). This problem is further exacerbated when biofilms that develop within the irrigation equipment act as a mechanism of growth and persistence for pathogens, as has been shown in potable water distribution systems (Block, 1992; Juhna *et al.,* 2007; Le Chevailler *et al.,* 1996; Mackerness *et al.,* 1993).

Polyethylene (PE) piping, which is commonly used in potable water distribution systems, is also primarily used in irrigated agriculture. Studies comparing PE to other materials used in water storage and distribution systems, showed that PE accommodated the incorporation of high numbers of microorganisms into biofilms that developed on their surfaces (Lehtola *et al.,* 2004; Momba and Kaleni, 2002; Momba and Notshe, 2003). The ability of a plumbing material to support biofilm formation is often encouraged by the ability of the material to supply nutrients, additional to the conditioning layer, for microbial growth (Momba *et al.,* 2000). A previous study found that polyethylene pipes were able to release significant amounts of phosphorus into the water and as a result, increase the microbial growth potential within the water distribution system (Lehtola *et al.,* 2004). Numerous studies have monitored the ability of polyethylene potable water distribution systems to support biofilm formation (Hallam *et al.,* 2001, Lehtola *et al.,* 2004; Lehtola *et al.,* 2006; Momba *et al.,* 2000; Ndiongue *et al.,* 2005). A study done by Mackerness *et al.* (1993) showed that municipal potable water contains sufficient nutrients and growth elements to support the incorporation of *E. coli* and coliforms into biofilms on the surface of water distribution systems. This was also confirmed by Le Chevallier *et al.* (1987) who found that biofilms were responsible for re-inoculating the bulk fluid of a potable water distribution system with coliforms that had persisted with the biofilm structure. Juhna *et al.* (2007), however, found that the *E. coli* that they were monitoring within a water distribution system biofilm was found not to be multiplying but rather just existing within the biofilm due to their scarce distribution.

The ability of pathogens to persist within biofilms in water distribution systems is dependent on the interaction of numerous factors such as microbial ecology, chemical influences, physical conditions and the operational factors of the distribution system (Berry *et al.,* 2006; Camper *et al.,* 1999). These include temperature effects, pH, support material, flow rates and the microbiological quality and organic substrata in the water (Camper *et al.,* 1999; Momba *et al.,* 2000). The use of contaminated water for irrigation purposes may lead to the incorporation of these pathogens into biofilm structures on the interior surface of the pipes, which contributes to contamination of the water long after the original water source is no longer contaminated. In addition to the detachment of pathogenic microorganisms, the detachment of other bacteria and organisms from biofilms can also result in the deterioration of the water not only aesthetically (Gibbs *et al.,* 2003), in taste and odour, but also in the microbiological quality of the water which could lead to adverse human health effects (Gibbs *et al.,* 2003; Huq *et al.,* 2008; Kerr *et al.,* 1999). Biofilm detachment can also be responsible for the clogging of tubes, valves, and nozzles which can render a system inoperative (Schmid *et al.,* 2004).

Studies have considered the role of biofilms in disrupting the functionality of irrigation systems through clogging of drippers and irrigation parts (Capra and Scicolone, 2007. However, to our knowledge no studies have been performed to monitor the rate of biofilm formation on PE pipes in an irrigation setting. In a study by Pachesky *et al*. (2011), the potential for heterotrophic biofilms in irrigation pipes to influence the microbiological quality of irrigation water was evaluated. It was concluded that the microbial quality of irrigation water can be changed significantly in an irrigation system. It was suggested that flushing of irrigation systems may be a useful practice to decrease the risk of fresh produce contamination.

Controlling crop contamination and biofilm formation in irrigation systems

The presence of biofilms, whether in irrigation systems or potable water systems, can have an effect on the efficiency of the operation of the system which can result in increased costs and loses in profitability (Bott, 1999). For the agricultural industry this can mean that the irrigation pipes have to be replaced, which results in losses in irrigation time and incurring unnecessary costs. Possible solutions to controlling the formation of biofilms in irrigation distribution systems could be the addition of chemical additives such as biocides or biodispersants to the water (Bott, 1999). The use of biodispersants is favoured over biocides as there are fewer risks associated with the contact of the chemicals with the crops. An alternative for the control of biofilms would be the use of physical methods such as the use of electric fields to keep cells in suspension and the application of ultrasonic waves to agitate the water (Bott, 1999). However, these would become tedious when considering the large size of farms and the amount of irrigation distribution piping that is used. Other techniques such as extensive heating of the water or UV irradiation would also be a waste of time and effort.

Biofilm formation in irrigation distribution systems cannot be completely averted. However, the risks associated with pathogen persistence in the biofilms can be minimised through adequate management of the water supplies distributed by the irrigation systems. Most water users can prevent or minimise contamination by keeping irrigation sources away from

livestock, such as cows and poultry, and ensuring that manure applications to fields have no potential of contaminating irrigation water sources (Steele and Odumeru, 2004). Restricting the use of poor quality irrigation water would be the most effective means of preventing crop contamination and potential persistence of pathogens in irrigation systems. This is however not always attainable as many irrigation schemes are located downstream of systems that experience upstream water quality deterioration activities (Department of Water Affairs and Forestry, 1996; James, 2006). If the water is of poor microbial quality, its use should be restricted to crops that are not likely to be consumed raw or a different irrigation scheme should be employed to minimise contact of water with the crops (Steele and Odumeru, 2004). Drip or surface irrigation poses the lowest risk of crop contamination.

Conclusions

Biofilm formation can be expected in all environments, even in those that are closely monitored, such as potable water distribution systems. Fully developed biofilms can be encountered in aqueous environments within a few hours after exposure and only require a few cells to attach to the surface. Multiplication within the biofilms and the additional attachment of cells contributes to the rapid formation of this protective structure that harbours 99.9% of the microbial microflora present in that environment.

Biofilm formation associated with water distribution systems and water storage devices are the predominant causes of water deterioration. Despite this, the extent of biofilm formation within irrigation systems has been underestimated and therefore very poorly studied with minimal evidence available for the microbial content of such systems. Water management systems are proposed as an alternative to on-farm treatment of irrigation water due to the complexity and the costs associated with treatment. Protection of the water source can minimise potential crop contamination. However, biofilm formation cannot be completely avoided. Despite this, future studies should still attempt to elucidate the extent of biofilm formation under current irrigation conditions and determine the potential of pathogens to persist within systems if an improvement in the state of irrigation water is to be made.

2.5 INTERNALISATION POTENTIAL OF HUMAN PATHOGENIC BACTERIA IN FRESH PRODUCE

The attachment of human pathogenic bacteria (*Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus*) to and colonisation of fresh produce surfaces have been studied and reported on extensively (Collignon and Korsten, 2010; Aruscavage *et al*., 2008; Ijabadeniyi *et al*., 2011). Additionally, numerous studies have shown the ability of these pathogens to enter the plants' tissues via different routes (Mililllo *et al*., 2008; Erickson *et al*., 2014; Deering *et al.*, 2011b; Chitarra *et al*., 2014; Wright *et al*., 2013; Mascaricin *et al*., 2014). Once internalised, human pathogens may move into the xylem (Guo *et al*., 2001), secondary xylem and axial and ray parenchyma, which store nutrients and water to sustain the viability of plants, and possibly, promote the survival of human pathogenic bacteria (Guo *et al*., 2001). Internalisation is a very important factor in the safety of fresh fruit and vegetables because current washing and surface sanitizers are ineffective at removing internal bacteria (Aruscavage *et al*., 2006). Consumption of the contaminated produce poses a

potential health risk for the consumer (Pachepsky *et al*., 2011). Approximately 20% of all verotoxigenc *E. coli* (VTEC) outbreaks were associated with contaminated fruit and vegetables (Wright *et al*., 2014).

Sixty and seventy per cent, respectively, of studies summarised in a review paper by Deering *et al*. (2011a) reported the internalisation of *E. coli* O157:H7 and *Salmonella* spp. in fresh produce (Table 3). In contrast, 40 and 30% of studies respectively reported that they found no evidence of internalisation of these pathogens. In our internalisation study, sterile distilled water was spiked with either one of the four human pathogenic bacteria (10^5 CFU/mL) and used to irrigate the lettuce seedlings grown in vermiculite and mature lettuce plants grown in a hydroponic system. Hydroponic systems were also used in other studies to investigate the internalisation potential of human pathogens in tomatoes (Guo *et al*., 2001), spinach (Warriner *et al.*, 2003), maize (Bernstein *et al*., 2007) and lettuce (Franz *et al*., 2007). Other internalisation studies used soil (Warriner *et al*., 2003; Bernstein *et al*., 2007; Mootian *et al*., 2009; Sharma *et al.*, 2009; Erickson *et al*., 2010a) or compost (Erickson *et al.*, 2010c) as growth medium, or directly inoculated leaf surfaces (Zhang *et al*., 2009) or seed (Jablasone *et al*., 2005; Miles *et al*., 2009). In a study by Quillam *et al*. (2012) twelve different lettuce cultivars were inoculated with a chromosomally *lux*-marked *E. coli* O157:H7 strain and cultivar specific phyllosphere and rhizosphere activity of the pathogen was observed.

Table 3: Summary of internalisation studies of *Salmonella* spp. or *E. coli* O157:H7 in plants according to Deering *et al*. (2011a).

Bacteria	**Plant**	**Source of contamination**	**Method**	**Internalised bacteria identified**
E. coli O157:H7	Apple	Wash water	Plating	Yes
Salmonella spp.	Tomato	Water (plants grown hydroponically)	Plating	Yes
Salmonella enterica	Tomato	Soil with *M. inconita* infection	Plating	No
E. coli	Spinach	Seed contamination	Plating and microscopy	Yes
E. coli	Spinach	Soil contamination	Plating and microscopy	No
E. coli	Spinach	Water (plants grown hydroponically)	Plating and microscopy	Yes
E. coli	Mung bean	Seedling contamination	Plating and microscopy	Yes
Salmonella s.v. Montevideo	Mung bean	Seedling contamination	Plating and microscopy	Yes
E. coli O157:H7	Oranges	Wash water	Plating	Yes
Salmonella spp.	Oranges	Wash water	Plating	Yes
E. coli O157:H7	Tomatoes	Wash water	Plating	Yes
Salmonella s.v. Typhimurium	Tomatoes	Wash water	Plating	Yes
Salmonella s.v. Enteritidis	Mangoes	Wash water	Plating	Yes
Salmonella spp.	Parsley	Wash water	Plating and microscopy	Yes
E. coli O157:H7	Spinach	Soil	Plating	Yes (roots only)
E. coli O157:H7	Spinach	Soil (following root injury)	Plating	No
E. coli O157:H7	Spinach	Soil with *Meloidogyne hapla* infection	Plating	No
E. coli O157:H7	Spinach	Leaf co-inoculation with *Pseudomonas syringae* DC3000	Plating	No
E. coli O157:H7	Cress, lettuce, radish, spinach	Seed contamination	Plating	Yes
Salmonella s.v. Typhimurium	Lettuce and radish	Seed contamination	Plating	Yes

Bacteria	Plant	Source of contamination	Method	Internalised bacteria identified
Salmonella s.v. Newport	Romaine lettuce	Soil	Plating	Yes
E. coli	Maize	Water (plants grown hydroponically)	Plating	Yes
E. coli O157:H7	Lettuce	Soil	Plating	Yes
Salmonella s.v. Typhimurium	Lettuce	Soil	Plating	Yes
Salmonella s.v. Typhimurium	Lettuce	Water (plants grown hydroponically)	Plating	Yes
Salmonella s.v. Typhimurium	Mango pulp	Wash Water	Plating	Yes
Salmonella s.v. Enteritidis	Almonds	Water	Swabbing	Yes
E. coli O157:H7	Lettuce	Wash water with vacuum cooling	Plating	Yes
E. coli	Lettuce	Wash water	Plating and microscopy	Yes
E. coli O157:H7	Tall fescue	Manure slurry applied to soil	Plating	Yes
Salmonella spp.	Tall fescue	Manure slurry applied to soil	Plating	No
Salmonella s.v. Montevideo	Tomato	Irrigation water	Plating	No
Salmonella s.v. Montevideo	Tomato	Seed	Plating	No
E. coli O157:H7	Spinach	Leaf infiltration	Plating	Yes
E. coli O157:H7	Lettuce	Soil	Plating	Yes
E. coli O157:H7	Lettuce	Irrigation water	Plating	Yes
E. coli O157:H7	Spinach	Soil	Plating	Yes (rare event)
E. coli O157:H7	Spinach	Soil	Plating	No
E. coli O157:H7	Spinach	Soil	Microscopy	Yes (roots only)
E. coli O157:H7	Spinach	Water (plants grown hydroponically)	Plating	Yes
E. coli O157:H7	Iceberg and romaine lettuce	Soil	Plating	No
E. coli O157:H7	Iceberg,	Leaves	Plating	No

Bacteria	Plant	Source of contamination	Method	Internalised bacteria identified
	romaine, leaf lettuce			
E. coli O157:H7	Iceberg, romaine, leaf lettuce	Soil	Plating	No
Salmonella spp.	Pecans	Water	Plating	Yes
E. coli O157:H7	Lettuce	Wash water	Plating	Yes
E. coli O157:H7	Spinach	Wash water	Plating	Yes
E. coli O157:H7	Spinach	Irrigation water	Plating	No
E. coli O157:H7	Spinach, lettuce, parsley	Compost applied to soil	Plating	No
Salmonella s.v. Newport	Tomato	Irrigation water	Plating	Yes

Methods used to show internalisation of *E. coli* O157:H7 and *Salmonella* spp. within plants are summarised in Table 4. Figure 1 is a schematic representation of a plant structure showing tissues in which internalized bacteria (red ovals) have been detected as reported in the publications listed in Table 2. The methods used for internalisation studies included the following: plating onto appropriate growth media, LM, scanning electron microscopy (SEM), TEM, immunolocalisation, fluorescence in situ hybridisation (FISH) and immunoblot tissue printing (Deering *et al.,* 2011a). In a study by Standing *et al.* (2013) methods selected to confirm internalisation included enumeration of viable bacteria by plating onto selective growth media, PCR confirmation of pathogen identity, LM as well as TEM. In order to verify the absence of the four human pathogens in uninoculated lettuce leaves, the natural viable background microflora was determined and the isolates identified using MALDI-TOF analysis. It is clear from published data that each method used to investigate potential internalisation of microbes has its limitations, however the use of a combination of methods supported the findings in the study by Standing *et al*. (2013). Concentrations ranging from as high as 10^9 CFU/mL of *E. coli* O157:H7 and *S.* Typhimurium (Franz *et al*., 2007) to as low as 10^2 CFU/mL of *E. coli* O157:H7 and *S.* Typhimurium (Jablasone *et al*., 2005) have been used to inoculate lettuce seeds in previous studies. Erickson *et al.* (2010b) reported no internalisation when lettuce leaves were inoculated with 10^6 log CFU/ml (4.4 log CFU per leaf) *E. coli.*

Table 4. Research examining the localisation of *Salmonella* spp. or *E. coli* O157:H7 within the plant organized by technique or method utilised (Deering *et al*., 2011a).

Method/technique	Bacteria detected	Plant species	Location on/ within plant or fruit
Direct tissue printing	*E. coli* O157:H7	Radish	Hypocotyl
Immunoblot tissue printing	*E. coli* O157:H7	Apple	Core
SEM	*E. coli* O157:H7	Radish	Stomata
	E. coli O157:H7	Spinach and lettuce	Vascular tissue, apoplast
	Salmonella s.v. Typhimurium	Lettuce	Sub-stomatal cavity
TEM	*E. coli* O157:H7	Apple	Cortex near cell wall
Confocal microscopy	*E. coli* O157:H7-GFP (plasmid)	Apple	Ventral cavity pericarp, locule pericarp, seed integuments, internal trichomes
	Salmonella s.v. Stanley-GFP (chromosomal)	Alfalfa sprouts	Root, hypocotyl, and cotyledons
	Salmonella s.v. Thompson-GFP (plasmid)	Cilantro	Leaf
	E. coli O157:H7-GFP (plasmid)	Lettuce	Subsurface tissue
	Salmonella s.v. Newport-GFP (plasmid)	*A. thaliana*	Root at lateral root junctions
	E. coli O157:H7-GFP (plasmid)	*A. thaliana*	Root at lateral root junctions
	FluoSpheres	Lettuce	Roots, stems, and leaves Subsurface tissue
	Salmonella s.v. Javiana-GFP, *Salmonella* s.v. Rubislaw-GFP, *Salmonella* s.v. Anatum-GFP (plasmid)	Parsley	Leaf
	E. coli O157:H7-GFP (plasmid)	Lettuce	Internal portions of leaf and stomata
	Salmonella s.v. Enteritidis-GFP	Almonds	Inner portions of hull and shell, kernel
	Salmonella s.v. Typhimurium-GFP (plasmid)	Lettuce	Parenchyma cells of the cortex, sub-stomatal cavity
	E. coli O157:H7-GFP (plasmid)	Spinach	Leaf
	Salmonella s.v. Typhimurium-GFP (plasmid)	Iceberg lettuce,	Leaf mesophyll

Method/technique	Bacteria detected	Plant species	Location on/ within plant or fruit
		arugula, romaine lettuce, tomato, parsley	
Immunolocalization	*E. coli O157:H7*	Radish	Xylem, hypocotyl
	E. coli O157:H7 (EM Level)	Radish	Xylem
	Salmonella s.v. Dublin	Lettuce	Root-shoot transition area
	Salmonella s.v. Typhimurium-GFP (plasmid)	Peanut	Epidermis, cortex, vascular tissue, pith
	E. coli O157:H7-GFP (plasmid)	Mung bean	Epidermis, cortex, vascular tissue, pith
FISH	*Salmonella* s.v. Typhimurium	Barley	Inner cortex of the root central cylinder, root hairs
Light Microscopy	*E. coli* — GUS	Spinach	Roots and hypocotyl
	Bioluminescent *E. coli* and *Salmonella* Montevideo	Mung bean	Apoplastic fluid
	E. coli — GUS	Mung bean	Hypocotyl
	E. coli O157:H7	Apple	Cortex, vascular tissue
	E. coli O157:H7-GFP (Chromosomal)	Spinach	Root hairs
	Salmonella enterica-GFP (plasmid) Bioluminescent *E. coli*	Tomato	Trichomes, leaves, calyxes, fruit

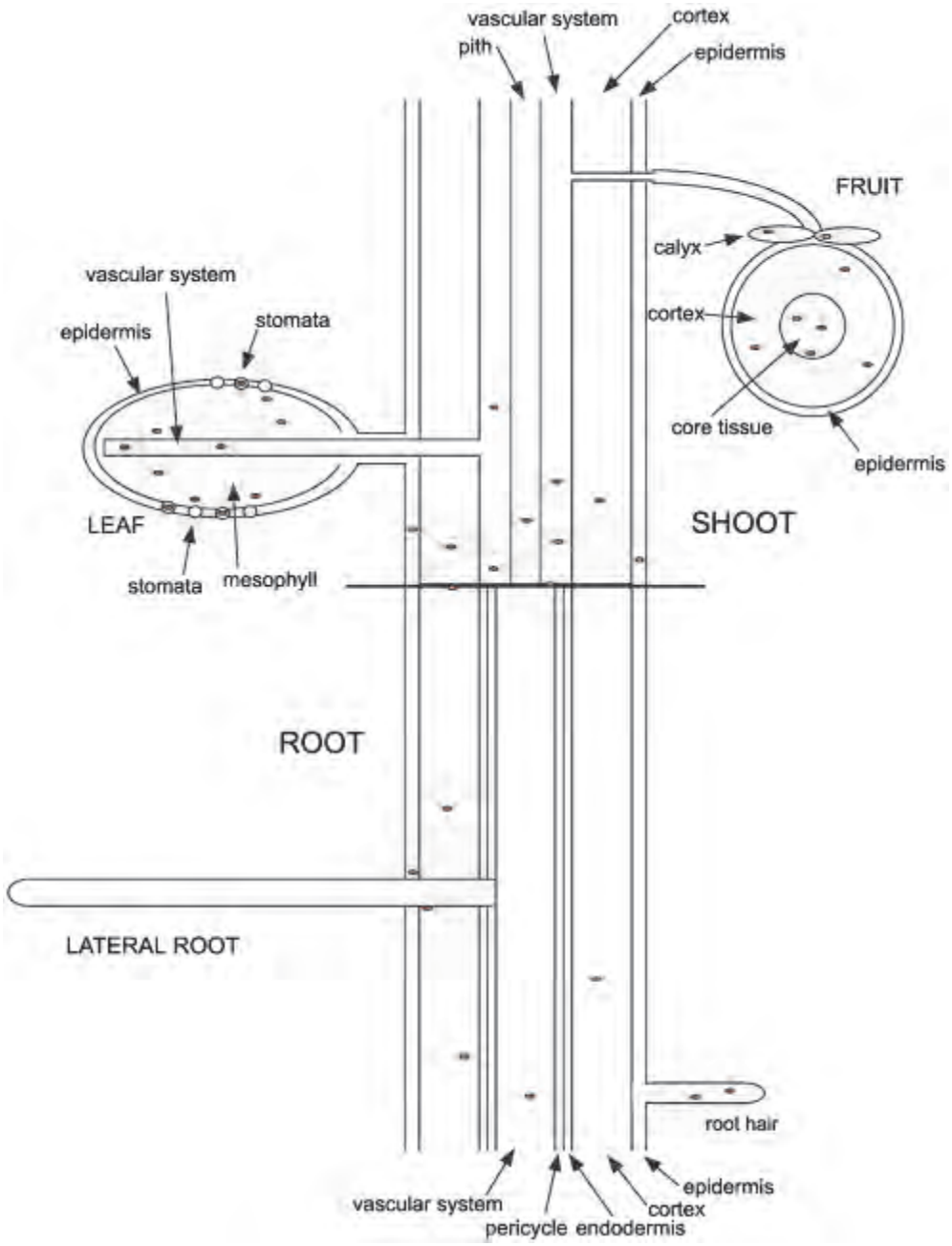

Figure 1 Schematic representation of plant structure showing tissues in which internalised bacteria (ovals) have been detected as reported in the publications listed in Table 2.

Two major routes of human pathogenic bacteria colonisation and internalisation have been reported to date: (1) entrance through natural plant openings such as stomata, lenticels, sites of lateral root emergence and/or wounds caused by biotic or abiotic factors; (2) following irrigation of plants, soaking of seeds or washing of fresh produce during processing after harvest [Fig. 2] (Hirneisen *et al.,* 2012; Deering *et al.,* 2011a). In the case of the latter, bacteria are pulled along with the movement of water as the water enters internal plant tissues. In a study by Standing *et al.* (2013), *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *S.* Typhimurium entered the plants through water uptake, as was evident due to the tracking of crystal violet dye uptake in the roots and stems

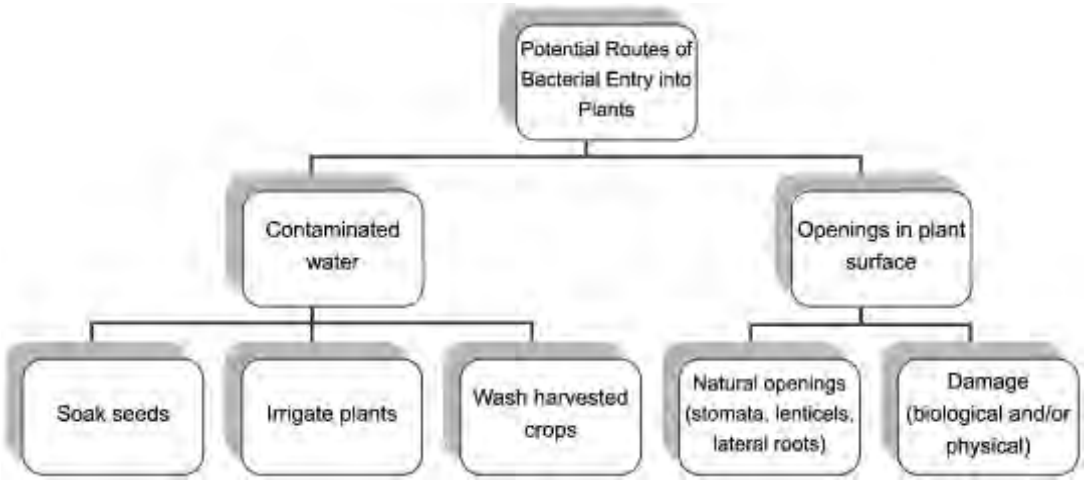

Figure 2 Major potential entry routes for bacteria into plant tissues.

Several mechanisms of human pathogen internalisation in plants have been postulated and have been extensively studied (Kroupitski *et al*., 2009). The first major obstacle for entry of human pathogenic bacteria into plant tissues is the rigid and chemically complex host cell wall (Aparna *et al*., 2009; Deering *et al*., 2011b). The ability of plant pathogens to produce enzymes such as cellulases, xylanases, pectinases and proteases that break down cell walls, has been reported to provide a point of entry for the pathogen (Aparna *et al*., 2009; Deering *et al*., 2011a). *Salmonella* is a member of the Enterobacteriaceae family that includes the soft rot *Erwiniae,* which produce cell wall degrading enzymes. A cellulase able to degrade cellulosic substrates has been isolated from *S.* Typhimurium (Yoo *et al.,* 2004). Additionally, Salmonella isolated from infected Arabidopsis plants were equally virulent for human cells and mice, which indicates that the Salmonella infection mechanisms for plants and animals are similar (Schikora *et al.,* 2011). *Salmonella* spp. induce phagocytosis in humans and once internalised, destroy the cell.
In contrast to the *Salmonella* infection mechanism, human pathogenesis studies showed that *E. coli* O157:H7 did not destroy the host cell by phagocytosis. *E. coli* O157: H7 adhere to the extracellular regions of the host cell, ultimately causing damage to the cell structure through complex processes, inflammation and inability of the cell to absorb nutrients, thereby enabling the pathogen to enter the host cell (Deering *et al*., 2011b).

The recent Listeriosis outbreak due to contaminated cantaloupe consumption in the USA (http://www.foodsafetynews.com/2011/10/cantaloupe-listeria-outbreak-84-sick-15-dead) emphasised the importance of assuring food safety in the supply chain. The seriousness of infections caused by *L. monocytogenes* has resulted in strict legislation regarding its presence on produce. Currently, the United States Food and Drug Administration (FDA) has a zero-tolerance policy for the presence of *L. monocytogenes* in ready-to-eat foods (Gandhi and Chikindas, 2007). Based on this policy, the detection of any *L. monocytogenes* in the food makes the product adulterated. The FDA is currently reviewing a petition made by trade associations to change the zero-tolerance for *L. monocytogenes* in foods that do not support the growth of the organism to a regulatory limit of 100 CFU/g. Concentrating on the number of *L. monocytogenes* present in a particular produce as opposed to just its presence alone may

be more effective in improving food safety and promoting public health (Gandhi and Chikindas, 2007).

A study undertaken by Jablasone *et al.* (2005) on the ability of *L. monocytogenes* to become internalised in lettuce and radish seedlings resulted in *L. monocytogenes* being restricted only to the surface of the plants. The presence of *L. monocytogenes* in certain produce and its absence in others may be explained by less contact between the plant and soil as *L. monocytogenes* is most commonly associated with plant matter and soil (Brackett, 1999). Absence of *L. monocytogenes* in some vegetables can also be explained by an antibacterial effect of some components of vegetables, as has been shown for carrots (Beuchat, 1996; Brackett, 1999). Furthermore, the background flora present on the produce may also have an inhibitory effect on *L. monocytogenes* (Johannessen *et al*., 2002). Flagella may also play a role in the internalisation of human pathogens in plants, as they can trigger Inducible Systematic Resistance (ISR) in plants whereby antimicrobials are released at the invasion site (Pieterse *et al*., 2001). ISR suppresses opportunistic saprophytes from invading the internal tissue of plants without leading to necrosis. Thus, *L. monocytogenes* lacking flagella has been found to be internalised in sprouting mung beans to a greater extent than the flagellated parental strain (Ibrahim *et al*., 2003). Using *L. monocytogenes* expressing green fluorescent protein (GFP), the pathogen was visualised in the intercellular spaces of *A. thaliana* leaves, suggesting internalisation through stomata (Milillo *et al*., 2008). The internalisation of *L. monocytogenes* in the lettuce plants was reported by Standing *et al.* (2013) and more recently by Chitarra *et al*. (2014).

Only a few studies have reported the isolation of *S. aureus* in lettuce, radish and seed sprouts, in which the levels varied between 7.7% and 24% (Beuchat, 1998). In another study carried out by Johannessen *et al*. (2002), staphylococci were isolated from approximately 20% of strawberries and mushrooms sampled, with only four of the isolates being identified as *S. aureus*. Although isolated from a number of vegetables, no outbreaks due to the consumption of vegetables contaminated particularly with *S. aureus* have been reported, as enterotoxigenic *S. aureus* does not compete well against other microorganisms normally present on raw fruit and vegetables (Beuchat, 1998). However, vegetable associated outbreaks due to *Staphylococcus* could occur under conditions that favour its growth and subsequent toxin production (Le Loir *et al*., 2003). There are no reports in the literature of *S. aureus* cellulosic degrading enzyme isolation and characterisation. However, a putative endo-1.4 beta-glucanase has recently been identified for *S. aureus* RF122 (EMBL Bank AJ938182). Interestingly, mutants of *S. aureus* strain with human pathogenesis genes inactivated, were attenuated in their ability to infect *A. thaliana*. These results indicate that as was observed for *Salmonella*, *S. aureus* infection mechanisms for plants and animals might be similar. Although *S. aureus* has not been implicated in foodborne disease outbreaks, comparison to infection mechanisms in humans could facilitate the elucidation of internalisation mechanisms of Gram-positive bacteria in plants.

2.6 The incidence and control of foodborne pathogens on postharvest fruits and vegetables

The high demand for healthier and more natural foods, minimal processing of fruits and vegetables, sources of produce, emerging pathogens and adaptation of foodborne pathogens to post-harvest processing have contributed to an increase in the incidence of human infections associated with fruits and vegetables (Beuchat, 2002). Produce, including raw and minimally processed fruits and vegetables is no longer considered low risk. In fact, it is ranked among the top five high-risk foods together with seafood, poultry, eggs and beef (DeWaal, 2003). In the United States alone, data from 1990 to 2003 indicated 82 outbreaks of foodborne infections that were linked to the consumption of produce (DeWaal, 2003).

According to DeWaal (2003), consumers feel ill-informed about new food technology methodologies and are concerned about the presence of harmful bacteria in their food. Apart from consumer concerns, the lack of adequate data on the prevalence and the effect of processing on foodborne pathogens associated with such foods, present a crucial public health risk. In South Africa, data on foodborne outbreaks is particularly scant. Due to the ever increasing population of immuno-compromised groups (e.g. HIV patients), who among other things are encouraged to consume fruits and vegetables, the implications of the occurrence of pathogens on postharvest produce could be grossly underestimated.

Statistics of foodborne outbreaks and illnesses from produce

There has been in increase in foodborne outbreaks linked to the consumption of fresh produce largely due to improvements in the surveillance and reporting systems (Fig. 3). Changes in production, processing, packaging, and marketing technologies globally have enabled supply of good quality fruits and vegetables year round but also enabled dissemination of foodborne pathogens across broad geographical borders (Beuchat, 2002). Of the five high risk foods, produce caused the highest number of illnesses (22%) from foodborne outbreaks in 1990 to 2005 (Centre for Science in the Public Interest (CSPI), 2007). In England and Whales, outbreaks linked to produce were 6.8% of the total outbreaks recorded in 1992 to 1999 (O'Brien *et al*., 2002).

It has been reported that outbreaks linked to produce are mainly caused by bacteria and viruses (CSPI, 2007). Data from the FDA indicates that 50% of produce outbreak in the United States (1998-2005) were attributed to foods from restaurants and other food establishments, while private homes accounted for 13% of the outbreaks (CSPI, 2007). Of the reported outbreaks, Norovirus accounted for 40%, *Salmonella* accounted for 18% and *E. coli* accounted for 8% of the produce outbreaks (CSPI, 2007). The frequency and size of produce outbreaks in the United States between 1990 and 2006 are shown in table 5.

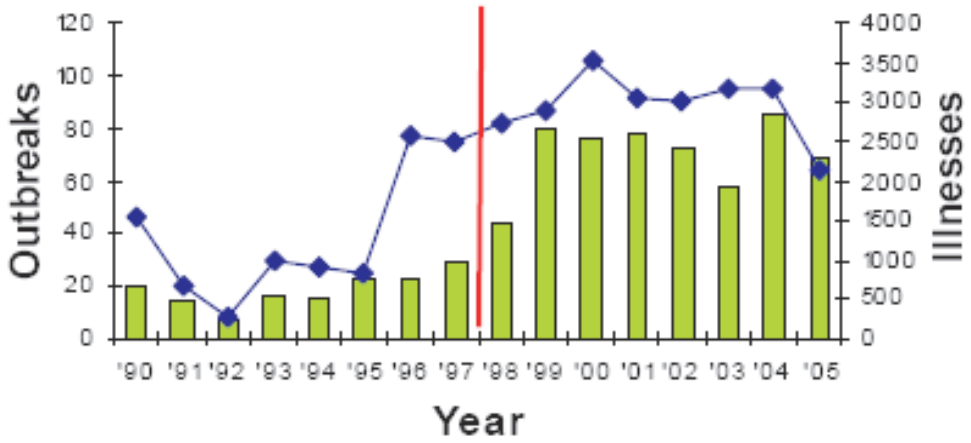

Figure 3: Foodborne outbreaks caused by produce consumption and illness linked to produce outbreaks in the United States from 1990 to 2005. Increase in outbreaks after 1997 is largely due to improved surveillance and increased reporting of disease (CSPI, 2007)

Table 5: Produce-pathogen combinations in outbreaks caused by produce in 1990-2005 in the United States

Rank	Food	Pathogen	Outbreaks	Associated Illnesses	Illnesses per outbreak
1	Greens-Based Salad	Norovirus	144	5,353	37.2
2	Lettuce	Norovirus	30	1,025	34.2
3	Sprouts	*Salmonella*	24	1,875	78.1
4	Fruit	Norovirus	22	1,636	74.4
5	Greens-Based Salad	*Salmonella*	20	1,033	51.7
6	Melon	*Salmonella*	16	1,137	71.1
7	Mushrooms	Chemicals/Toxins	16	82	5.1
8	Greens-Based Salad	*E. coli*	15	791	52.7
9	Lettuce	*E. coli*	14	382	27.3
10	Potato	*Salmonella*	14	206	14.7

Adapted from CSPI (2007)

Twenty-seven produce related disease outbreaks were reported in Canada from 2001 to 2009 (Fig. 4). Approximately 1549 cases of illness were reported of which 66% were due to bacterial infections. *Salmonella* accounted for 50% of the outbreaks, *E. coli* for 33% including two deaths due to *E. coli* O157:H7 and Shigella for 17%. Produce linked to foodborne disease outbreaks included spinach, lettuce, mung bean sprouts, Roma tomatoes, carrots, cantaloupe, Spanish onions, onion sprouts and fruit salad.

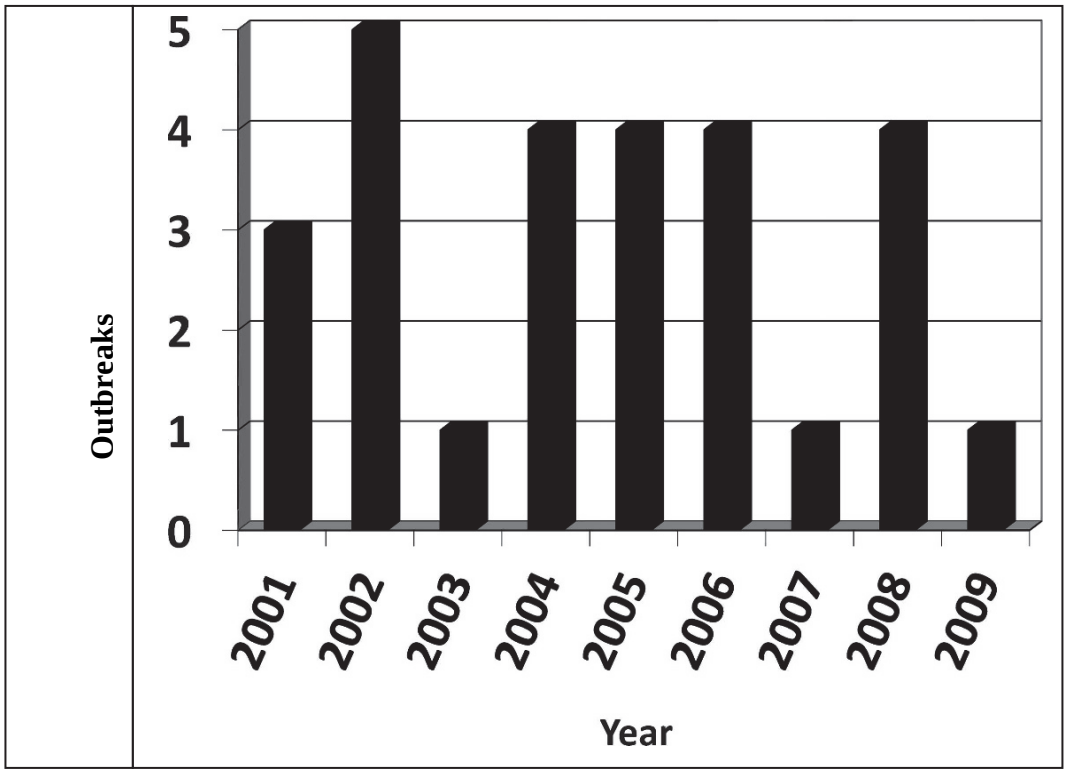

Figure 4: Canadian produce outbreaks, 2001 through 2009 (Kozak *et al*., 2012).

Incidence of foodborne infections associated with produce

Advances in epidemiologic and surveillance programs, particularly the use of molecular methods for detection of pathogens, have enabled the association of foodborne pathogens with raw and processed fruits and vegetables (Beuchat, 2002). Several factors affect the occurrence and behaviour of pathogens on fruits and vegetables. These include the surface morphology of produce providing attachment opportunities to pathogens, internal tissue composition, internalisation of pathogens into fruits and vegetables and metabolism of vegetable leaves, florets, stems, tubers and fruits that may enhance or retard survival and growth of food pathogens (Beuchat, 2002).

Spoilage bacteria as well as yeasts and moulds dominate the microflora of fresh produce but pathogens including bacteria, viruses and parasites have been isolated from a variety of produce. Among these, *Salmonella* species, enterotoxigenic and enterohaemorrhagic *Escherichia coli, Shigella, Yersinia, Listeria monocytogenes, Campylobacter* spp., *Bacillus cereus*, *Clostridium* and *Aeromonas hydrophilia* appear to be common inhabitants of fresh produce (Burnett and Beuchat, 2001). Norwalk virus and parasites such as *Giardia lamblia* and *Cryptosporidium* have also been isolated. In recent years, *Salmonella* and *E. coli* have been the most common bacterial pathogens linked to produce related foodborne outbreaks (Olsen *et al.*, 2000). It should however be noted that pathogenic microorganisms occur infrequently and generally in low numbers on fruits and vegetables. Notwithstanding, pathogens with a low infectious dose are expected to be absent, since fruits and vegetables are typically consumed raw or minimally processed.

Several associations of pathogens with fruit and vegetable types have been made (Doyle and Erickson, 2008) but such associations vary widely depending on factors such as location, sanitation methods, processing, handling, storage and distribution. For example, *Salmonella* has been associated with several vegetables including mixed salads and sprouts and Norovirus with greens based salads (Doyle and Erickson, 2008). Assessment of the global food outbreak data indicated high prevalence (40%) of Hepatitis A virus associated with produce related outbreaks (Greig and Ravel, 2009).

E. coli O157:H7 was implicated in an outbreak associated with the consumption of unpasteurized apple juice in USA in 1996 (Beuchat, 2002). In October 2006, *E. coli* O157:H7 contamination of pre-packaged spinach caused a multistate outbreak in the USA (CDC, 2006). Out of the 205 persons infected, 102 were hospitalized, 31 developed haemolytic uremic syndrome (HUS) and five died (CSPI, 2007). Another *E. coli* O157:H7 outbreak from contaminated iceberg lettuce was reported in the Midwest and Northeast states of the US in November and December 2006 (CSPI, 2007). The outbreak caused a total of 150 illnesses. *Shigella* in baby carrots caused an outbreak in the US in 2007 (CSPI, 2007). Immediately after the multi-state spinach related *E. coli* outbreak in the United States, two separate outbreaks caused by *Salmonella* contaminated tomatoes were reported. These tomatoes were served in restaurants (CSPI, 2007).

The occurrence of pathogens on fruits and vegetables vary widely. For example, Abadias *et al.* (2008) reported the absence of *E. coli, Salmonella* and *L. monocytogenes* on fresh cut ready-to-eat fruits and vegetables. However, in the same study, soybean and alfalfa sprouts had high incidence of *E. coli* (40%) and low incidence of *Salmonella* (1.3%) and *L. monocytogenes* (0.7%).

In a study conducted in UK, relatively high levels of *E. coli* and *Staphylococcus aureus* were isolated from salad vegetables collected from take-away kebab restaurants (Meldrum *et al.*, 2009). A similar study conducted in India indicated the presence of *E. coli*, *S. aureus*, *Salmonella*, and *Enterobacter* from a variety of fruits, vegetables and sprouts purchased from street vendors (Viswanathan and Kaur, 2001). Lin *et al.*, (1996) also reported low incidence of *E. coli* and *L. monocytogenes* on vegetable salads from a local supermarket and two fast food restaurants. Christison *et al.* (2008) conducted a microbiological survey of ready-to eat foods at four delicatessens and the associated production surfaces in Johannesburg, South Africa. In their study, *L. monocytogenes* and *Salmonella* were detected in salads samples. *E. coli* and *coliforms* were also isolated from food preparation surfaces (Christison *et al*., 2008).

McMahon and Wilson (2001) studied the occurrence of *Aeromonas* spp. on organic vegetables purchased from retail supermarkets in Ireland. These commercially packaged vegetables included carrots (whole and cut types), mushrooms, tomatoes, cherry tomatoes, pepper, courgette, asparagus, onions, potatoes, alfalfa sprouts and watercress, whole lettuce, broccoli, cabbage, celery, cauliflower and turnip. Of all these vegetable samples, 645 of which were ready-to-eat, *Salmonella* and *E. coli* O157:H7, *Campylobacter* and *Listeria* spp. were not detected. Several strains of *Aeromonas* including *A. hydrophilia*, *A. schubertii*, *A. trota*, *A. caviae*, and *A. veronii* biovar *veronii* were detected on 34% of the vegetable samples

tested. Xanthopoulos *et al*. (2009) isolated *Aeromonas* spp. from 61% of minimally processed salad types composed of spinach, sprouts, cabbage, mushrooms, rocket, valerian and multi-salad packs in Greece. Of the *Aeromonas* spp. isolated, 80% were identified as *A. hydrophilia*. In the same study, *Yersinia enterolitica* were also isolated from minimally processed vegetables, though at a low incidence (7.7%). The presence of *Aeromonas* spp. and *Yersinia* spp. on vegetables presents a consumer risk due to their possible pathogenicity and the ability to survive and grow at chilling temperatures to cause salad spoilage (Xanthopoulos *et al*., 2009).

Frozen raspberries have also been implicated in foodborne outbreaks involving Calicivirus and Hepatitis A. Norovirus has been shown to be prevalent on greens-based salad (Doyle and Erickson, 2008). *Cryptosporidium parvum* was implicated in an outbreak in USA associated with the consumption of unpasteurized apple cider (Burnett and Beuchat, 2001).

Sources of postharvest contamination and factors affecting survival of pathogens

Postharvest handling of produce offers various opportunities for contamination. Potential sources of postharvest contamination due to direct contact with pathogens include human handling, transport containers and vehicles, storage conditions, insects, dust, rinse water, ice and processing equipment (Brackett, 1999; Burnett and Beuchat, 2001, Warriner *et al*., 2009). Indirect factors causing contamination are due to adaptation to stressful environments during processing, storage or distribution that affect survival, growth or virulence of contaminating pathogens (Beuchat, 2002). *E. coli* O157:H7 and *Salmonella* are typical examples of enteropathogens that adapt to low pH to enhance broad spectrum tolerance to other unrelated ecological stresses (Parry-Hanson *et al.,* 2009).

Processing of fresh produce involving a chain of physical operations introduce several opportunities for produce contamination. Operations such as cutting of lettuce, apples and pears, shredding of carrots and cabbage, dicing of tomatoes or pealing of carrots and oranges disrupt the structural integrity of produce, removes the physiological barrier that prevents bacterial internalization, creates a large surface area for attachment and cut surfaces also exude nutrients needed for bacterial proliferation (Doyle and Erickson, 2008). Studies have indicated that while *E. coli* O157:H7 and *L. monocytogenes* preferentially attaches to cut or injured surfaces of produce, *Salmonella* attaches to either cut or intact surfaces (Takeuchi *et al*., 2000). Other reports indicate that *E. coli* O157:H7 attachment on lettuce is merely through entrapment. Regardless of the process, bacteria that become internalized through attachment to injured or cut surfaces are resistant to sanitizing solutions (Takeuchi and Frank, 2000).

Globalization of agriculture and food supply, which allows centralization of food production and processing in different parts of the world, enables dissemination of pathogens across geographic borders. Large scale production and processing of fruits and vegetables can also easily spread pathogens along the processing chain into large volumes of processed food (CSPI, 2007). Cross contamination could occur via direct contact of contaminated with uncontaminated produce or through contaminated food contact surfaces, wash water and human handling. Martínez-Gonzales *et al.* (2003) reported contamination of wet or dry lettuce

with high numbers of *C. jejuni* and *Salmonella* after contact with contaminated steel surfaces 1 to 2 hours after contamination of the contact surface.

Processing methodologies and their effect in reducing bacterial numbers

Several new processing methodologies are being investigated to extend shelf life and improve the microbiological safety of fresh produce. Bacterial, viral and parasitic contamination of produce is predominantly on the surface, though internalization is also likely. A simple rinsing procedure to remove dirt and bacterial contamination is insufficient to reduce high bacterial numbers on the surface of produce (Basset and McClure, 2008).

The vacuum, steam vacuum (VSV) surface intervention is a new technology used to reduce bacterial numbers on food surfaces. Kozempel *et al.* (2002) proved that VSV technology can be used to destroy bacteria on the surface of produce without causing thermal defects on produce surface. This technology makes use of steam to destroy bacteria on the surface of food. However, the presence of air and water on the surface of produce insulate bacteria to such an extent, that the amount of time required to kill the bacteria on the surface will thermally damage the surface of produce (Kozempel *et al*., 2002). The VSV surface technology removes the insulation with vacuum, followed by a quick burst of condensing steam which is quickly removed once again by vacuum to cool down the product surface. This technology caused a reduction of *L. innocua* on grapefruits of up to 4 log CFU/ml (Kozempel *et al.*, 2002). Though this process is not suitable for all produce types, it has been successfully applied to cucumber, carrot, avocado, kiwi, mango and pawpaw (Kozempel *et al*., 2002).

Postharvest washing of fruits and vegetables is a traditional intervention that has long been used to reduce the bacterial load on produce surface. The efficacy of washing depends on the produce type, bacterial types and populations, as well as the washing method (Bassett and McClure, 2008). For example, rinsing of apples has been shown to reduce *Salmonella* and *E. coli* by 0.5 log CFU/g but had no effect on *L. monocytogenes* population. Soaking with spray water for 1 to 10 min prior to rubbing and rinsing of apples caused a 3 log CFU/g reduction of *Salmonella* but did not have any additional effect on *E. coli* (Beuchat *et al*., 1998). Depending on the surface structure, washing may not be as effective in reducing protozoan populations compared to bacteria on fruits and vegetables. A study on the effect of washing on *Toxoplasma gondii* demonstrated that raspberries retained more oocysts than blueberries due to the hair-like projections on the surface of raspberries (Kneil *et al.*, 2002). Although wash water reduces microbial counts on produce surface, it has also been reported to cause cross-contamination of produce. For example, Ilic *et al*. (2008) reported a 26% increase in spinach samples that tested positive for coliforms after washing. Contamination could be due to low temperature infiltration of contaminated wash water into the vascular system of spinach (Warriner *et al.*, 2009).

Commonly used disinfectants such as liquid chlorine and hypochlorite have been shown to effect up to 2 log reduction of microbial numbers on produce at concentrations ranging 50 to 200 ppm (Basset and McClure, 2008). Chlorine dioxide (Wu and Kim, 2007), acidified

sodium chlorite (Allende *et al.*, 2009), slightly acidic electrolyzed water (Koide *et al*., 2009) and ozone (Selma *et al.*, 2008) have all been shown to have variable efficacies in reducing artificially inoculated pathogens on produce surface. However, actual reduction levels on naturally contaminated produce surfaces typically applied in industry is *ca* 2 log CFU/g irrespective of sanitizer application (Doyle and Erickson, 2008).

Modified atmosphere packaging (MAP) has long been used to improve the keeping quality of food. Zagory (1999) discussed the effect of MAP on microbial quality and shelf life of fresh produce. His paper suggested that although MAP may retard growth of spoilage bacteria, it does not have any inhibitory effect on pathogens such as facultative anaerobe *E. coli* O157:H7 (Abdul-Raouf *et al*., 1993) and microaerophilic *L. monocytogenes*. This is supported by Bennik *et al*. (1996) who demonstrated that modified atmospheres of 0, 1.5 and 21% O_2, in combination with 0 or 20% CO_2 and made up to 100% with N_2, delayed growth of spoilage bacteria but failed to inhibit *L. monocytogenes* on chicory endive. It is therefore imperative to avoid contamination of produce before or during produce processing (Lynch *et al.*, 2009).

Alternative processing methodologies including surface pasteurization, ionizing irradiation and ultraviolet rays have been suggested. Surface pasteurization of fruits and vegetables with steam, hot water or chlorine dioxide gas on hard surface produce can reduce the microbial loads on the surface (Warriner *et al*., 2009). However, post pasteurization contamination with pathogens will enhance survival and growth due to loss of competitive natural flora (Stringer *et al*., 2007). Several investigations into the use of gamma irradiation to decontaminate fruits and vegetables have indicated that although gamma irradiation can be applied in reducing microbial counts on fresh and processed fruits and vegetables, the maximum dosage allowed on produce (1 kGy) is insufficient to cause significant reduction of microbial numbers (Zagory, 1999). For that reason, it is suggested that ionizing irradiation be used in conjunction with other preservation treatments such as chemical sanitizers and chilled storage (Kamat *et al*., 2003). UV treatment has been used as an alternative to chemical disinfection of fruits and vegetables. UV-C light at a wavelength of 253.7 nm has been shown effective in reducing counts of *Salmonella* and *E. coli* O157:H7 on apples, tomatoes and lettuce). Nonetheless, stationary phase bacterial cells and spores are resistant to UV light. The application of hydrogen peroxide in combination with UV energy has been used to decontaminate surface and internalized pathogens (Hadjok *et al*., 2008).

The concept of biological control of pathogens on fruits and vegetables is gaining popularity in the produce industry (Sharma *et al*., 2009). Microbial antagonists typically suppress the activities of spoilage and pathogenic bacteria by competitive exclusion. It has been used to delay fungal deterioration of many fruits but few studies have applied bio-protective bacteria to prevent or inhibit pathogens in produce. Janisiewicz *et al*. (1999) demonstrated that *Pseudomonas syringae* can inhibit growth of *E. coli* O157:H7 in apple wounds. In another study, Leverentz *et al*. (2006) showed that strains of *Candida*, *Gluconobacter*, *Discophaerina* and *Metschnikowia* can inhibit *Salmonella* and *L. monocytogenes* in fresh-cut apples. Recently, a study by Trias *et al*. (2008) showed that *Leuconostoc mesenteriodes* and *L. citreum* strains isolated from fresh-cut vegetables had bactericidal activity on *L.*

monocytogenes in apple wounds and on fresh-cut lettuce due to the production of organic acids, hydrogen peroxide and bacteriocins.

Low temperature is the most important factor in maintaining the microbial quality of fresh cut products (Gonzalez-Aguilar *et al.,* 2004). A cold chain should be maintained throughout the unit operations involved in preparation right up to consumption. Ideally temperatures should not exceed 5°C ^{0}C, although preferably they should be closer to 1°C (Scifo *et al*., 2009). In a study carried out by Gonzalez-Aguilar, 2004, when temperature was increased from 5^{0}C to 10°C, the number of microorganisms increased by more than 3 log CFU/g and therefore lowering temperature is a more critical factor than MAP in reducing microbial counts. Low temperatures and MAP are however weak hurdles against psychrotrophic pathogenic microorganisms like *L. monocytogenes* (Scifo *et al*., 2009).

2.7 *LISTERIA MONOCYTOGENES* AND VEGETABLES AND EFFECT OF MINIMAL PROCESSING ON SURVIVAL OF THE PATHOGEN

During 2007, a study was initiated by the Water Research Council (WRC) of South Africa to determine whether irrigation water, with high levels of bacterial contamination, contributes to the bacterial contamination on vegetable produce in the field. Several rivers in the Western Cape, Gauteng, Mpumalanga and North West provinces were identified as sources of irrigation water for food crops. The water from rivers such as the Berg River, Bree River, Olifants River and Wilge River, along with the produce from the fields being irrigated with water from these sources, was analysed for microbial contamination. The results obtained by Ijabadeniyi *et al*. (2008) after investigation of the Gauteng, Mpumalanga and North-West provinces, confirmed the presence of pathogens, including *E.coli, S. aureus, L. monocytogenes* and *Salmonella* in the water and also on the irrigated produce. *L. monocytogenes* specifically, was detected in 37.5%, 37.5% and 62.5% of water samples from the Loskop canal, Olifants and Wilge River respectively and on 25% and 75% of cauliflower and broccoli samples respectively, on one of the farms. These figures were similar for produce from all of the investigated sites. Lettuce and spinach from other sources were also analysed in the study.

Broccoli harvested from the field was found to contain pathogens, of which *L. monocytogenes* was one (Ijabadeniyi *et al*., 2008). *L. monocytogenes* is a Gram-positive, asporogenous organism which is widely distributed throughout the environment and has been isolated from various plant and animal products associated with foodborne illness outbreaks. Ingestion of the organism can cause the disease listeriosis (Peiris, 2005). Listeriae are not fastidious. They can survive in faeces, soil, milk, water, silage and on plants. *L. monocytogenes* is known to survive under certain extreme conditions, having the ability to multiply in high salt (up to 10% sodium chloride) and at temperatures as low as 0.5°C (Peiris, 2005). The pathogen grows best at neutral to slightly alkaline pH and has an optimum growth temperature of between 30 and 37°C. The growth of *Listeriae* is enhanced in an atmosphere with reduced oxygen and 5 to 10% carbon dioxide (Pearson and Marth, 1990) and they are able to grow for extended periods under adverse conditions. (Peiris, 2005). The versatility, adaptability and

resistance of the pathogen are important factors in minimally processed vegetables as its phsychrotrophic properties render refrigeration temperatures insufficient to ensure the safety of stored food against *L. monocytogenes* (Lee *et al*., 2007). Furthermore, broccoli is a winter crop and therefore grows in environmental conditions of relatively low temperature (ideally between 18 and 23°C) and humidity depending on the region of produce, further explaining why *L. monocytogenes* is a factor to consider in the cultivation of this vegetable (Pearson and Marth, 1990).

As the incidence of pathogens such as *L. monocytogenes* on broccoli has been confirmed by the aforementioned study (Ijabadeniyi *et al*., 2008), and by other reports (Beuchat, 1996), the question now remains to what extent the pathogen present in the irrigation water attaches to broccoli and whether it survives and proliferates on broccoli under environmental conditions in the field. *L. monocytogenes* has been found to rapidly attach to and multiply on plant surfaces and colonize intercellular spaces where it may be protected from sanitation treatments, which poses a problem during processing treatments (Milillo, 2008). *L. monocytogenes* may attach to plant surfaces at the stomata, broken trichomes or cracks in the cuticle (Frank, 2001).

After harvesting, broccoli undergoes minimal processing procedures including washing and packaging under modified atmosphere. Modified atmosphere packaging, in combination with refrigeration, is increasingly being employed as a mild preservation technique to ensure quality and shelf life, as the demand for fresh, convenient, minimally processed vegetables increases. The fresh nature of these products, together with the mild processing techniques and subsequent storage conditions have presented organisms such as *L. monocytogenes* with new potential infection opportunities and vehicles (Francis *et al*., 1999). The investigation of the effect of these processing procedures on the growth of *L. monocytogenes* on broccoli is therefore an area of further interest.

Listeria monocytogenes and vegetables

L. monocytogenes causes human listeriosis and, according to the United States Centre for Disease Control, as on the 25th April, 140 cases of listeriosis were reported in the year 2009, with an average of 11 cases being reported per week over the 5 preceding years (CDC, 2009). The consumption of contaminated food is considered as the primary source of infection in epidemics. The infective dose varies depending on the immune status of the host, virulence of the organism, the type and amount of food consumed as well as the concentration of the pathogen in the food. Consumption of food contaminated with levels as low as 10^2 to 10^4 cells per gram of food has been reported to cause disease (Peiris, 2005). The incubation period for illness varies from days to weeks and in listeriosis outbreaks, infection has been reported to develop 24 hours after ingestion of the contaminated food (Peiris, 2005). Although rare when compared to other food-borne diseases, listeriosis often leads to severe consequences (Buchrieser, 2006).

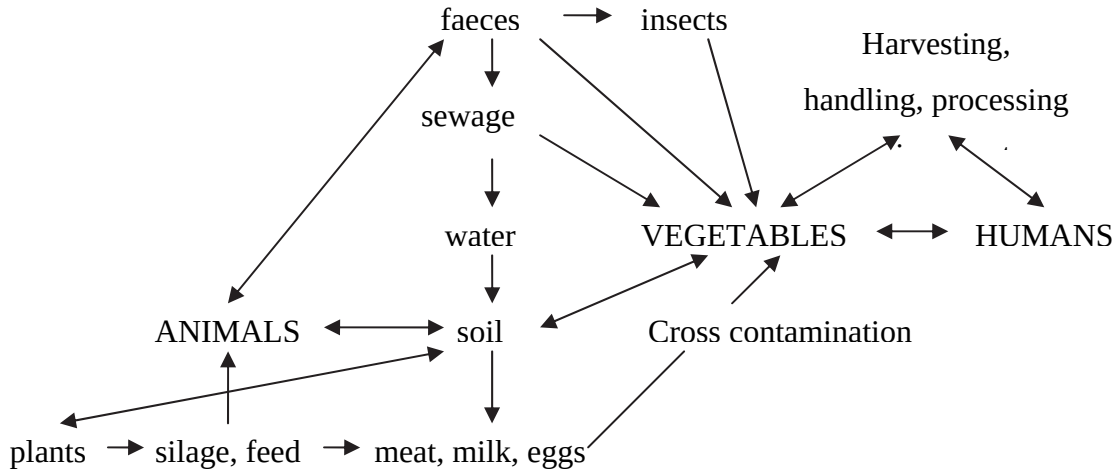

Figure 5: Potential pathways for transmission of *L. monocytogenes* to humans via vegetables (figure adapted from Beuchat, 1996)

Various outbreaks of listeriosis, including an outbreak of *L. monocytogenes* in 1979, involving 23 patients from eight hospitals have been reported (Beuchat,1996). After investigation, the only common foods found to have been served amongst the patients were certain raw vegetables and it was concluded that the consumption of these raw vegetables could have caused the listeriosis outbreak. Another case of listeriosis reported that *L. monocytogenes* was isolated from commercially prepared, unopened coleslaw after prolonged refrigeration and also found on various other forms of fresh produce, including celery, carrots and cucumber (Beuchat, 1996). Potential pathways for transmission of *L. monocytogenes* to humans via vegetables (figure adapted from Beuchat, 1996) are depicted in Figure 5.

A survey of 1000 samples of 10 types of fresh produce in the USA has revealed the presence of *L. monocytogenes* on cabbage, cucumbers, potatoes and radishes (Heisick *et al.,* 1989b). In another survey, prepared mixed salads and two individual salad ingredients were found to contain *L. monocytogenes*. The higher rate of contamination of prepared salads was attributed to cross contamination of the pathogen during chopping, mixing and packaging. *L. monocytogenes* was detected in 11 of 25 samples of fresh cut vegetables in The Netherlands and it was reported that 7 of 66 samples of salad vegetables and prepared salads produced in Northern Ireland contained the organism (Beuchat, 1996). Products such as vegetables are traditionally considered low risk, but have recently been linked to listeriosis transmission. Despite the fact that on several occasions vegetable consumption have been linked to listeriosis outbreaks, incidence rates reported in these studies have been, in general, below 10%, despite the ubiquity of this microorganism, and the fact that vegetables seem to be a good substrate for growth of *L. monocytogenes* (Aguado *et al*., 2004). One such vegetable substrate is broccoli. Broccoli (*Brassica oleracea* var *botrytis)* is a member of the cabbage family, grown in winter with a water requirement of between 30 and 38 mm of water per week. Most broccoli harvested in the United States is sold as fresh produce. The surface of a broccoli floret is rough and the crevices in the broccoli structure (Frank, 2001) retain water and aid in attachment of organisms to the crop, therefore making this vegetable a possible substrate for organisms such as *L. monocytogenes* (Stine *et al*., 2005).

Attachment, growth and survival

Microorganisms attach to surfaces by means of fimbriae (pili), fibrils and flagella interacting with surfaces by means of electrostatic, hydrogen bonding and hydrophobic forces, followed by production of exocellular binding polymers (Frank, 2001). Attachment to broccoli is possible due to the large contact surface of the vegetable and the crevices in the broccoli, which retain water, providing more contact time and so aiding attachment to the rough surface of the broccoli (Stine, *et al*., 2005). Attachment takes place at the stomata, broken trichomes or cracks in the cuticle. The surface layers of a plant are illustrated in Fig. 6 (Frank, 2001).

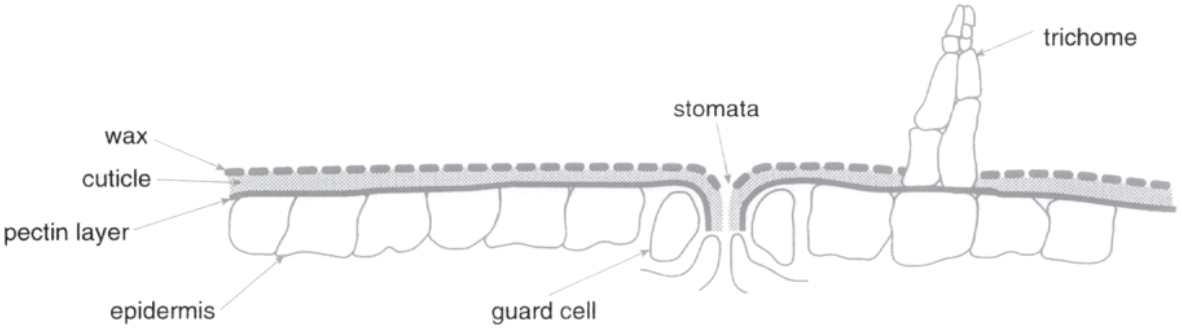

Figure 6: Surface layers of a plant (Frank, 2001).

Milillo *et al.* (2008) assessed the ability of *L. monocytogenes* to attach to and grow on *Arabidopsis thaliana*, a well characterized plant model. This study was performed in response to various lines of evidence indicating that *L. monocytogenes* contamination of fruits and vegetables may contribute to the burden of human listeriosis infections. The ability of *L. monocytogenes* to survive and multiply under various stress conditions, including those likely encountered on plant surfaces contributes to the risk of fresh produce contamination. The *Arabidopsis thaliana* plant is a small flowering plant popularly used as a model organism in plant biology and genetics, as its small genome sequence has been completed and can be used to study and represent plant interactions with microorganisms. Using this plant model system to gain a better understanding of pre-harvest interactions between *L. monocytogenes* and plants, their data indicated that *L. monocytogenes* is able to rapidly attach to and multiply on *A. Thaliana* after inoculation of leaves (Milillo *et al*., 2008).

Food that is held in prolonged cold storage before distribution may allow proliferation of the psychrotrophic *L. monocytogenes* while the number of competing microorganisms decreases (Pearson and Marth, 1990). During a study of the behaviour of *L. innocua* during production of parsley, the organism was found to survive better in winter, indicating an important influence of temperature (Girardin *et al*., 2005).

Confocal laser scanning microscopy (CLSM) has been widely used in microstructural studies of food products as CLSM can provide more information about microbial viability and identity, and produce 3-D images of samples. Also, samples are easily prepared for CLSM and the morphology of microbes and surfaces is fully maintained. (Han *et al*., 2000).

Surrogate organism: *Listeria innocua*

Listeria innocua is an organism found widely and naturally in the environment, including soil. It is closely related to the food-borne pathogen *Listeria monocytogenes* (Buchrieser, 2006), but is non-pathogenic in character (Girardin *et al*., 2005). It is thus often used as surrogate organism to study *L. monocytogenes* (O'Brien *et al.*, 2006) without the safety risk. *L. innocua* lacks the 10-kb virulence locus that engenders pathogenicity to *L. monocytogenes* and this explains why *L. innocua* does not infect humans or animals and is regarded as non-pathogenic (Hof and Hefner, 2005).

Effect of Minimal processing

Consumers increasingly demand foods which retain their natural flavour, colour and texture and contain fewer additives such as preservatives. These minimal processing technologies are designed to limit the impact of processing on nutritional and sensory quality and to preserve food without the use of synthetic additives. (Ohlsson and Bengtsson, 2002). The disadvantage, however, is the increased risk of the survival of certain harmful organisms, in particular *L. monocytogenes*, as this organism can survive in extreme environments, including at refrigeration temperatures (Peiris, 2005) and under the oxygen concentrations within modified atmosphere packaging (MAP) (Francis *et al*., 1999).

Fresh produce is packaged under conditions of modified atmosphere to delay ripening and reduce respiration and ethylene production in order to increase shelf life and quality. It has been found that optimal MAP conditions for broccoli are a concentration of 5% CO_2 and below 2% O_2 (Zagory and Kadel, 1988; Ishikawa *et al.*, 1998). According to Ishikawa *et al.* (1998), these MAP conditions can be obtained by packaging in an LDPE (Low Density Polyethylene) film with properties as indicated in Table 6, or by gas flushing, with optimal gas concentrations as indicated in Table 7. The behaviour of *L. monocytogenes* on raw broccoli under modified atmosphere gas packaging was investigated by Beuchat (1996) and MAP appeared to have little or no effect on the rate of growth of the organism on the vegetable.

Table 6: Film properties for optimal package conditions of broccoli

Film	**Thickness (µm)**	**Gas transmission rate (cc/day/atm)**			**Surface area (m^2)**
		N_2	**O_2**	**CO_2**	
LDPE	29 µm	1100	5700	27 500	0.1998

Table 7: Oxygen, carbon dioxide and nitrogen concentrations for controlled atmosphere storage

Gas concentration (%)		
O_2	**CO_2**	**N_2**
2	5	93

L. monocytogenes remained viable on Brussels sprouts dipped for 10s in water containing 200 µg/ml of chlorine and it was concluded that hypochlorite was ineffective in removing *L. monocytogenes* from contaminated vegetables (Beuchat, 1996). Furthermore, as *L.*

monocytogenes attaches to the broccoli, also in crevices of the structure (Frank, 2001), it resists from being washed off by water and is so also protected from the effect of chlorination (Francis *et al*., 1999).

2.8 MICROBIAL SOURCE TRACKING USING FAECAL INDICATOR BACTERIA

Determining the correct source of faecal contamination is important in order to maintain quality of surface water bodies. Faecal indictor bacteria such as faecal coliforms, *E. coli* and faecal streptococci have been used to indicate faecal contamination in food and the environment. What is important for public health is distinguishing faecal bacteria from human and animal sources (Vantarakis *et al*., 2006).

Using different methods for identifying sources of faecal indicator bacteria is termed as MST (Whitlock *et al*., 2002). However the complexity of environmental conditions has hindered the application of a single method to all settings (Jan and Sadowsky, 2007). MST is a wide field and keeps changing continuously as newer methods are found to improve on reliability of results (United States Environmental Protection Agency (EPA), 2005). Microbial source tracking methods that have been applied to water sheds include phenotypic and genotypic methods (Da-Silva *et al*., 2011; Vantakaris *et al*., 2006; Burnes, 2003; Mohapatra and Mazumder, 2007).

Among the phenotypic methods used for microbial source tracking, antibiotic resistance patterns of faecal coliforms have been commonly used in water sheds (Burnes, 2003). The use of antibiotics is either based on Multiple Antibiotic Resistance (MAR) index or Antibiotic Resistance Analysis (ARA) (Olivas and Faulkner, 2008). MAR involves testing *E. coli* or faecal coliform resistance to multiple antibiotics at a single concentration while ARA profiles are developed by testing the same faecal indicators to multiple antibiotics at increasing concentrations (Olivas and Faulkner, 2008).

Use of antibiotics is based on measurable differences in resistance among bacteria isolated from faeces of humans and animals allowing for discrimination (Whitlock *et al*., 2002). Da-Silva *et al*. (2011) used MAR to determine the anthropogenic influence at different points along a river in Brazil. Higher resistance to antibiotics was noted in *E. coli* recovered from dense than in sparsely populated areas (Da Silva *et al*., 2011). *E. coli* from humans have higher resistance to antibiotics than animal isolates (Vantakaris *et al*., 2006). Use of antibiotic based methods has been shown to provide good indication of possible sources of faecal contamination in water sheds (Burnes, 2003; Vantakaris *et al*., 2006). The popularity of this method in source tracking studies is due to its ease to carry out, negating the need for expensive equipment (Vantakaris *et al*., 2006). Some of the drawbacks linked to use of antibiotics is that application in big water sheds might not give a good indication of faecal sources due to within-source variation. Better results are obtained if complimented with a finger printing method (Jan and Sadowsky, 2007). The use of a wider variety of antibiotics in a study has also been noted to improve discrimination among isolates (Vantarakis *et al*., 2006).

Genotypic methods used for source tracking in water sheds include; Repetitive Extragenic Palindromic (REP)-PCR, Amplified Fragment Length Polymorphism (AFLP) and Pulsed-Field Gel Electrophoresis (PFGE) (Seurinck *et al*., 2005). These methods have successfully been used in water sheds to help show genetic relatedness among faecal indicator bacteria and the source of contamination (Seurinck *et al*., 2005).

Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR) is a genotypic fingerprinting method that generates specific strain patterns by amplifying repetitive elements in the *E. coli* genome (Mohapatra and Mazumder, 2008). Rep-PCR involves the use of repetitive elements such as repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus sequences (ERIC) and Box sequences which have been reported as highly evolutionary conserved areas as they are sites for essential protein-DNA interaction (Suerinck *et al*., 2005). Mohapatra and Mazumder (2008) compared the efficiency of 5 different Rep-PCR methods to differentiate faecal *E. coli* strains according to their source of origin (human or animal). Ateba and Mbewe (2014) showed that *E. coli* O157:H7 isolates from a specific farm in South Africa, food products from supermarkets in a particular city or water, grouped together following cluster analysis of Enterobacterial Repetitive Intergenic Consensus (ERIC) – PCR profiles.

AFLP is a genotypic fingerprinting method that uses a combination of genomic DNA digestion with resolution enzymes and PCR with short adaptors fixed to the digested fragment end providing sufficient length of known sequence for primers that are to be used for PCR (EPA, 2005). AFLP was used to determine the genetic relatedness of *E. coli* isolates recovered from closely related points within a domestic setting in rural South Africa (Du Preez *et al*., 2008). Possible routes for contamination within a rural household setting were determined (Du Preez *et al*., 2008).

PFGE involves pulse field gel electrophoresis of total genomic DNA after restriction enzyme digestion using infrequently cutting enzymes (EPA, 2005). PFGE was used to show genetic relatedness among *E. coli* from a water source and associated sediment (Lu *et al*., 2004). PFGE was found to have high resolution, although this made it harder to discriminate between isolates hence the study recommended using a complimentary method especially with studies involving large water sheds (Lu *et al*., 2004).

The major drawbacks of using genotypic methods is the intensive laboratory work involved and expensive equipment which makes them beyond reach for most laboratories (Field *et al*., 2003; Seurinck *et al*., 2005). Suggestions have been made that future microbial source tracking methods should be easy to carry out and combine the advantages of both phenotypic and genotypic characters (Scott *et al*., 2002).

Therefore a study that combines use of these two (phenotypic and genotypic) inherent characteristics of faecal indicator bacteria would provide higher resolution for analysis and more reliable information relating to sources of faecal contamination. Phenotypic (antimicrobial) and genotypic (virulence gene such as shiga toxin [*stx1& 2*] producing *E. coli* [STEC] prevalence, DNA fingerprinting) characterisation of *E. coli* (including O157:H7) isolates from different sources, i.e. irrigation water, soil, animal and human faeces, meat and

lettuce have been used to determine if any of these can act as a source of faecal contamination (Ateba and Bezuidenhout, 2008; Ateba and Mbewe 2011; Aijuka *et al.*, 2014; Holvoet *et al.*, 2013).

In April 2013, the European food safety authority (EFSA) published a scientific opinion on shiga-toxin producing *E. coli* (STEC)-seropathotype (also known as vero-toxin producing *E. coli* VTEC-seropathotype) and scientific criteria regarding pathogenicity assessment of foodborne bacteria (http://www.efsa.europa.eu/en/efsajournal/doc/3547.pdf accessed March 2014). Although it is not possible to predict the potential of STEC to cause disease, characterisation at the molecular level may be a common approach to assist the Competent Authorities (CA) and European Union (EU) Member States (MS) in conducting a risk assessment (according to Article 14 of Regulation (EC) No178/2002) when a STEC positive result is obtained and taking action to prevent unsafe food from being placed on the market. For each STEC positive result, both the type of analytical result and the risk profile of the food should be taken into account. Risk assessment may start taking into account the type of food, the availability of traceability information, the consumption habits and the consumer awareness level according to Cen ISO/TS 13136:2012 EURL-adapted version (http://www.iss.it/vtec/index.php?lang=2&anno+2014&tipo=3).

2.9 MICROBIAL SOURCE TRACKING POTENTIAL USING PHYLOGENETIC ANALYSIS OF NOROVIRUSES AND HEPATITIS VIRUSES

This is an updated review on viral pathogens, providing detailed information on morphology and physicochemical properties, genome structure and organisation, the genetic diversity, laboratory diagnostic detection methods, etc. and phylogenetic analysis to establish a possible link between viruses isolated from irrigation water and associated fresh produce. In a recent publication by Wong *et al.* (2014) the application of enteric viruses for faecal pollution source tracking has been reviewed extensively.

Enteric viruses are well recognized foodborne pathogens that infect and replicate in the gastrointestinal tract of humans causing gastroenteritis and hepatitis (Fong and Lipp, 2005). The viruses are mainly transmitted through the faecal-oral route, directly from person-to-person or indirectly via ingestion of contaminated food and water (Kocwa-Haluch, 2001; Bosch, 1998). Infected individuals shed the viruses in high numbers in faeces which are spread in the environment when water comes into contact with the faecal matter (Fong and Lipp, 2005; Rodríguez-Lázaro *et al.*, 2012).

Viruses are comprised of a diverse group of families including noroviruses (NoVs) and hepatitis A (HAV) (Carter 2005) which have been identified as the most common causes of foodborne illnesses (Koopmans and Duizer, 2004; Rodríguez-Lázaro *et al.*, 2012). These viruses are non-enveloped viruses with RNA genomes and very stable in the environment, enabling their survival in the environment (Fong and Lipp, 2005; Rodríguez-Lázaro *et al.*, 2012).

NOROVIRUSES

Norovirus

Noroviruses were previously referred to as “Norwalk” or “Norwalk-like viruses” and “small round structured viruses” (SRSVs) (Glass *et al*., 2009). Initially, electron microscopy (EM) was used to diagnose NoVs in faecal specimens (Figure 7) (Kapikian *et al*., 1972; Hutson *et al*., 2004). The development of the reverse transcriptase-polymerase chain reaction (RT-PCR) allowed NoVs to be cloned and sequenced from the faecal specimens (Le Pendu *et al*., 2006). Classification of NoVs was historically based on cross-challenge studies in volunteers and analysis of cross-reactivity by immuno-electron microscopy (Wyatt *et al*., 1974; Rydell, 2009). The current classification of NoVs relies on sequence similarity on the capsid protein (Zheng *et al*., 2006; Rydell, 2009). Noroviruses belong to the *Caliciviridae* family and the genus *Norovirus* (Rodríguez-Lázaro *et al*., 2012).

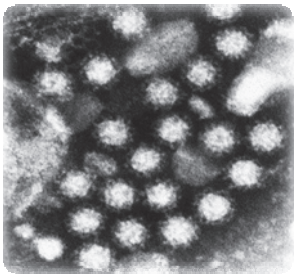

Figure 7: Electron micrograph of norovirus virions (Le Pendu *et al*., 2006).

Morphology and physicochemical properties

The NoV virion consists of a capsid and nucleic acid, and does not contain an envelope (Morillo *et al*., 2011). The nucleocapsid is icosahedral in symmetry and between 27 and 30 nm in diameter (Rodríguez-Lázaro *et al*., 2012). The capsid of NoVs is comprised of 180 copies of a single protein and has a T=3 icosahedral symmetry with 90 dimers of the major capsid protein VP1 and a few copies of VP2 (Hardy 2005; Scipioni *et al*., 2008).

The surface of NoVs shows 32 cup-shaped depressions and protruding arches (Scipioni *et al*., 2008). The major capsid protein subunit consists of an S and P domain which stand for shell and protruding, respectively (Figure 8) (Okoh *et al*., 2010). The P domain is composed of subdomains P1 and P2; P1 forms the middle subdomain while P2 forms the distal subdomain (Okoh *et al*., 2010). The S domain plays a role in the formation of the icosahedral shell and the P domain plays a role in the formation of the protrusions emerging from the shell (Okoh *et al*., 2010). The P1 and P2 subdomains have been reported to play a role in antigenicity and in helping NoVs bind to cellular receptors (Okoh *et al*., 2010).

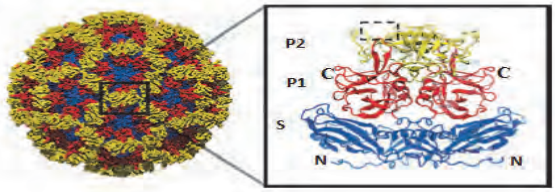

Figure 8: Schematic representation of the norovirus viral particle. Three dimensional representation of the NoV particle showing a detailed view of an individual monomer that forms the NoVs capsid. The subunit is composed of the protruding (P) domain (subdivided into sub-domains P1 and P2) and the shell (S) domain.

Genome structure and organisation

The virus genome of NoVs is protein-linked at the 5' end (covalently linked to VPg) and polyadenylated at the 3' end, is comprised of a positive-sense single stranded RNA that is approximately 7.7 kilobases (kbs) in size, and organised into three open reading frames (ORFs) (Hardy 2005). The guanine + cytosine (G+C) content of the NoV genome is between 45% and 56% (Morillo *et al*., 2011).

Norovirus open reading frames

(i) Open reading frame 1 encodes a large non-structural polyprotein (approximately 195 kDa) that is processed by a proteinase into intermediate precursors and final non-structural protein products (e.g. nucleoside triphosphatase (NTPase), VPg, proteinase and polymerase) that are involved in virus transcription and replication;

(ii) ORF2 encodes VP1 (60 kDa) (major capsid protein);

(iii) ORF3 encodes VP2 (minor structural protein) (Le Pendu *et al*., 2006; Bok *et al*., 2009; Morillo *et al*., 2011) (Figure 9).

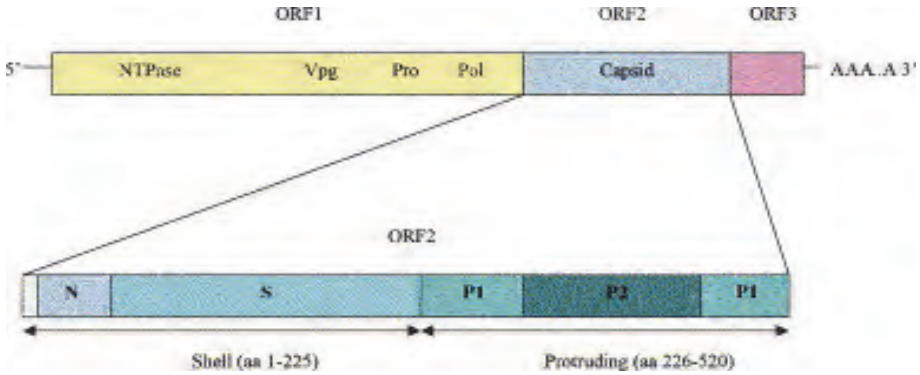

Figure 9: Schematic representation of the NoV viral genome organization (Le Pendu *et al*., 2006).

Norovirus genetic diversity

The genus *Norovirus* comprises of viruses capable of infecting humans, pigs, cattle and mice species (Patel *et al*., 2009). Due to the accumulation of point mutations associated with error-prone RNA replication and recombination between two related viruses, a great diversity of NoV strains exists and the strains are divided into genogroups (Said *et al*., 2008; Glass *et al*., 2009). There are 5 genogroups (G) of NoVs ranging from group I through V (GI-V) (Glass *et al*., 2009) (Figure 10). The groups are further subdivided into genotypes and strains based on sequence diversity in the capsid protein VP1 and genetic analysis of the RdRp (Iritani *et al*., 2008; Rocha-Pereira and Nascimento, 2012). Isolates within the same genogroup have an amino acid sequence difference of about 43% in the major capsid protein while isolates from different genogroups have a difference of up to 61% (Rydell, 2009). Each genogroup consists of 1 to 19 different genotypes (Atmar, 2010).

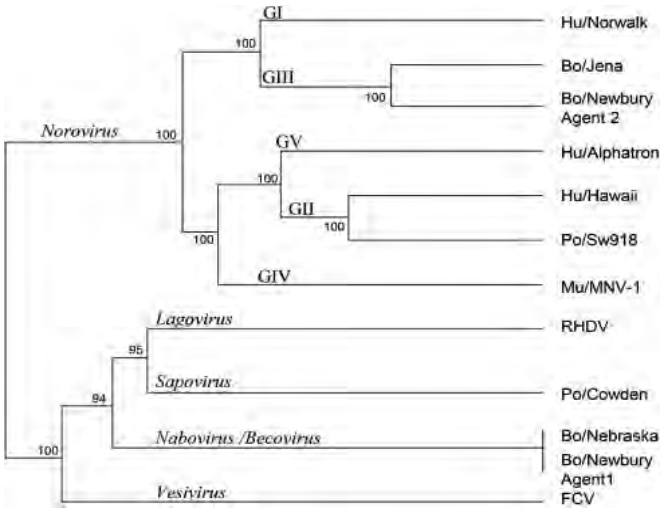

Figure 10: Phylogenetic tree showing various genera of Caliciviridae (Scipioni *et al.*, 2008).

Thirty three NoV genotypes have been found to exist; genogroups I through V consist of 9, 19, 2, 2, and 1 genotypes, respectively (Figure 11) (Atmar, 2010). Only GI, GII, and GIV are able to cause human infections (Zheng *et al.*, 2006). Genogroups III and V strains cause infections in cows and mice; GV are also capable of causing infections in cats and dogs; strains from GII cause infections in pigs (Glass *et al*., 2009). Strains of genogroup II, genotype 4 (GII.4) have been reported to be the cause of the majority of cases and outbreaks of NoVs in the last decade and the rapid evolution of the GII.4 variants or antigenic drifts of NoVs have led to the emergence of novel strains associated with global epidemics (Glass *et al*., 2009; Koo *et al*., 2010). To date, no NoV animal strains have been identified in people as NoVs infections that are specific for animals have been reported to be unable to infect humans (Atmar, 2010).

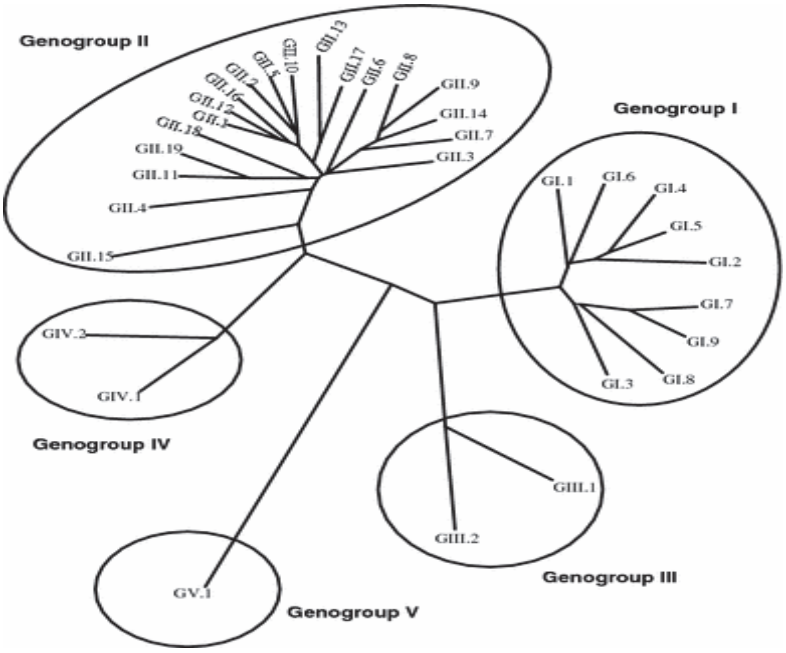

Figure 11: Schematic representation of genogroups and genotypes of NoVs (Atmar, 2010).

However, a risk of zoonotic transmission between human and animal NoV strains could possibly exist. In such an event a novel virus with a new host tropism and increased or decreased pathogenicity may arise (Almanza *et al*., 2008). Swine NoVs are genetically and antigenically related to the most prevalent human NoVs strain (GII) (Van der Poel *et al*., 2000; Wang *et al*., 2005; Mattison *et al*., 2007). Mattison *et al*., (2007) demonstrated the presence of NoV GII.4 human-like strains in pigs whereas Wang *et al*. (2005) identified a potential human-swine recombinant NoV strain that could infect gnotobiotic pigs. The possibility for pigs as potential reservoirs for the emergence of new human NoVs strains therefore exists (Van der Poel., 2000; Wang *et al*., 2005; Mattison *et al*., 2007; Almantza *et al*., 2008).

Pathogenesis

Humans contract NoVs via the faecal-oral route and since NoVs are acid-stable, the viruses pass through the stomach and replicate in the small intestines (Lopman *et al*. 2002). The small intestinal mucosa incurs lesions as a consequence of the replicating viruses (Lopman *et al*., 2002). The mucosa lining becomes inflamed and absorptive cells develop an abnormal appearance as a result (Lopman *et al*., 2002). Infection with the viruses also causes blunting of the villi, shortening of the microvilli, dilation of the endoplasmic reticulum, swollen mitochondria, and intracellular oedema (Lopman *et al*., 2002).

Clinical features

Norovirus infections can be asymptomatic and such infections have been reported in nearly one-third of exposed persons (Koo *et al*., 2010). Norovirus infections are characterised by diarrhoea, projectile vomiting, abdominal pain, malaise, and a low-grade fever (Said *et al*., 2008). Infection of NoV is often brief with vomiting or diarrhoea as the predominant symptoms (Le Guyader *et al*., 2006). The viruses replicate in the intestine of the infected individual during 2 to 3 days of clinical disease, which leads to watery diarrhoea and progeny viruses being shed in large quantities in the stool (Le Guyader *et al*., 2006). Disease is self-limiting lasting 24-60 hours in majority of cases (Koo *et al*., 2010). Noroviruses are very contagious owing to a low infectious dose of approximately 18 viral particles and levels of shedding > 1 x 10^{10} RNA copies per gram of stool (Siebenga *et al*., 2009).

Laboratory diagnosis

A diagnostic algorhythm of suspected NoV infection was developed in 1982 and the following criteria was established: stool cultures must be negative for bacterial pathogens, vomiting reported in about 50% of cases, an incubation period of 24-48 hours, and a mean or median illness duration of 12-60 hours (Said *et al.,* 2008). Norovirus investigation has come a long way since the gastroenteritis outbreak in 1968 (Table 8). Challenges that have been encountered with NoV investigations are that NoVs do not grow in cell or organ culture and no small animal model for NoV infection and gastrointestinal disease has been derived (Hutson *et al*., 2004; Said *et al*., 2008). The study of NoV infections included the use of techniques such as electron microscopy, immune electron microscopy (IEM), or enzyme immunoassays (EIAs) with reagents from volunteer studies (Wolfaardt *et al*., 1997; Said *et al*., 2008).

Table 8: Advances in the detection of NoVs (Glass *et al*., 2009)

Year	Investigator, reference	Detection method	Sensitivity	Comment
1972	Kapikian	EM/IEM	~10^{6-7}	Detection of Norwalk virus and antibody
1978	Greenberg	RIA		Immunoassays for antibody / antigen based on reagents from human volunteers
1992	Jiang	RT-PCR	~10^{2-4}	Beginning of molecular diagnostics
1992	Jiang	EIA for serology		VLPs used as synthetic antigens
1995	Ando	RT-PCR with multiple primers and probes	~10^{2-4}	RT-PCR detection extended to characterise strains with probe hybridization
1995	Jiang	EIA for antigen detection	~10^{4-6}	Antibodies to VLPs formatted into immunoassay

EM = electron microscopy; EIM = immune EM; RIA – radioimmunoassay; RT-PCR – reverse transcription-polymerase chain reaction; EIA = enzyme immunoassay; VLPs = virus-like particles

Epidemiology

Noroviruses are the common cause of epidemic acute gastroenteritis worldwide and are distinguished from other agents of viral acute gastroenteritis by the fact that people of all ages are susceptible to infection, particularly adults and the elderly (Taylor *et al*., 1997; Wobus *et al*., 2006). Noroviruses are responsible for nearly half of all gastroenteritis cases and constitute 90% of non-bacterial cases of gastroenteritis in the world (Bok *et al*., 2009). The frequent implication of NoVs in foodborne and waterborne gastroenteritis outbreaks is due to NoVs having a high environmental stability and a very low infectious dose (Fong and Lipp 2005). Outbreaks are often witnessed in crowded locations, old age homes, cruise ships, restaurants, schools and health care institutions (Butt *et al*., 2004; Wobus *et al*., 2006). Norovirus outbreaks have a serious cost impact on the health care institutions resulting from closure of wards, increased length of hospitalisation, hiring of extra personnel, and the use of extra supplies (Siebenga *et al*., 2009). The cost incurred by the United Kingdom National Health Service due to NoV infections in hospitals amounts to an estimated amount of £115 million (Thorne *et al*., 2012).

The incidence of NoV illness is high in many countries. Fifty percent of gastroenteritis outbreaks recorded in England and Wales, The Netherlands, Germany, Finland, Sweden, and USA, were found to have resulted from NoV infections (Lopman *et al*., 2002). A population study in the Netherlands indicated that an estimated number of 500 000 NoV cases occurred

among a population of 15.7 million in 1999 (Siebenga *et al*., 2009). A study conducted in England between 1993 and 1996 identified NoVs as the most common pathogen in cases of intestinal infectious disease (Siebenga *et al*., 2009). It is estimated that 23 million people are affected yearly in the United States (USA) by NoVs and as such NoVs are regarded as the leading cause of viral gastroenteritis (Said *et al*., 2008). A study in the USA indicated that 50 000 people are hospitalised annually as a result of NoV infections (Mead *et al*., 1999).

Zahorsky gave the first description of the "winter vomiting disease" in 1929 which was again reported in Norwalk, Ohio, USA, in 1968 after an outbreak of gastroenteritis at an elementary school (Hutson *et al*., 2004). At the time of the gastroenteritis outbreak, there was no evidence that proved viruses to be the cause. It was only proven in 1972 from the faecal specimens collected during the outbreak that the causal agent was the NoV (Kapikian *et al*., 1972).

Reports of gastroenteritis outbreaks have shown human NoVs as an important cause of infection in many countries (Romani *et al*., 2012). Noroviruses were documented as being the cause of most foodborne outbreaks in Europe and the US for a number of years (Baert *et al*., 2008). Outbreaks frequently occur in restaurants, hotels, day care centres, schools, nursing homes, cruise ships, swimming pools, hospitals, and military installations (Lou *et al*., 2011). The genogroup II-genotype 4 (GII.4) NoVs are reported to have been the dominant circulating strain since the early 1990s and an unusually high number of outbreaks of gastroenteritis has resulted due to NoVs GII.4 as witnessed in 2002 after the emergence of the GII.4 NoVs variant and the epidemic gastroenteritis around the world in the winter of 2002/2003 (Allen *et al*., 2009).

The first report of a NoV associated infection in South Africa (SA) was in 1993 (Taylor *et al*., 1993; Mans *et al.*, 2013). Two outbreaks of viral gastroenteritis were reported: the first outbreak was associated with consumption of meals from a congress that members of the department of Medical Virology, University of Pretoria, had attended while the second episode was associated with consumption of food at a Christmas buffet that had been attended by some of the members that were involved in the first outbreak (Taylor *et al*., 1993). Symptoms of both cases of gastroenteritis included diarrhoea or vomiting or both, abdominal cramps with or without associated headache, myalgia or fever within 24 to 72 hours after the congress (Taylor *et al*., 1993). In both cases, the NoVs were identified in stool samples by EM (Taylor *et al*., 1993).

A study on the incidence and seroprevalence in Pretoria (SA) indicated a large portion of the population to have had exposure to NoV infections (Taylor *et al*., 1997; Mans *et al.*, 2010). The study revealed 57% of children to have been exposed to NoV infection by age 1-2 years and 62% of the population to have been seropositive by age 40 (Taylor *et al*. 1997; Mans *et al*., 2010). The study by Mans *et al*. (2010) involving hospitalised paediatric patients with viral gastroenteritis showed NoV to have a prevalence of 14% with rotaviruses having a prevalence of 24%. Diarrhoea is a leading cause of death in young children in developing countries, but relatively little is known about the etiological role played by NoVs, but estimates indicating that > 1.1 million hospitalisations and almost 220 000 deaths occur among children < 5 years old each year in those countries (Siebenga *et al*., 2009). Despite

these findings and others presented before the study, NoVs are still not routinely tested for and infections are still under-reported (Mans *et al*., 2013).

Studies report the true incidence of NoV infections to be unknown as many cases of gastroenteritis go unreported. It has been estimated that more than 90% of outbreaks of acute non-bacterial gastroenteritis are caused by human NoV, but the percentage may be underestimated due to the large number of asymptomatic NoV infections and lack of proper detection methods (Lou *et al*., 2011). Despite the significant economic impact and considerable morbidity caused by human NoVs, no drug or vaccine is available to treat or prevent human NoVs disease (Wobus *et al*., 2006). Noroviruses are classified as class B biological agents due to their high infectivity and stability and the suddenness of outbreaks and the debilitating nature of the disease (Wobus *et al*., 2006).

HEPATITIS A VIRUS

Hepatitis A virus was first reported in 1973 after it was visualized from faecal samples collected from human volunteers using IEM (Figure 12) (Feinstone *et al*., 1973). The virus was successfully propagated in cell cultures in 1979 (Provost and Hilleman, 1979).

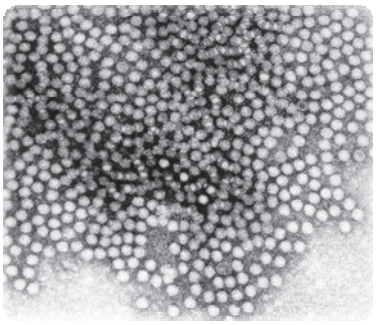

Figure 12: Hepatitis A virus particles visualized by immunoelectron microscopy (Blacklow 1996).

Studies were undertaken to reverse transcribe and clone the HAV RNA genome (Najarian *et al*., 1985). A breakthrough was reached when the complete nucleotide sequence of HAV was established by Cohen *et al*., (1987). Methods for the inactivation of HAV that were in development soon led to the discovery of the HAV vaccine in the 1990s (Martin and Lemon, 2006).

Hepatitis A virus belongs to the *Picornaviridae* family and has been recognized since the Middle Ages (Carter, 2005). Primates were previously identified as their only natural host (Fiore, 2004). Recently, a new virus referred to as the duck hepatitis virus or DHV has been described that is specific for ducks and has also been classified as a member of *Picornaviridae* constituting the genus *Avihepatovirus* (Pan *et al.,* 2011). Human HAV was initially classified in the genus *Enterovirus* as a member of the family *Picornaviridae* but now

belongs to the *Hepatovirus* genus as the only member, due to the virus having unique properties in relation to its genetic structure and replication procedure (Carter, 2005).

Morphology and physicochemical properties

The infectious particle of HAV consists of a capsid protein and an RNA genome (Totsuka and Moritsugu, 1999). The mature particle has a buoyant density and sedimentation of 1.33 g/cm^3 in CsCl solutions and 160 S in sucrose solutions respectively (Totsuka and Moritsugu, 1999). Virion capsid polypeptides of HAV have molecular masses (M_r) of 32 000, 26 000, 22 000 and 10 000 (Ticehurst *et al*., 1983). Physicochemical features include: extreme stability in elevated temperatures and low pH, slow and inefficient replication in cell culture, no obstruction of cellular protein synthesis and no induction of cytopathic effects, a protracted and asynchronous uncoating process, lack of a myristate moiety on viral protein VP0, a small VP4 capsid protein (Totsuka and Moritsugu 1999). The hepatitis A virus capsid is spherical and icosahedral in structure (27-29 nm in diameter) and non-enveloped (Rodríguez-Lázaro *et al*., 2012). The virus particle consists of a positive sense single-stranded RNA genome that is approximately 7.5 kb in length (Rodríguez-Lázaro *et al*., 2012).

Genome structure and organisation

The HAV genome is divided into:

(i) the 5' non-coding region (NCR) that makes up about 10% of the genome,
(ii) a single ORF that consists of 2227 amino acids that encode all the viral proteins,
(iii) a short 3' NCR followed by a poly (A) tail (Figure 13) (Yi and Lemon 2002; Sánchez *et al*., 2007).

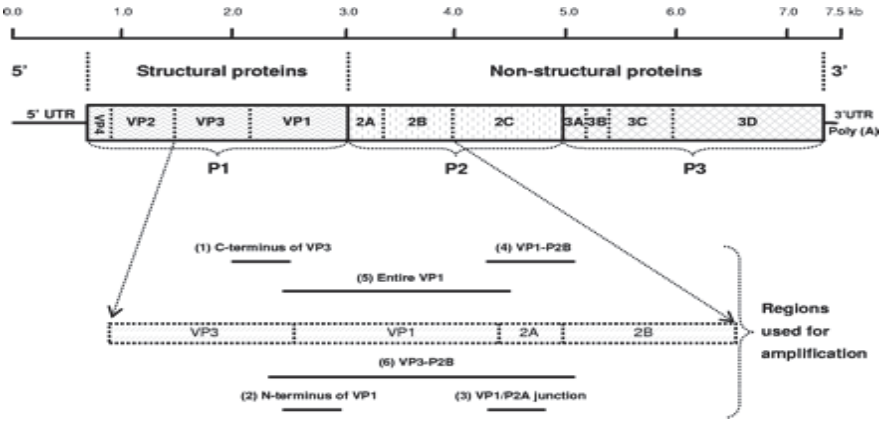

Figure 13: Schematic representation of the HAV genome organisation (Nainan *et al*., 2006)

The 5' NCR

The 5' NCR has complex secondary and tertiary structures and also contains signals needed for HAV RNA replication. The region also directs the initiation of translation of the viral polyprotein via an internal ribosome entry site (IRES) within its sequence (Yi and Lemon 2002). The 5' NCR is approximately 734-704 nucleotides in length and has a protein VPg covalently linked to its terminus (Cuthbert 2001; Totsuka and Moritsugu 1999). The nucleotide region 1-95 of the 5' terminus contains six major structure domains. Domain I has

a hairpin structure. Domain II has two stem loop structures which are followed by a polypyrimidine tract (Totsuka and Moritsugu 1999). Four other domains (III-IV) are spread in the nucleotide 155-734 region (Totsuka and Moritsugu 1999).

The open reading frame

The ORF is 2.225-2.227 nucleotides in length and comprises of the P1, P2, and P3 regions (Cuthbert, 2001; Sánchez *et al*., 2007). The P1 region encodes the structural proteins VP1, VP2, VP3 and a putative VP4, while the P2 and P3 regions encode non-structural proteins associated with replication (Nainan *et al*., 2006). VP1 encodes viral proteins and VP2 and VP3 encode the non-structural proteins (*et al*., 2005). The non-structural polyproteins P2 and P3 consist of 2A, 2B, 2C and 3A, 3B, 3C, 3D respectively (Yi and Lemon, 2002). The protein 2A, 2B, 2C encodes 45, 251 and 335 amino acids respectively (Yi and Lemon, 2002). The 2A and 3C serve an enzymatic role during processing of HAV (Totsuka and Moritsugu, 1999). The translated 2A regions act as an intermediary: the proteins are partially located on the surface (VP1) with some assembled inside the virion (Totsuka and Moritsugu, 1999). Proteins 2B and 2C play a role in the replication of HAV RNA (Totsuka and Moritsugu, 1999). Protein 2C has a nucleoside triphosphate motif: 2B acting on its own or with 2C directs membrane rearrangements essential for RNA replication (Yi and Lemon, 2002).

The proteolytic activities of the encoded viral proteins process the HAV polyprotein into precursor intermediates and mature proteins (Yi and Lemon, 2002). Protein 3B (also known as VPg) is covalently linked to the 5' end of the genomic RNA and serves as the protein primer for RNA synthesis (Cristina and Costa-Mattioli, 2007). Protein 3C is a proteinase (designated $3C^{pro}$) and is the only one encoded by the virus; it is responsible for most cleavages in the proteolytic processing of the protein (Yi and Lemon, 2002). Protein 3D (designated $3D^{pol}$) is the RNA dependent RNA polymerase (Yi and lemon, 2002).

The 3' NCR

The 3' NCR is made up of 40-80 nucleotides that are attached to a poly-A tract (Cuthbert, 2001; Totsuka and Moritsugu, 1999). The 3' NCR consists of two stem loop structures and a pseudoknot structure with the adjacent coding nucleotides of the 3D region (Totsuka and Moritsugu, 1999). The structures of the 3 NCR and those of the 5' terminus (i.e. domain I, domain II and the polypyrimidine tract) are important for RNA synthesis (Totsuka and Moritsugu, 1999). Domains III and VI of the 5' NCR serve as the internal ribosome entry site (IRES) (Totsuka and Moritsugu, 1999).

HAV genetic diversity

The VP1/2A region of the HAV genome is most commonly used in defining HAV genotypes since HAV has a high degree of amino acid sequence conservation, thus reflecting a high degree of conservation in the VP1/2A junction region as well (Chironna *et al*., 2003; Endo *et al*., 2007). Genetic analyses based on phylogenetic analysis of the 168-nucleotide fragment encompassing the VP1/2A junction, and the full length VP1, VP2 and VP3 of 152 strains of HAV from various parts of the world, have allowed characterisation of the HAV strains and grouping into different genotypes and subgenotypes (Figure 14) (Robertson *et al*., 1992; Chironna *et al*., 2003; Costa-Mattioli *et al*., 2003). Thus, HAV was classified into genotypes I, II, III and VII that were associated with humans, and IV-VI that were isolated from simian

species that had developed a hepatitis A-like illness (Costa-Mattioli *et al*., 2003). An HAV genotype can be defined as a group of viruses with > 85% nucleotide sequence identity (Robertson *et al*., 1992; Costa-Mattioli *et al*., 2003). The HAV genotypes were further classified into subgenotypes (Cao *et al*., 2011). The nucleotide sequence of HAV strains differs at 15-25% of positions within the 168 nucleotide (nt) sequence of the VP1/2A junction region of the genome while the subgenotypes have a sequence variability of < 7.5% (Stene-Johansen *et al*., 2005; Endo *et al*., 2007).

Currently, six genotypes of HAV, i.e. genotypes I-VI, have been defined based on analysis of the 900 nucleotides of the complete VP1 protein (Desbois *et al*., 2010). Genotypes I, II, III have been further subdivided into subtypes IA, IB, IIA, IIB, and IIIA, and IIIB, respectively (Endo *et al*., 2007). Genotype VII was reclassified within the genotype II clade as subgenotype IIB, thus constituting to genotype II having only two strains IIA and IIB (Debois *et al*., 2010; Cao *et al*., 2011).

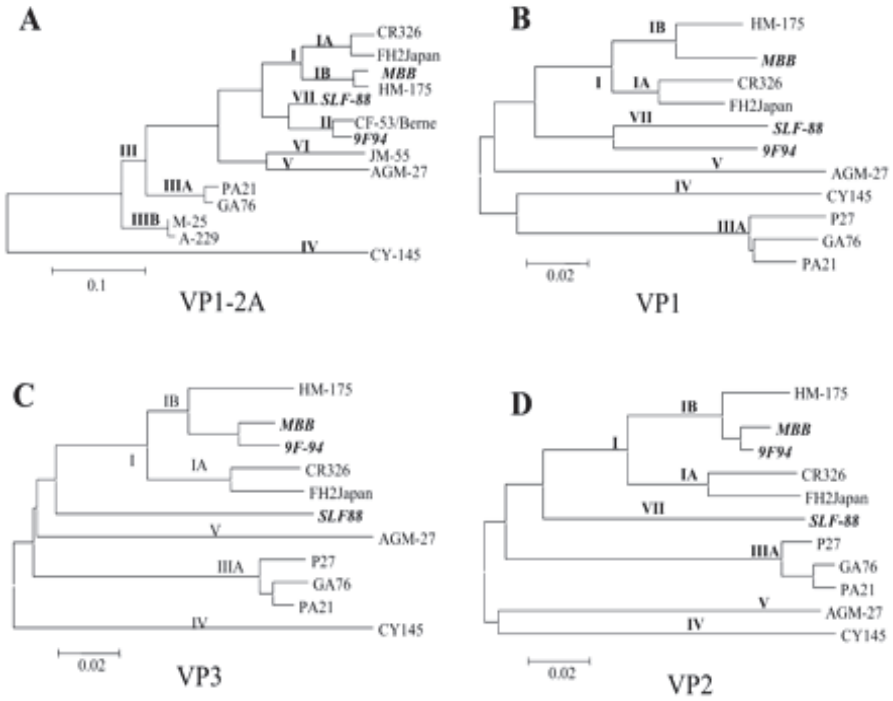

Figure 14: Phylogenetic tree of different genetic regions of HAV (Costa-Mattioli *et al*., 2003)**.**

The majority of strains studied belong to genotype I as it is the most prevalent of the HAV strains, i.e. comprises at least 80% of circulating human strains (Stene-Johansen *et al*., 2005; Cao *et al*., 2011). Genotype III has also been well studied and found to include strains that have been recovered from both humans and non-human primates (Stene-Johansen *et al*., 2005). Despite all the various strains of HAV, only one serotype has been described thus far (Desbois *et al*., 2010).

Distribution of HAV genotypes

Different HAV genotypes have a different geographic distribution (Figure 15) (Cristina and Costa-Mattioli 2007). Distribution of HAV genotypes show that genotype I is prevalent worldwide as it has been reported in America, Brazil, Europe and other parts of the world (Kulkarni *et al*., 2009). Genotype IA is reported to be more common than IB (Endo *et al*., 2007; Desbois *et al*., 2010). Western Europe and the US are areas of low HAV endemicity and have subgenotype IA as the dominant strain but other genotypes and subtypes have been witnessed (Desbois *et al*., 2010).

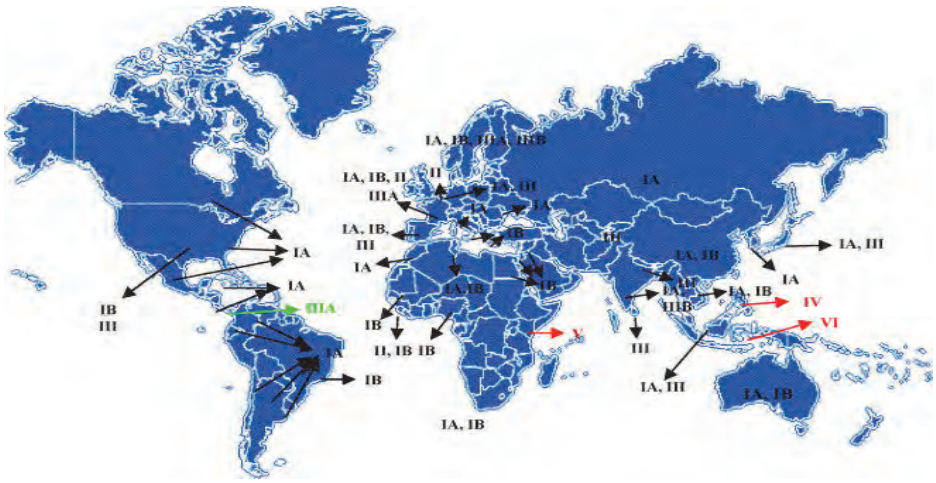

Figure 15: Distribution of different HAV genotypes and subgenotypes (Cristina and Costa-Mattioli, 2007).

Other countries in which the HAV subgenotype IA has been found include China, Japan, the former USSR, and Thailand (Costa-Mattioli *et al*., 2003). Cases of genotype II are very few while genotype III has been reported to be endemic in Southeast and Central Asia (Kulkarni *et al*., 2009). Outbreaks of subgenotype IIIA has been associated among intravenous drug users in Sweden, Norway, the United Kingdom (UK), and Estonia (Stene-Johansen *et al*., 2005). Only a few cases have been associated with IIIB (Stene-Johansen *et al*., 2005). Co-circulation of IA and IB subgenotypes has been reported in Brazil, France, and South Africa as well as the US but most IB isolates were found to be from travellers returning from other countries (Cristina and Costa-Mattioli, 2007).

Pathogenesis

Studies involving experimental infection of nonhuman primates and natural infection of humans have assisted greatly in understanding the pathogenic events that occur during HAV infection (Nainan *et al*., 2006). The hepatitis A virus replicates primarily in the liver (Nainan *et al*., 2006). HAV travels to the gastrointestinal tract where it is taken up and thence the liver where it is replicated and shed in bile (Figure 16) (Wei and Kniel, 2010). Stool samples of infected individuals have been found to contain up to 10^9 infectious particles per gram HAV (Fiore, 2004; Wei and Kniel, 2010).

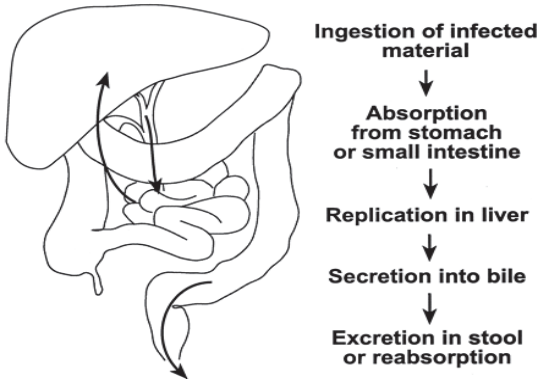

Figure 16: Graphic representation of the HAV infection cycle (Cuthbert 2001)

The incubation period of HAV infection is 3-5 weeks, with an average of 28 days before the onset of symptoms (Nainan *et al*., 2006). High concentrations of HAV are found in the faeces toward the end of the incubation period. Children shed HAV in the faeces for as long as a few months after the onset of illness; this shedding process does not last as long in adults (Leung *et al*., 2005).

Laboratory diagnosis

Studies involving experimental infection of non-human primates and natural infection of humans have assisted greatly in understanding the pathogenic events that occur during HAV infection (Nainan *et al*., 2006). Hepatitis A virus can be propagated in cell cultures derived from the primates with reason efficiency and no cytopathic effects (Lemon, 1997). Common cells used in culturing HAV include African green monkey cells and foetal rhesus kidney cells (Lemon, 1997). Hepatitis a virus of human origin is said to require an extensive adaptation period before it grows in cell culture, and once adapted, HAV produces a persistent infection and becomes attenuated (Nainan *et al*., 2006).

The humoral immune response plays a major role in the diagnosis of HAV infection (Nainan *et al*., 2006). Laboratory diagnosis of HAV relies on specific serological tests for detection of anti-HAV IgM (de Paula, 2012). Immunoglobulin M anti-HAV is used as the primary marker of acute infection (Nainan *et al*., 2006). The presence of IgM antibodies in the early phase of infection renders the antibodies important for diagnosis (de Paula, 2012). A number of methods have been used to detect HAV and include radio-immunoassay, bent assay, immunoblotting and dot blot immunogold filtration (Nainan *et al*., 2006). These methods detect anti-HAV IgM in blood as the antibodies act as primary markers of acute infection (Nainan *et al*., 2006). Immunoglobulin M antibodies to HAV infection are present in almost all individuals (> 99%) at the time of infection and anti-HAV IgM response usually peaks within the first month of illness and usually declines within 6 and 12 months (Lemon, 1997). Thus, IgM antibodies produced in response to HAV infection are detectable soon after infection and can remain detectable for nearly 6 months (Hussain, 2011). The anti-HAV IgG, on the other hand, appears early after infection and can remain detectable for a lifetime and provides lifelong immunity (Hussain, 2011).

Other methods of detecting HAV have been proposed and these include the method developed by Archibald *et al*. (1986) which is based on detection of antibodies in saliva. The method was developed in response to challenges in collecting blood samples, especially in children, when compared to saliva samples which are easy to collect.

Molecular methods are the most sensitive for screening HAV clinical specimens (de Paula, 2012). Methods involve the use of RT-PCR and provide a means of detecting HAV RNA in blood earlier than antibodies (de Paula, 2012). Also, molecular testing does not require a lengthy incubation period needed for viral isolation from cell culture and technical expertise (Costa *et al*., 2013).

Epidemiology

Hepatitis A can be a seriously debilitating disease progressing from a non-specific illness with fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice (Gensberger and Kostić, 2013). Hepatitis A represents around 50% of the total hepatitis cases worldwide and although it is self-limiting and rarely causes death, may incapacitate patients for several months (Gensberger and Kostić, 2013). An estimated 1.5 million new HAV infections occur annually (Traoré *et al*., 2012). Hepatitis A is usually underreported as infections are often unnoticed, thus the epidemiology of hepatitis A relies on the measurement of humoral antibodies (Melnick, 1995). Seroprevalence studies have been carried out to determine data on the endemicity of HAV infections (Poovorawan *et al*., 2002). Three epidemiological patterns of endemicity exist around the world, namely “low”, “intermediate”, and “high” (Taghavi *et al*., 2011).

Areas of high endemicity have a prevalence of HAV immunoglobulin G (IgG) antibodies that reaches 90% in adults, with most children having been infected by the age of 10 years; 50%-60% of adults and 20%-30% of 10 year old children infected in areas of intermediate endemicity; only 30% of adults have HAV antibodies in areas of low endemicity (Nelson, 2006).

All three endemicities are dependent on age and level of hygiene (Taghavi *et al*., 2011). More than 70% of children below the age of 6 years are asymptomatic and if illness occurs, it is not usually associated with jaundice (Poovorawan *et al*., 2002; Nelson, 2006). Older children and adults are usually symptomatic and more than 70% of the HAV infections are associated with jaundice (Poovorawan *et al*., 2002). Areas of high endemicity include most of Africa, Asia and Central and South America (Poovorawan *et al*., 2002).

Transmission occurs through the faecal-oral route directly through contact with infected individuals and indirectly through ingestion of contaminated food or water (Fiore, 2004; Taylor, 2011). Other modes of transmission include exposure to contaminated blood or blood products (Fiore 2004). Following ingestion, HAV travels to the gastrointestinal tract where it is taken up, and then travels to the liver where it replicates and gets excreted, i.e. in bile. Stool samples of infected individuals have been found to contain up to 10^9 infectious particles per gram HAV (Fiore, 2004; Wei and Kniel, 2010).

Infections are usually asymptomatic and prevalent among children as seen in the developing world, but can progress from a non-specific illness with fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice. Hepatitis A virus constitutes 50% of total hepatitis cases worldwide (Koopmans and Duizer, 2004; Bosch, 1998). It is endemic in lower socio-economic communities in South Africa (Taylor, 2011).

Sanitation is a principal risk factor of HAV as it influences the number of infections (Melnick, 1995). Hepatitis A virus infections occur early in life where sanitation is poor and living conditions are crowded (Melnick, 1995). Improvements in personal hygiene and environmental sanitation lead to a declined natural immunity against HAV (Poovorawan *et al*., 2002). Immunisation with vaccines also lead to low incidences of HAV infection as seen in countries with effective immunisation programmes such as the US. The number of HAV cases in the US has been reduced by 92% and the infection rate is one case per 100 000 persons per year (Rodríguez-Lázaro *et al.*, 2012). Similar situations have been reported in countries like Canada, Australia, Japan and New Zealand (Rodríguez-Lázaro *et al.*, 2012).

Other risk factors of HAV include sharing the same household with a patient with hepatitis, homosexual activity, close contact with young children attending day-care centres, and international travel to regions where hepatitis A is endemic (Lemon, 1997). Intravenous drug use has also been associated with hepatitis A, as witnessed in Sweden (Lemon, 1997). Hepatitis A virus infections have a fatality rate that is lower than 0-1% (Sánchez *et al*., 2007).

Examples of outbreaks involving HAV include an outbreak associated with ingestion of contaminated green onions in Pennsylvania which resulted in three deaths among 601 cases reported, indicating the potential of HAV infections in causing death (Sánchez *et al*., 2007). Another outbreak of hepatitis A was among patrons who had eaten menu items containing green onions, which identified the onions as the source of infection. The study reports that the green onions were contaminated with HAV before reaching the restaurant. All the employees were ruled out as transmitters as they showed no serologic evidence of recent HAV infection (Newell *et al*., 2010). The epidemic of HAV infection in Shanghai, China, in 1988 was caused by the ingestion of raw clams and resulted in 32 deaths with a total of 292 301 people affected and serves as an example of a large outbreak caused by HAV infection (Nelson, 2006).

ROLE OF NoVs AND HAV IN FOOD- AND WATERBORNE OUTBREAKS

Enteric virus infections originate from a number of sources and humans become exposed through various routes (Bosch, 1998; Okoh *et al*., 2010). Water used for drinking and recreational purposes as well as those used for agricultural purposes, such as crop irrigation, and food processing, may become contaminated with enteric viruses (Gensberger and Kostić, 2013). Food becomes contaminated upon contact with enteric virus-contaminated water, e.g. food crops grown in land irrigated with wastewater and/or fertilised with sewage, sewage-polluted recreational water, and contaminated drinking water (Bosch, 1998).

Shellfish have been implicated in outbreaks of NoVs and HAV, and this is because shellfish are filter feeders (Okoh *et al*., 2010). Filter feeding results in the bio-concentration of the environmentally stable NoVs and HAV in the digestive glands and gills of shellfish (Okoh *et al*., 2010). Human exposure to NoVs and HAV results when shellfish are consumed and this is due to viruses being stored in the edible tissues of the shellfish (Bosch, 1998; Okoh *et al*., 2010). Most enteric virus outbreaks involve shellfish grown and harvested in virus contaminated water (Bosch, 1998). Reports state that virus contamination of shellfish is found with a high frequency due to regular sewage contamination (Newell *et al*., 2010). Human exposure is increased when shellfish are consumed raw or only lightly cooked (Okoh *et al*., 2010).

Noroviruses and HAV have been well documented in fresh produce (Newell *et al*., 2010). Pre-harvest contamination occurs when fresh produce is subjected to irrigation with wastewater, when fertilised with sewage sludge or when the area of the field in which the fresh produce is located is polluted with faecal matter (Okoh *et al*., 2010). Using wastewater requires adequate treatment of the water prior to its use in crop irrigation (Hussain, 2011). Treatment of wastewater is vital as contaminants are removed through this process, which would otherwise result in viral illness outbreaks (Hussain, 2011). Produce that is to be consumed raw should be washed thoroughly to ensure the complete removal of possible viral contaminants, especially if it was irrigated with wastewater or fertilised with sewage sludge (Hussain, 2011). An outbreak of gastroenteritis was reported in Israel at a military camp due to consumption of vegetable salads, proving the need for adequate washing of produce prior to consumption (Okoh *et al*., 2010).

Infected food handlers also play a role in the transmission of enteric viruses (Newell *et al*., 2010). Post-harvest contamination of food occurs as a result of human handling (Hussain, 2011). Contaminated harvesting equipment, transport containers, contaminated aerosols, wash and rinse water or cross contamination during transportation and storage are ways in which post-harvest contamination can occur (Hussain, 2011). Hands of harvesters and handlers are a source of contamination of produce (Hussain, 2011). Sanitary hand washing facilities are essential and food harvesters and handlers should be encouraged to use them (Hussain, 2011). Access to proper toilet facilities and clean water near the field are also essential (Hussain, 2011).

Wastewater treatment processes have been seen to only eliminate between 50% and 90% of viruses present in wastewater (Okoh *et al*., 2010). Bacterial indicator organisms are relied on for monitoring of the wastewater treatment systems (Okoh *et al., 2010*). However, infectious viruses have been isolated from water that met bacterial indicator standards, proving the use of bacterial indicators to be insufficient in monitoring wastewater quality because bacterial and viral contaminations are not associated and linked (Okoh *et al*., 2010). Viral pathogens are host specific and have thus been considered as possible tools for testing the efficacy of wastewater treatment systems (Fujioka and Yoneyama, 2002; Thurston-Enriquez *et al*., 2003).

Assessment of water quality should comprise bacterial indicators as well as viral pathogens as pathogenic viruses are more resistant than bacterial indicators during wastewater treatment

(Fujioka and Yoneyama, 2002; Thurston-Enriquez *et al*., 2003). Enteric viruses have a tolerance for pH levels between 3 and 10, and can survive under low temperatures for extended periods and these periods are longer than those for faecal coliform (Kocwa-Haluch, 2001).

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CHAPTER 3

IMPACT OF ENVIRONMENTAL PARAMETERS ON THE GROWTH KINETICS OF MICROBIAL CONTAMINANTS

3.1 INTRODUCTION

Fresh produce is of importance to national food supply and population health while fresh fruit exports constitute a major part of the economy, representing the third biggest earner of foreign exchange. South Africa has over the past 100 years built an international reputation as a major competitor on the export markets delivering top quality safe produce. As one of the leading countries in international food trade, South African farmers have taken measures to comply with new stringent good agricultural practices and food safety requirements. Yet unprecedented challenges facing the quality and quantity of our national water supply have resulted in additional risks for farmers reliant on irrigation of cultivated crops.

The deteriorating quality of water used for domestic, recreational and agricultural purposes is a well-known fact and has raised public health concerns (Chigor *et al*., 2013; CSIR 2010). Due to water scarcity, many farmers (rural and commercial) resort to using surface water for irrigation of fresh produce (Nevondo and Cloete, 1999). The microbiological quality of the water is compromised by inadequate sanitation systems and inadequate management of municipal sewage works. In addition, the growing number of informal settlements and challenges associated with growing urbanisation contribute to the stress on water networks (Nevondo and Cloete, 1999). World-wide dietary guidelines recommend an increase in the daily consumption of raw fresh fruit and vegetables (Beuchat, 2002). This emphasises the need for measures to ensure the microbiological safety of fresh produce for consumption, especially since HIV/AIDS affects a large proportion of the population in South Africa, leading to suppressed immune systems (Gemmell and Schmidt, 2012 and 2013).

Vegetables can be contaminated with pathogenic and/or spoilage bacteria during production from many sources, including manure fertilizers, irrigation water and during food processing by contaminated equipment and food handlers (Martinez-Sanchez *et al*., 2006). Whilst water is essential for crop growth, it is also an exceptional medium for microbial proliferation and is therefore recognized as one of the main sources of contamination associated with crop production. Water is used for irrigation, fertilizer and pesticide application, soil amendments preparation, equipment and facility washing, hydro-cooling and icing in cultivation and post-harvesting processes. The use of contaminated water for irrigation purposes therefore poses a great risk to the consumer.

Factors that influence the growth kinetics of bacteria in water include the pH and temperature of the water (Cheng *et al*., 2013). Organic waste from water treatment plants acts as a food source for waterborne bacteria. Bacteria decompose these organic materials using dissolved oxygen in the water. Biochemical oxygen demand (BOD) is a measure of the amount of oxygen that bacteria will consume while decomposing organic matter under aerobic

conditions. If effluent with high BOD levels is discharged into a stream or river, it will accelerate bacterial growth in the river and consume the oxygen levels in the river. Furthermore, it has been reported that salt has the ability to reduce or inhibit microbial growth due to the associated changes in water activity and ionic strength. The pH of water affects the solubility of many toxic and nutritive chemicals, therefore the availability of these substances to aquatic organisms is affected (Cheng *et al.*, 2013). As acidity increases, most metals become more water soluble and more toxic. Toxicity of cyanides and sulfides also increases with a decrease in pH (increase in acidity). Nitrogen occurs predominantly as nitrate in irrigation water. The ammonium form is usually a result of contamination with wastewater. Ammonia, however, becomes more toxic with only a slight increase in pH. It has been reported that elevated iron concentrations increased the survival rate of *S. typhimurium* as well as the efficiency of invasion during infection.

It is known that fresh produce harbour large natural microflora of which we have a limited understanding in terms of diversity and richness. We also have limited knowledge of foodborne pathogens that may occur on these surfaces and the factors that influence their composition and survival (PLOS ONE | www.plosone.org 1 March 2013 | Volume 8 | Issue 3 | e59310). A range of environmental factors can shape microbial community composition, including pH and moisture availability which can vary across produce types. Likewise, differences in growing conditions, transport as well as storage procedures and -conditions could influence the diversity and composition of produce-associated microbial communities.

Most of these natural microfloras on fresh produce surfaces represent epiphytic organisms with only a small number being plant pathogens (Beuchat, 2002*;* Lindow *et al.*, 2003). Plant and important human pathogenic microorganisms (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus*) affect the microbiological quality and safety of fresh produce (Johnston *et al*., 2006). Plant pathogenic microorganisms have been linked to the increased occurrence and ability of human pathogenic microorganisms to attach to and survive on fresh fruit. This can be ascribed to the presence of plant pathogenic microorganisms in the subsurface and surface tissues which can alter the pH of the plant tissues, allowing the growth of human pathogenic microorganisms to commence (Conway *et al*., 2000; Deering *et al.*, 2011a).

Contamination with these human pathogens can happen at any time throughout the pre- and post-harvest stages of production and marketing. In an effort to increase food safety, different methods for disinfection of these produce must therefore be researched and implemented. Washing as well as sanitizing treatments have been shown to improve product quality and safety by reducing microbial populations (Sapers, 2001). Chlorination has been used in routine washing steps, to treat post-harvest cooling water (Suslow, 2000). The inhibitory effect of chlorine solutions on microbial cells depends on the amount of free chlorine in the solution, therefore these chlorine washing solutions must be routinely checked (WHO, 1998). An alternative disinfectant solution, such as hydrogen peroxide has been recognized as being safe for food applications since it produces no residue as it is rapidly decomposed by an enzyme (catalase) to water and oxygen. Hydrogen peroxide at 0.5% concentrations has been shown to inhibit the “development of post-harvest decay” that is caused by numerous fungi

(Bachmann and Earles, 2000). Besides commercially used methods for washing of fresh produce, consumers use household methods such as washing with tap water or a salt solution to remove contaminants from fresh produce. Since temperature plays an important role in the growth, survival and death of an organism, control of storage temperatures can aid in the elimination of foodborne pathogens and therefore add to the assurance of food safety. High storage temperatures for fresh produce have been shown to promote the growth of various microbes, which in turn promotes the subsequent spoilage of the produce (Carlin *et al*. 1995; Hassenberg and Idler, 2005). However, refrigeration is and has been the key method for controlling the rate of deterioration of fresh produce by means of reducing the respiration rate of the product and by slowing the growth rate of spoilage microbes (Fonseca and Rushing, 2006).

Surfaces within the food production arena can harbour microorganisms (Duffy *et al*., 2005). Studies have demonstrated that foodborne bacterial pathogens do not need a constant high nutrient source to survive for extended periods (Kusumaningrum *et al.*, 2003). Presence of these foodborne pathogens within the food production arena might lead to the contamination of foods and of contact surfaces in such environments. *E. coli, L. monocytogenes* and *S. aureus* have been shown to be transferred to contact surfaces and food through exposure to other contaminated surfaces or hands. Floors can also become contaminated with microorganisms being carried in on the soles of shoes of people moving through the food production or storage environments. The presence and survival of these organisms, as has been demonstrated for *L. monocytogenes* within a packhouse and cold storage facility, can lead to biofilm formation on inert surfaces. The formation of biofilms makes it increasingly difficult to remove these microorganisms and can then lead to chronic contamination of the food production environment (Hood and Zottola, 1997).

Haemorrhagic *E. coli* is an organism, often foodborne due to faecal contamination that can lead to a haemorrhagic colitis, haemolytic uremic syndrome (Mead and Griffin, 1998) or thrombotic thrombocytopenia purpurea (Willshaw *et al.*, 2000). *E. coli* can survive at temperatures ranging from 7^{o}C to 50^{o}C and can even survive at refrigeration and freezing temperatures for a period of time (Adams and Moss, 2000; Willshaw *et al.*, 2000). Unlike *E. coli* O157:H7, *L. monocytogenes* is a psychrotrophic organism that can grow at temperatures as low as 0^{o}C, even if nutrients are scarce (Adams and Moss, 2000). *L. monocytogenes* can cause mild to severe meningitis, especially in patients with a compromised immune system (Bremer *et al.*, 2003).

Salmonella species cause a wide range of gastrointestinal infections ranging from an asymptomatic carrier to severe diarrhoea (Adams and Moss, 2000). *Salmonella* species are not psychrotrophic organisms, but rather grow at temperatures between 5^{o}C and 48^{o}C. Some *Salmonella* spp. are able to survive freezing temperatures, depending on the physiology of the organisms (D'Aoust, 1991). Similarly, *S. aureus* grows at temperatures between 7^{o}C and 48^{o}C and is able to survive freezing temperatures. *S. aureus* is regarded as a minor foodborne pathogen (Mataragas *et al.*, 2008) and is an indicator of poor personal hygiene. However *S. aureus* can cause a range of infections, among these are cutaneous infections, organ infections and toxinoses (Novick *et al.*, 2001). *S. aureus* can also survive on surfaces with little or no

nutrients as this organism is adapted to surviving on the human skin (Adams and Moss, 2000).

As was reported regarding bacteria, fresh produce can not only become contaminated with *Cryptosporidium* oocysts during production but also during processing from contaminated wash water, the food production environment and infected food handlers. Knowledge of the factors affecting *Cryptosporidium* oocysts viability on fresh produce, might contribute to implementing suitable food safety assurance technologies. The robust structure of the oocysts makes it difficult to kill with disinfectant commonly used in fresh produce processing such as chlorine. It was found that *Cryptosporidium* oocysts need to be exposed to 80 ppm. free chlorine for 2 hours to be inactivated (Korich *et al*., 1990). However, the oocysts and cysts are temperature sensitive. Oocysts in water or milk died when subjected to high-temperature-short-time pasteurization treatment of 71.7˚C for 15 seconds. Holding the oocysts at 45˚C for 20 min was also sufficient to render the oocysts non-infective. However, another study found that treatment at 60˚C for 45 seconds or 75˚C for 20 seconds was required to kill oocysts present on the surface of meat. This suggests that the medium in which oocysts are found affects their susceptibility to heat treatment. Fayer and Nerad (1996) found that cryogenic freezing at -70˚C for 1 hour, -20˚C for 24 hours or -15˚C for 168 hours was sufficient to render the oocysts non-infectious. Slow freezing was found less effective and 152 hours at -22˚C was required to kill 90% of oocysts (Robertson *et al.,* 1992).

Recent data indicates that noroviruses (NoV), hepatitis A virus (HAV) and to a lesser extent human rotavirus (HRV) are the leading causes of foodborne illness in industrialized countries (Butot *et al*., 2007). Berry fruits have been associated with outbreaks of gastroenteritis and hepatitis worldwide (Le Guyader *et al*., 2004). Strawberries are more prone contamination than raspberries as they are grown closer to the ground than raspberries and are therefore more prone to contamination with irrigation water. In addition, strawberries cannot be harvested mechanically, resulting in greater contact with food handlers (Verhaelen *et al.*, 2012). In the US, the consumption of imported contaminated strawberries lead to two multi-state outbreaks of hepatitis A (Niu *et al*., 1992), while raspberries have been more often implicated in NoV-associated outbreaks of gastroenteritis (Le Guyader *et al*., 2004; Sarvikivi *et al*., 2011). Noroviruses, specifically NoV GI, are more persistent on raspberries than on strawberries, thus explaining that raspberries are more frequently implicated in foodborne outbreaks of gastroenteritis than strawberries (Verhaelen *et al*., 2012). This difference in viral persistence was more noticeable at 21ºC than at 4ºC and 10ºC (Verhaelen *et al*., 2012). An optimized viral recovery method is necessary to accurately determine the persistence of different enteric viruses on berry fruits and other fresh produce. This information will provide a better understanding of viral and food characteristics that influence the persistence of viruses on fresh produce and could form the basis of hurdle technologies to improve food safety in the supply chain.

Recent studies supported by the Water Research Commission (Projects: K5/1226 & K5/1773) have shown that the national water supply is contaminated and may pose a potential risk for the fresh produce export industry (including fruit and vegetables) and national safe food

supply. In order to support and strengthen the findings of previous research projects the WRC decided to introduce another support project that will focus on the risks in the supply chain.

3.2 RESEARCH REPORTS

3.2.1 IMPACT OF ENVIRONMENTAL AND NUTRITIONAL PARAMETERS ON THE SURVIVAL AND GROWTH KINETICS OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* TYPHIMURIUM

Specific aim

The aim of this study was to investigate the effect of NaCl, pH, iron and a combination of all three on the fate and survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in irrigation water.

Experimental procedures

Cultures

American Type Culture Collection cultures namely *E. coli* O157:H7 (ATCC 35150) and *Salmonella* Typhimurium (ATCC 14028) were used in this study. All cultures were maintained lyophilized and stored at -70^{o}C with subcultures on standard 1 medium (Merck, Johannesburg, South Africa) prepared 24 hours prior to use. Cultures were used to inoculate five replicates of 100 mℓ Tryptone Soy Broth (TSB) (Merck) for each pathogen and were subsequently incubated at 37^{o}C for 18h to achieve a concentration of 8 log CFU/mℓ. Cultures were centrifuged at 5000 r.p.m. and washed twice with sterile distilled water and finally re-suspended into 1% (w/v) Peptone Buffered Water (Merck) to obtain a known concentration of cells.

Effect of pH, sodium chloride, iron and nitrate on the growth and survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in irrigation water

The stationary phase cells were inoculated at 2 log/mℓ into TSB with the required sodium chloride, iron and nitrate concentrations and combinations thereof (Jordan and Davies, 2001). For growth at different pH values the cells were inoculated into TSB media adjusted to pH 4, 7 and 9 respectively. The inoculated cultures were incubation at 37°C for 0-10 days. The number of cells in the different media was calculated at each time point by serially diluting the sample and plating onto Standard 1 medium (Merck). All growth experiments were repeated at least three times. Values shown are the mean of these experiments.

Results

Effect of sodium chloride on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

This study revealed that the survival of both *E. coli* O157:H7 and *S.* Typhimurium decreased proportionally with an increase in the concentration of NaCl (Fig. 17A and B). At NaCl concentrations of 5% and 3%, the growth of *E. coli* O157:H7 was inhibited at days 9 and 10

respectively, and *S.* Typhimurium at days 7 and 9 respectively. Generally, *S.* Typhimurium survived better than *E. coli* O157:H7 at all 3 NaCl concentrations. It is important to note that at a concentration of 1% NaCl no inhibition of growth was detected for both organisms after 10 days (Fig. 17 A and B).

It has been reported that salt has the ability to reduce or inhibit microbial growth due to the associated changes in water activity and ionic strength (Jay, 1992). According to Ingram and Kitchell (1967), relatively low concentrations of salt stimulate the growth of microorganisms whilst high concentrations inhibit them. Sodium chloride stress results in an increased lag phase which results in a progressive decrease in the protein content of the bacteria subjected to growth at high NaCl concentrations (3 to 5%). Arnold and Kasper (1995) also reported that stationary phase *E. coli* O157:H7 cells synthesize stress protective proteins under conditions of high salinity. The results of this study support the observations made by previous researchers on the effect of NaCl on bacteria.

A B

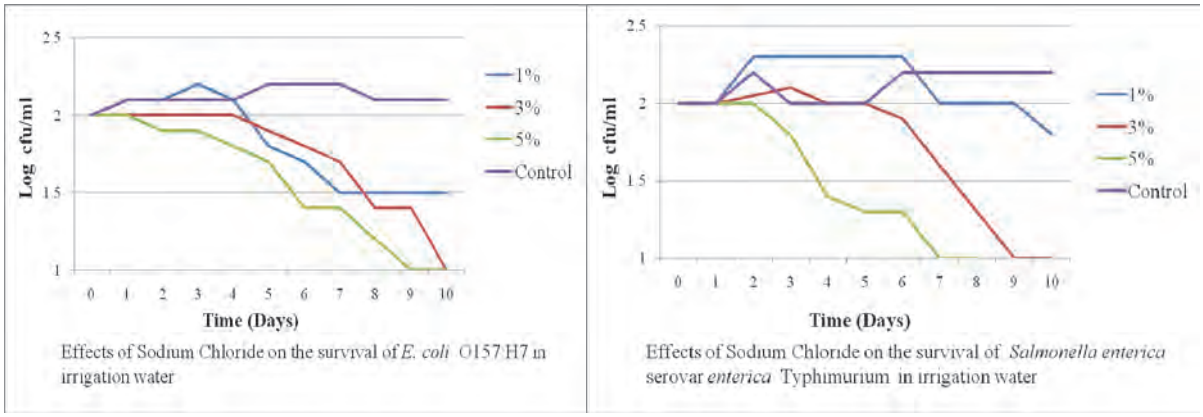


Figure 17 Effect of sodium chloride on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

Effect of pH on the growth and survival of *E. coli O157:H7* and *S.* Typhimurium in irrigation water

In this study, it was found that both *E. coli* O157:H7 and *S.* Typhimurium survived best at pH 7 as well as in the autoclaved irrigation water used as control, with a pH of 7.08 (Fig. 18A and B). An alkaline pH (pH 9) favoured the survival of both bacteria over that of an acidic pH (pH 4). *S. typhimurium* survived better than *E. coli* O157:H7 at pH 9 (Fig. 18 A and B).

Protein concentrations in bacteria are known to be highest at an acidic pH and this may be attributed to the production of acid shock proteins or stress protective proteins that assist with the limited survival of the bacteria. It is known that these organisms are capable of activating the acid tolerance response system which induces pH homeostasis and protein repair systems, thus allowing for survival at low pH conditions. pH is an important parameter, influencing the ability of pathogens to cause disease in humans.

A B

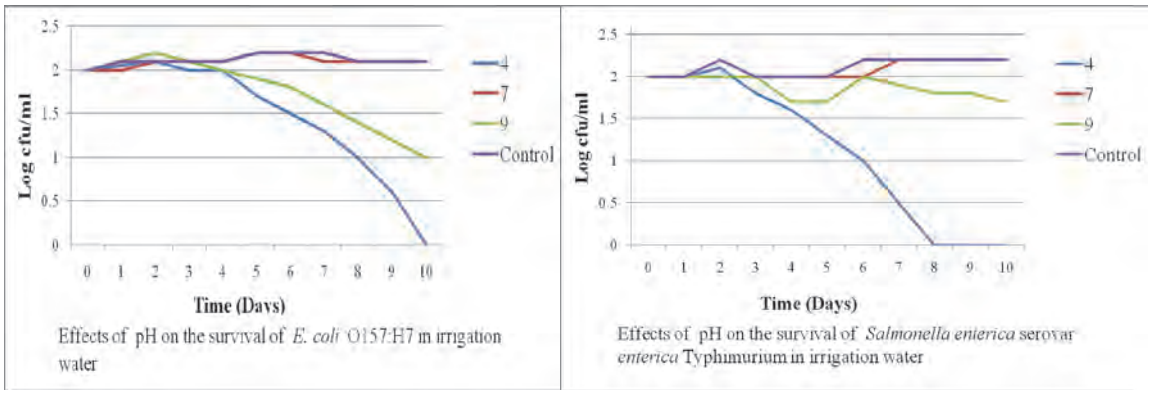


Figure 18 Effect of pH on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

Effect of iron on the growth and survival of *E. coli O157:H7* and *S.* Typhimurium in irrigation water

Iron is essential to all microorganisms but does pose problems of toxicity and poor solubility. As the iron concentration increased from 10 mg/ℓ to 30 mg/ℓ, the survival of *E. coli* O157:H7 increased proportionately. There was a progressive increase in the bacterial population under all iron concentrations from day 1, with 30 mg/ℓ iron resulting in the highest increase (Fig. 19A and B). These findings are of major concern since Patel (2004) reported that an increase in the iron concentration markedly stimulated the toxigenicity of some strains of *E. coli* O157:H7.

A similar trend was seen for *S.* Typhimurium in which high iron concentrations also favoured growth. This correlates with the findings of Foster (2001) who reported that elevated iron concentrations increased the survival rate of *S.* Typhimurium as well as the efficiency of invasion during infection. Bacteria have evolved various mechanisms to counter the problems imposed by their iron dependence, allowing them to achieve effective iron homeostasis under a range of iron regimes. Bacterial iron storage proteins (ferritin) provide intracellular iron reserves for use when external supplies are restricted and iron detoxification proteins (Dps) are employed to protect the chromosomes from iron reduced free radical damage.

A B

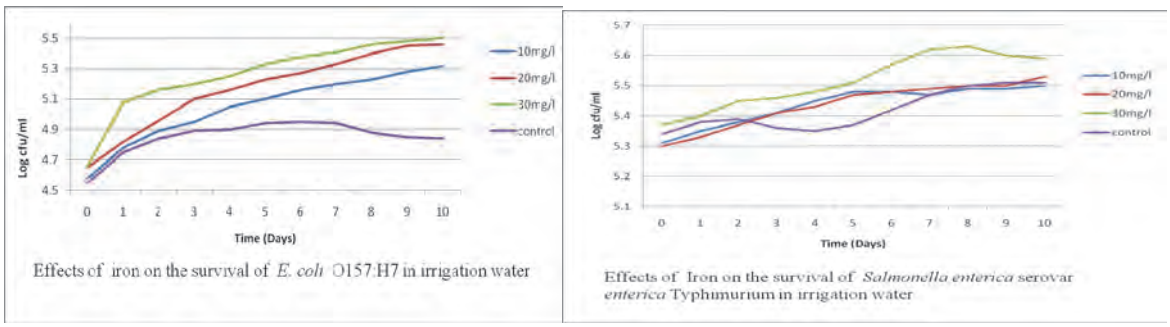


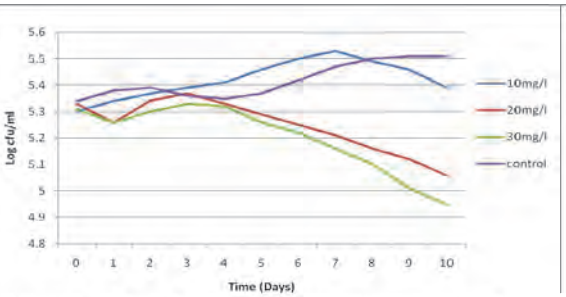
Figure 19 Effect of iron on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

Effect of nitrate on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

Nitrates and nitrites occur widely in foods and drinks. The contamination of groundwater with nitrates poses potential adverse effects to communities or people who depend solely on these sources of water (Okafor and Ogbonna, 2003). In this study *E. coli* O157:H7 survived best at low concentrations of nitrate (10 mg/ℓ); however, there was a decrease in growth after day 5 (Fig. 20 A and B). At higher nitrate concentrations, survival decreased from day 4 and day 2 at 20 and 30 mg/ℓ, respectively. In the control irrigation water which contained 6 mg/ℓ nitrate, *E. coli* O157:H7 survival was found to be most favourable with a gradual increase in growth throughout the survival study (Fig. 20A and B). This correlates with the findings of Whiting and Golden (2003), who reported that an increase in nitrate concentration decreases the survival time of *E. coli* O157:H7 and *S.* Typhimurium.

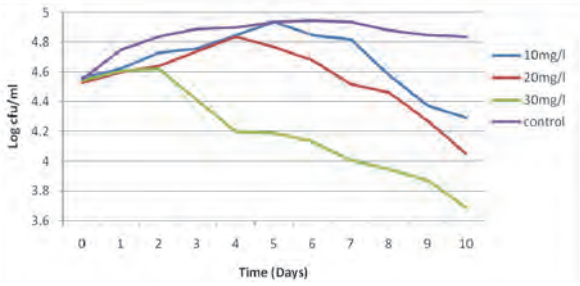
S. Typhimurium survived equally well when the irrigation water was supplemented with low concentrations of nitrate as compared to the higher nitrate concentrations. This could be due to the presence of nitrate reductase as nitrate has been known to be converted to nitrite thereby reducing the survival times at higher nitrate concentrations. Large amounts of proteins were produced at low nitrate concentrations for both *E. coli* O157:H7 and *S.* Typhimurium. This could be attributed to the presence of nitrate transport proteins (NarK and NarU), which are responsible for the transportation of nitrate throughout the cell thereby preventing accumulation.

A



Effects of nitrate on the survival of *Salmonella enterica* serovar *enterica* Typhimurium in irrigation water

B



Effects of nitrate on the survival of *E. coli* O157:H7 in irrigation water

Figure 20 Effect of nitrate on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

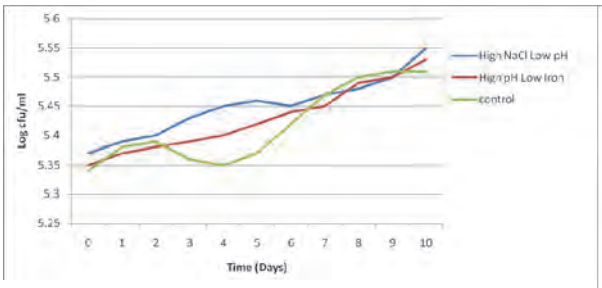
Effect of the combination of pH, NaCl and Iron on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water

The combination of a high salt concentration (5%) and low pH (pH 4) produced a synergistic effect on the survival of both organisms, as compared to the individual parameters. It was found that both *E. coli* O157:H7 and *S.* Typhimurium survived and increased in growth throughout the study period of 10 days (Fig. 21A and B). This effect may occur as a result of the high salt concentration which enhances recovery and growth of the acid stressed cells. NaCl is known to reverse the inhibitory effects of acid and enables growth under low pH

conditions. It has been reported by Jordan *et al*. (2002) that the addition of NaCl results in an improved recovery of stressed cells. This correlates with the findings of Casey and Codon (2002) who found that NaCl decreases the bactericidal effects of low pH.

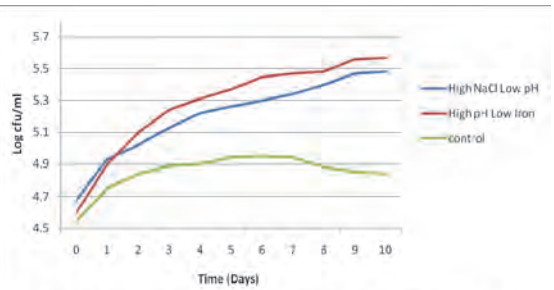
The second combination of physicochemical factors tested was high pH (pH 9) and low iron concentration (10 mg/ℓ). The results demonstrated that *E. coli* O157:H7 survived well under these conditions as compared to the individual conditions (Fig. 5A and B). It has been reported that the period of survival increased with an increased pH and added iron concentrations (Patel *et al.,* 1995). This study also showed that *E. coli* cells survived at pH 9 for up to 11 days but when iron was added, survival increased up to day 30. Like many other organisms, *E. coli* O157:H7 and *S.* Typhimurium produce siderophores, which serve to solubilize iron and transport it to the cell under conditions of limited iron availability thus allowing an increase in cell growth.

A



Effects of combination factors on the survival of *Salmonella enterica* serovar *enterica* Typhimurium in irrigation water

B



Effects of combination factors on the survival of *E. coli* O157:H7 in irrigation water

Figure 21 Effect of the combination of pH, NaCl and Iron on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water.

3.2.2 THE EFFECT OF IRRIGATION METHODS (DRIP, SPRINKLER AND FLOOD) ON THE SURFACE CONTAMINATION OF FRESH PRODUCE

Specific aim

To investigate if the irrigation method used contributes to the contamination of lettuce and tomatoes with pathogenic microorganisms implicated in foodborne disease outbreaks.

Materials and methods

Cultures

American Type Culture Collection (ATTC; Manassas, VA, USA) cultures *Salmonella* Typhimurium (ATCC 14028) and *Listeria monocytogenes* (ATCC 19115) were used in this study. All cultures were maintained as lyophilised stocks and stored at -70° C with subcultures prepared 24 hours prior to use on Standard 1 medium. A single colony of each respective bacterium was subsequently inoculated into sterile distilled H_2O and shake incubated at 200 rpm at 37° C for 18 hours to obtain a final concentration of 10^8 CFU/mℓ. The concentration was confirmed by dilution plating onto selective agar Xylose lysine deoxycholate agar for S. Typhimurium Listeria agar for *L. monocytogenes* (both from Oxoid).

Sterile distilled water containing full strength nutrient solution and the respective bacterial inoculum (10^5 CFU/mℓ) was used to water the experimental lettuce and tomato plants weekly for three weeks.

Sampling

Three replicates were sampled at each time point. Three twenty-five grams sub-samples of each replicate were weighed into a sterile polyethylene bag containing 225 mℓ of 0.1% peptone buffered water (BPW) (Merck, Darmstadt, Germany) and macerated for 5 min at 230 rev/min in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK).

Microbiological analysis

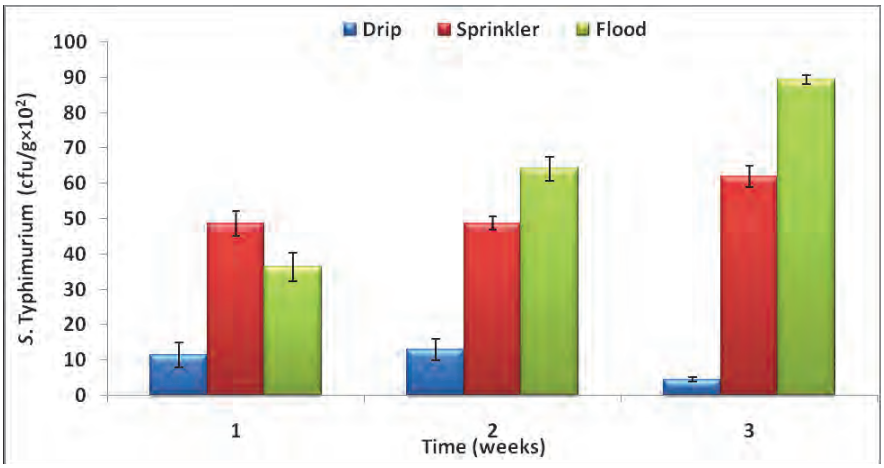
Each macerated lettuce and tomato sample was serially diluted and plated onto the appropriate selective media for enumeration purposes. The data was transformed to log CFU/g for the lettuce and tomato samples respectively.

Results, Discussion and Conclusions

The effect of irrigation methods on the surface contamination of lettuce leaves

The population of *S.* Typhimurium and *L. monocytogenes* on the surface of lettuce leaves irrigated via different irrigation methods over a period of three weeks are shown in Figures 22A and B, respectively. Flood irrigated lettuce leaves showed the highest levels of contamination with a maximum of 3.95 log CFU/g for *S.* Typhimurium and 4.33 log CFU/g for *L. monocytogenes* obtained by the third week. This was followed by sprinkler irrigation that resulted in a contamination level of 3.79 log CFU/g for *S.* Typhimurium and 4.04 log CFU/g for *L. monocytogenes* on lettuce leaves. On the other hand, drip irrigated lettuce leaves showed a minimal amount of contamination, resulting in >19-fold reduction for *S.* Typhimurium and >53-fold reduction of *L. monocytogenes* as compared to flood irrigated lettuce leaves. The population of *L. monocytogenes* was two-fold greater than that observed for *S.* Typhimurium on lettuce leaves (Fig. 22A and B).

A



B

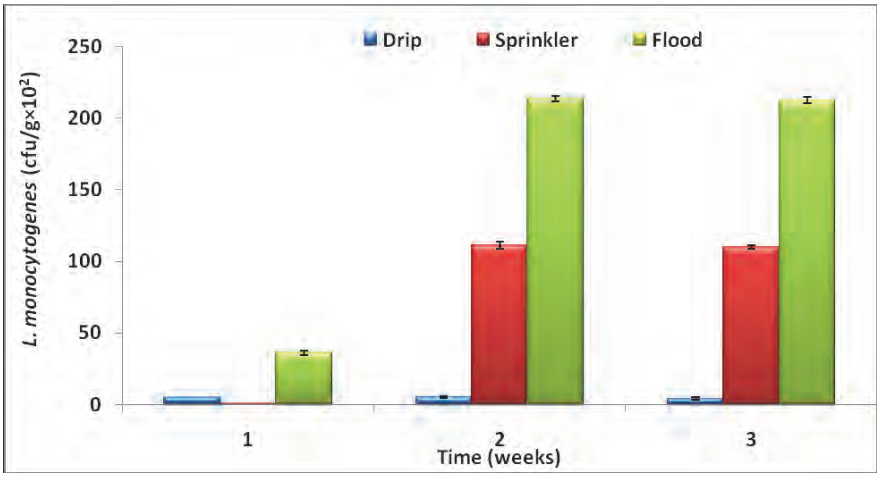
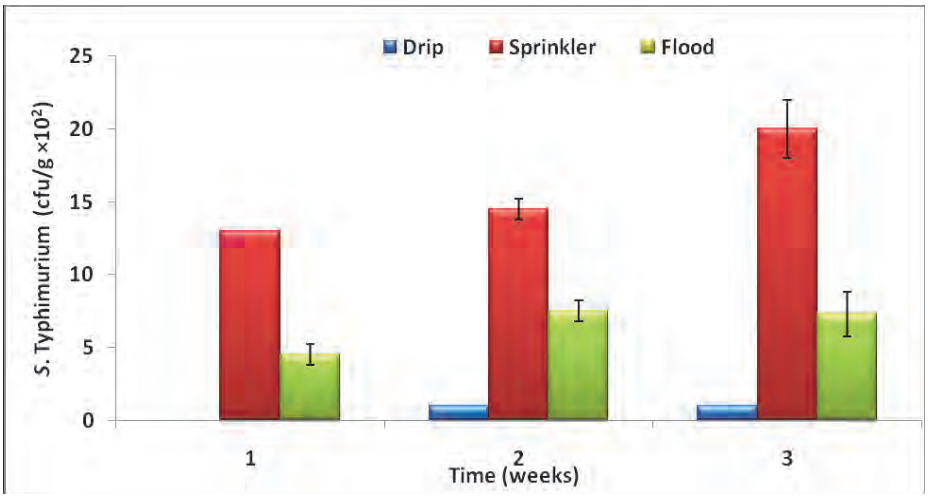


Figure 22 The population of *S.* Typhimurium (A) and *L. monocytogenes* (B) on the surface of lettuce leaves irrigated via different irrigation methods.

The effect of irrigation methods on the surface contamination of tomatoes

The degree of contamination by *S.* Typhimurium (Fig. 23A) and *L. monocytogenes* (Fig. 23B) on the surface of tomatoes irrigated via different irrigation methods was assessed over the three week duration. From the results obtained, the highest numbers of *S.* Typhimurium (3.16 log CFU/g) and *L. monocytogenes* (3.21 log CFU/g) were observed on sprinkler irrigated tomatoes; followed by flood irrigated tomatoes with a contamination level of 2.87 log CFU/g of *S.* Typhimurium and 2.17 log CFU/g of *L. monocytogenes.* Drip irrigation caused minimal contamination of tomatoes and resulted in >14-fold reduction of *S.* Typhimurium compared to sprinkler irrigation, with no contamination by *L. monocytogenes* observed in weeks one and two, and minimal contamination of 2 log CFU/g at week three (Fig. 23B).

A



B

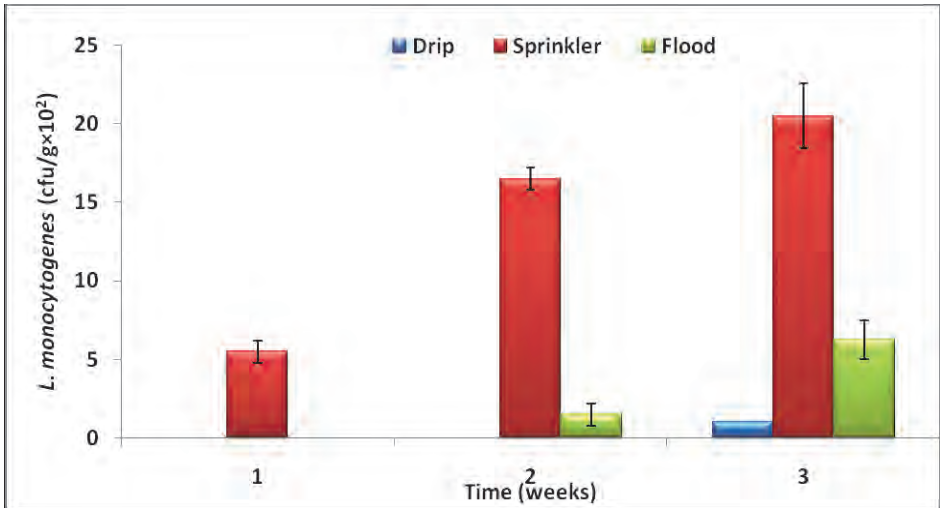


Figure 23 The population of *S*. Typhimurium (A) and *L. monocytogenes* (B) on the surface of tomatoes irrigated via different irrigation methods.

Conclusion

A range of irrigation methods such as drip, flood, sprinkler and sub-irrigation can be utilized for cultivating fresh produce and the choice of irrigation method plays a pivotal role in the transference of contaminants to fresh produce (Ibekwe *et al*., 2004; Stine *et al.*, 2005; Wachtel *et al.,* 2002). Drip or surface irrigation can minimize contact of crops with contaminants present in irrigation water, compared with sprinkler irrigation, because the edible portions of plants are not in direct contact with water (Steele and Odumeru, 2004). *E. coli* O157:H7 has been found in dust, suggesting that furrow and drip irrigation water contaminated with pathogens could have the potential for contaminating the crops through dust or mud dispersal in the field (Cooley *et al*., 2007). Previous reports suggested that irrigation methods that do not allow for direct contact of the contaminated water with the edible regions of the plant facilitate a lower risk of contamination (Fonseca *et al.,* 2011). This is evident in the results of the current study. Drip irrigation allows for minimal contact between contaminated irrigation water and edible regions of the plant and is also water efficient. On the other hand, flood irrigation of lettuce plants are more susceptible to surface contamination via irrigation because they are low-lying plants and have a large surface area and deep crevices. Therefore,

irrigation water quality should not be compromised and more stringent post-harvest treatments of fresh produce should be implemented. This will enable farmers to apply good agricultural practices to considerably reduce fresh produce contamination, thus protecting the consumers by minimizing the risk of foodborne illness outbreaks.

3.2.3 INTERNALISATION POTENTIAL OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, *SALMONELLA ENTERICA* SUBSP. ENTERICA SEROVAR TYPHIMURIUM AND *STAPHYLOCOCCUS AUREUS* IN LETTUCE SEEDLINGS AND MATURE PLANTS

Standing T-A, Du Plessis EM, Duvenage S and Korsten L (2013) Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. *Journal of Water and Health*, Volume 11.2, page 210-223 (doi: 10.2166/wh.2013.164)

Abstract

The internalisation potential of Listeria monocytogenes, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium in lettuce was evaluated using seedlings grown in vermiculite in seedling trays as well as hydroponically grown lettuce. Sterile distilled water was spiked with either one of the four human pathogenic bacteria (105 CFU/mL) and used to irrigate the plants. The potential for pathogen internalisation was investigated over time using light microscopy, transmission electron microscopy and viable plate counts. Additionally, the identities of the pathogens isolated from internal lettuce plant tissues were confirmed using PCR with pathogen-specific oligonucleotides. Internalisation of each of the human pathogens was evident in both lettuce seedlings and hydroponically grown mature lettuce plants. To our knowledge, this is the first report of *S. aureus* internalisation in lettuce plants. In addition, the levels of background microflora in the lettuce plants were determined by plate counting and the isolates identified using matrix-assisted laser ionisation-time of flight (MALDI-TOF). Background microflora assessments confirmed the absence of the four pathogens evaluated in this study. A low titre of previously described endophytes and soil inhabitants, i.e. *Enterobacter c*loacae, *Enterococcus faecalis*, *Lysinibacillus fusiformis*, *Rhodococcus rhodochrou*s, *Staphylococcus epidermidis* and *Staphylococcus hominis* were identified.

3.2.4 GROWTH DYNAMICS OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SEROVAR TYPHIMURIUM AND *STAPHYLOCOCCUS AUREUS* UNDER DIFFERENT NUTRIENT AND TEMPERATURE CONDITIONS

Specific aim

The aim of this study was to determine how temperature and nutrient fluctuating conditions affect the growth of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*

Typhimurium and *Staphylococcus aureus*, and to determine if these pathogens are able to survive and potentially grow on peaches and plums during simulated local transit conditions.

Materials and Methods

Cultures

American Type Culture Collection cultures namely *E. coli* O157:H7 (ATCC 35150), *L. monocytogenes* (ATCC 19115), *S. enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028) and *S. aureus* (ATCC 12600) were used as reference cultures in this study. All cultures were maintained lyophilized and stored at -70^{o}C with subcultures on Standard 1 medium (Merck, Johannesburg, South Africa) prepared 24 hours prior to use. Cultures were used to inoculate five replicates of 100 mℓ Tryptone Soy Broth (Merck) for each pathogen and were subsequently incubated at 37^{o}C for 18 hours to achieve a concentration of 8 log CFU/mℓ. Cultures were centrifuged at 5000 r.p.m. and washed twice with sterile distilled water and finally re-suspended into 1% (w/v) Peptone Buffered Water (Merck). Cultures were also serially diluted to obtain an inoculum concentration of 7 log for fruit inoculation to achieve a concentration of 5 log CFU/cm^{2}. Cultures were further diluted to obtain a concentration of 6 log CFU/mℓ, which was used to inoculate the nutrient-rich and -poor broths to achieve a 3 log CFU/mℓ concentration which would achieve a final concentration of 3 log CFU/mℓ. In addition, cultures that were serially diluted to 5 log CFU/mℓ were used to inoculate a 12.25cm^{2} section of a floor tile to achieve a final concentration of 3 log CFU/section to determine the survival of each pathogen on a nutrient-free surface. Concentrations were confirmed by serial dilution and subsequent plating in duplicate.

Growth of foodborne pathogens under various growth conditions

- **Nutrient-rich and nutrient-poor**

Forty-eight sterile 100 mℓ Tryptone soy broth, (consisting of 1.5 g of peptone and 0.5 g of sodium chloride per 100 mℓ) called nutrient-rich broths and 48 sterile 0.3 (w/v) Tryptone soy broth (consisting of 0.15 g of peptone and 0.05 g of sodium chloride per 100 mℓ) called nutrient-poor were both divided into four sets of twelve. Three of the twelve sets were inoculated each with 500 µl of 6 log concentration of one pathogen, resulting in three replicates with *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus*. Following inoculation the final concentration of each culture in the broth was confirmed to be 3 log CFU/mℓ. Each set was subsequently incubated at 0.5^{o}C, 4^{o}C, 21^{o}C and at fluctuating temperatures for six days, with daily agitation. Fluctuating temperatures were as follows; 21^{o}C for 24 hours (day 0 to day 1), 0.5^{o}C for 48 hours (day 1 to day 3), again at 21^{o}C for 24 hours (day 3 to day 4) and finally at 4^{o}C for 48 hours (day 4 to day 6), these fluctuating temperatures represent the local supply chain temperatures for peaches and plums. Daily, 1 mℓ of the solution was removed from all 48 broths and serially diluted with subsequent plating onto Baird-Parker medium for *S. aureus*, Oxford Listeria Selective Agar for *L. monocytogenes*, Levine Eosin-Methyl Blue Agar for *E. coli* O157:H7 and XLD Agar for *Salmonella* Typhimurium (all supplied by Merck). Following 24 hour incubation, counts were recorded and transformed to log (x+1) CFU/mℓ.

- **Fruit**

Peaches (*Prunus persica* cv. Excellence) and plums (*Prunus domestica* cv. Flavour King) were aseptically hand harvested at optimum maturity from two commercial farms in the North-West Province and Northern Province, respectively. The full experiment was repeated on two separate occasions. Fruits of a uniform size and weight and without pest, disease or damage were used in this study. Harvested fruits were bagged in paper bags and transported to the laboratory in cooler boxes and stored at 4^{o}C overnight (approx. 12 to 15 h). Collected fruits consisted of 24 peaches (five replicates for four time intervals selected and four negative controls) and 24 plums (five replicates for four time intervals and four negative controls) were used to quantify the pathogen titre following inoculation. Fruits were all washed using 0.05% (v/v) Sodium hypochlorite for 30s, rinsed twice with sterile distilled water and allowed to air dry before inoculation.

Spot inoculation, using 50µl of culture, for the quantification of pathogen titres was carried out on five fruits per time interval (0d, 1d, 4d, and 6d). Spot inoculation was carried out ensuring that cultures were not mixed. Following spot inoculation the final concentration of each culture on the fruits was confirmed to be 5 log CFU/fruit with serial dilutions as described before. The viable *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium and *S. aureus* titres remaining on these fruits following various time intervals (0d, 1d, 4d, and 6d) was determined. Fruits were incubated overnight at 21^{o}C where after fruits were transferred to -0.5^{o}C for two days to mimic the on-farm storage of fruit. Fruits were then transferred to 21^{o}C to for two days to mimic the transportation and market sale of fruit at room temperatures. Fruits were subsequently transferred to refrigeration temperature (4^{o}C) for one day to simulate the home storage conditions of fruit.

Fruits were removed from the incubation storage areas at the different time intervals (0d, 1d, 4d and 6d) and washed to determine the bacterial titre present on the fruit. Fruits were washed in 500 mℓ quarter strength Ringer's solution amended with 0.02% Tween-80 (Sigma, Johannesburg) in the Ultrasonic Bath (Labotec, Johannesburg) for 30s. The Ringer's solution was subsequently filtered through a 0.45µm nitrocellulose membrane. The membrane was subsequently used for serial dilution and plating in duplicate onto selective agar specific for the four pathogens [Baird-Parker medium for *S. aureus*, Oxford Listeria Selective Agar for *L. monocytogenes*, Levine Eosin-Methyl Blue Agar for *E. coli* O157:H7 and XLD Agar for *Salmonella* Typhimurium] was performed and counts recorded. Volume displaced (vd) for each fruit was recorded and converted to cm^2 using the following equation: $A=4.84[(vd)^{1/3}]^2$ (De Jager, 1999*)*. Counts were converted to log CFU/cm^2 and transformed to log (x+1) CFU/cm^2.

- **Nutrient-free floor tiles**

Two vinyl composition tiles were cut into 3.5 x 3.5 cm blocks. All blocks were dip sterilised in 70% ethanol for 1 min. and subsequently allowed to air dry in the laminar flow cabinet. Sixty-three of these tile section blocks were subsequently spot inoculated with 50µl of 5 log

CFU/mℓ of each culture. Spot inoculation was carried out to ensure that cultures did not mix. Following spot inoculation with *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* resulted in a final concentration of 3 log CFU/tile. The concentration of the inoculum was confirmed to be 3 log CFU/tile by serial dilution and plating. The 63 tile blocks were then divided into three sets according to temperature for incubation, placed into sterile storage containers and incubated at 0.5^{o}C, 4^{o}C and 21^{o}C mimicking the temperatures at which peaches and plums are stored, for six days. Three tiles per day per temperature were removed from each temperature and placed into sterile 100 mℓ containers. Five grams of sterile 0.55µm glass beads (Glass World, Johannesburg) with 1 mℓ 0.1% peptone buffered water (Merck) was added to the 12.25 cm^2 tile block and subsequently shaken at 5 kHz for 10 minutes. Subsequently 8 mℓ of 0.1% peptone buffered water was added to the block. The solution was then further diluted as necessary and plated onto selective media. Following 24 hour incubation, counts were recorded and transformed to log (x+1) CFU/tile.

- **Statistical Analysis**

All experiments were repeated. Results obtained for each repeat were analysed together, therefore 10 replicates were analysed (two repeats of five replicates). Statistical analysis was performed on log values. Data were analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary United States of America). A one-way analysis of variance was used to determine the difference in pathogen titres present on fruit surfaces. Means were analysed using the least significant difference (using the Fischer test) at a 5% level of significance.

Results

- **Growth dynamics of foodborne pathogens under fluctuating temperatures**

When growing in nutrient-rich broth under fluctuating temperature conditions, *E. coli* O157:H7 titres exhibited an overall increase in titres from day 0 to day 6 (P<0.0001) (Table 9). On day 1, 2 and 3 the *E. coli* O157:H7 titres where the highest of all six days tested when incubated in a nutrient-rich broth under fluctuating temperatures (Table 9). On the day of inoculation the *E. coli* O157:H7 titres were the lowest of all six days (Table 9). Following the initial inoculation, broths were moved to 21^{o}C therefore there was the significant increase in titre from day 0 to day 1 (P<0.0001) (Table 9). Following the constant titre levels on day 1, day 2 and day 3, there was a significant decrease in titre on day 4 (P<0.0001) (Table 9). *E. coli* O157:H7 titres recovered on day 5 to titre levels comparable to day 1 and remained constant to day 6 (P<0.0001) (Table 9).

When growing in nutrient-poor broth under fluctuating temperature conditions, *E. coli* O157:H7 titres exhibited an overall increase in titres from day 0 to day 6 (P<0.0001) (Table 9). In nutrient-poor broth, *E. coli* O157:H7 titres were at the lowest level on the day of inoculation (day 0) with a significant increase in titre on day 1 (P<0.0001) (Table 9). *Escherichia coli* O157:H7 titres on day 1, day 2, day 4, day 5 and day 6 were not significantly different (P<0.0001) (Table 9). From day 2 to day 3 there was a significant increase in *E. coli* O157:H7 titre, with a significant decrease in titre from day 3 to day 4 (P<0.0001) (Table 9).

When growing in a nutrient-rich broth under fluctuating temperatures conditions, *L. monocytogenes* titres demonstrated an overall increase from day 0 to day 6 ($P<0.0001$) (Table 10). The biggest significant increase in titre of *L. monocytogenes* was recorded after one day in the nutrient-rich broth, following the 21°C incubation ($P<0.0001$) (Table 10). The titres recorded on day 1 were maintained until day 2, where after there was a significant decrease in *L. monocytogenes* titre recorded on day 3 due to the 0.5°C incubation ($P<0.0001$) (Table 10). *Listeria monocytogenes* titres recorded on day 3 were maintained until the completion of the study on day 6, following a two day incubation at 21°C and a further day at 4°C ($P<0.0001$) (Table 10).

In the nutrient-poor broth the titres of *L. monocytogenes* fluctuated according to the temperature and availability of nutrients but there was an overall increase in titres from day 0 to day 6. On day 0, the titre of *L. monocytogenes* in the nutrient-poor broth was the lowest ($P<0.0001$) (Table 10). Following the 21°C incubation from day 0 to day 1, titres increased significantly ($P<0.0001$) (Table 10). Interestingly, following the 0.5°C incubation on day 2 and day 3, there was a significant increase in *L. monocytogenes* titres to the highest level recorded in nutrient-poor broth under fluctuating temperatures for six days ($P<0.0001$) (Table 2). Following day 3 there was a significant decrease in *L. monocytogenes* titres recorded on day 4, with a further decrease in titre on day 5 ($P<0.0001$) (Table 10). *L. monocytogenes* titres on day 5 and day 6 were not significantly different ($P<0.0001$) (Table 10).

S. Typhimurium demonstrated an overall growth from day 0 to day 6 when incubated in a nutrient-rich broth at fluctuating temperatures ($P<0.0001$) (Table 11). The lowest *Salmonella* Typhimurium titres were recorded on day 0 ($P<0.0001$) (Table 11). Following the first 24 hour incubations at 21°C there was a significant increase in *Salmonella* Typhimurium titres incubated in the nutrient-rich broth ($P>0.0001$) (Table 11). A subsequent significant increase was seen from day 1 to day 2 following one day at 0.5°C, titres recorded on day 2 were the highest recorded over the six-day period ($P<0.0001$) (Table 11). Following an additional 24 hours at 0.5°C, titres on day 3 were significantly lower than on day 2 ($P> 0.0001$) (Table 11). *L. monocytogenes* titres on day 4 were not significantly different to those on day 3 ($P<0.0001$) (Table 11). Following two days at 21°C, titres significantly increased on day 5 and these titres were maintained to day 6, after the 24 hours at 4°C ($P< 0.0001$) (Table 11).

An overall increase in *S.* Typhimurium titres was recorded from day 0 to day 6 incubated at fluctuating temperatures in a nutrient-poor broth. *S.* Typhimurium titres increased significantly within the first 24 hours following the 21°C incubation ($P<0.0001$) (Table 11). These titres remained constant until day 4 following 48 hours of 0.5°C incubation and 24 hours of 21°C incubation ($P<0.0001$) (Table 11). Titres on day 4 and day 5 were not significantly different but titres on day 5 were significantly higher than titres recorded on day 3 ($P<0.0001$) (Table 11). Following the 24 hour incubation at 4°C the *Salmonella* Typhimurium titres again significantly decreased ($P<0.0001$) (Table 11). Titres on day 1, day 2, day 3, day 4 and day 6 were not significantly different ($P<0.0001$) (Table 11).

Titres of *S. aureus* demonstrated an overall increase from day 0 to day 6 when incubated at fluctuating temperatures in a nutrient-rich broth ($P<0.0001$) (Table 12). The lowest titres for all six days were recorded on day 0 ($P<0.0001$) (Table 12). Following 24 hour incubation at 21^{o}C, *S. aureus* titres reached the highest titre recorded on all six days ($P<0.0001$) (Table 12). Titres recorded on day 1 and day 2, following day 2, remained constant until day 6 ($P<0.0001$) (Table 12).

S. aureus titres in a nutrient-poor broth incubated at fluctuating temperatures demonstrated a systematic and overall increase from day 0 to day 6 ($P<0.0001$) (Table 12). Titres on day 1 were significantly higher than on day 0 ($P<0.0001$) (Table 12). Day 1 titres were maintained until day 3 ($P<0.0001$).Titres on day 4 were significantly higher than titres on day 1, day 2 and day 3 ($P<0.0001$). Titres on day 5 and day 6 were not significantly different, but were significantly higher than titres on day 4 ($P<0.0001$) (Table 12).

Table 9 Growth dynamics of *Escherichia coli* O157:H7 under varying temperature conditions in a nutrient-rich and nutrient-poor broth and on a nutrient-free tile

Nutrient Conditions	Day	Temperature Conditions			
		Fluctuating	0.5°C	4°C	21°C
Nutrient Rich	0	4.91 **c** [w]	4.87 **ab**	4.86 **b**	4.85 **d**
	1	8.99 **a**	5.26 **a**	5.57 **a**	8.95 **bc**
	2	9.03 **a**	3.62 **c**	5.36 **a**	9.07 **abc**
	3	9.03 **a**	4.77 **b**	5.42 **a**	8.89 **c**
	4	8.79 **b**	4.45 **b**	5.15 **ab**	9.00 **abc**
	5	9.03 **a**	3.82 **c**	5.54 **a**	9.16 **a**
	6	9.03 **a**	3.84 **c**	5.51 **a**	9.10 **ab**
	LSD[x]	0.1761	0.4501	0.4675	0.1864
	***P* value**[y]	<0.0001	<0.0001	0.0587	<0.0001
Nutrient Poor	0	4.97 **d**	4.97 **ab**	4.97 **a**	4.97 **d**
	1	8.33 **c**	3.38 **c**	4.27 **b**	8.13 **c**
	2	8.40 **bc**	4.12 **b**	4.02 **bc**	8.21 **c**
	3	9.36 **a**	3.28 **cd**	3.83 **bc**	8.44 **b**
	4	8.50 **bc**	2.96 **de**	3.46 **c**	8.59 **ab**
	5	8.61 **b**	2.99 **de**	3.90 **bc**	8.63 **a**
	6	8.53 **bc**	2.77 **e**	3.85 **bc**	8.60 **a**
	LSD	0.2328	0.3267	0.6783	0.1532
	***P* value**	<0.0001	<0.0001	0.0112	<0.0001
Nutrient Free	0	ND[z]	0.90 **a**	0.90 **a**	0.90 **a**
	1	ND	0.96 **a**	0.64 **a**	0.96 **a**
	2	ND	0.96 **a**	0.64 **a**	0.00 **a**
	3	ND	0.96 **a**	0.64 **a**	0.32 **a**
	4	ND	0.64 **a**	0.00 **a**	0.32 **a**
	5	ND	0.96 **a**	0.00 **a**	0.32 **a**
	6	ND	0.32 **a**	0.00 **a**	0.00 **a**
	LSD	-	1.2493	1.3128	1.1598
	***P* value**	-	0.8818	0.5661	0.3063

[w]:Values followed by the same letter in bold type means that the two values are not significantly different according to the Fischer Test (P<0.05); [x]: LSD is the least significant difference within one temperature condition; [y]: *P* value is significant if the value is less than 0.5;[z]: ND means that the specific value is not available because it was not included in the present study.

Table 10 Growth dynamics of *Listeria monocytogenes* under varying temperature conditions in a nutrient-rich and nutrient-poor broth and on a nutrient-free tile

Nutrient Conditions	Day	Temperature Conditions			
		Fluctuating	**0.5°C**	**4°C**	**21°C**
Nutrient Rich	**0**	4.98 **c** [w]	4.95 **c**	4.99 **e**	4.93 **e**
	1	9.08 **a**	5.48 **c**	6.07 **d**	8.93 **ab**
	2	9.05 **a**	5.83 **b**	6.61 **cd**	9.19 **a**
	3	8.31 **b**	5.93 **ab**	6.39 **cd**	8.72 **bc**
	4	8.40 **b**	6.15 **ab**	7.36 **bc**	8.24 **d**
	5	8.34 **b**	6.23 **ab**	7.74 **ab**	8.44 **cd**
	6	8.25 **b**	6.33 **a**	8.54 **a**	8.23 **d**
	LSD[x]	0.3575	0.4549	1.0502	0.3492
	***P* value**[y]	<0.0001	0.0002	0.0002	<0.0001
Nutrient Poor	**0**	5.19 **e**	5.19 **b**	5.19 **e**	5.19 **b**
	1	8.48 **b**	5.28 **ab**	5.21 **e**	8.35 **a**
	2	9.11 **a**	5.23 **b**	5.62 **d**	8.29 **a**
	3	9.09 **a**	5.22 **b**	3.99 **f**	8.22 **a**
	4	7.92 **c**	5.35 **ab**	6.41 **cd**	8.38 **a**
	5	6.89 **d**	5.36 **ab**	6.69 **bc**	8.35 **a**
	6	6.90 **d**	5.49 **a**	7.15 **a**	8.28 **a**
	LSD	0.1316	0.2247	0.2127	0.1667
	***P* value**	<0.0001	<0.0001	<0.0001	<0.0001
Nutrient Free	**0**	ND[z]	2.65 **a**	2.65 **a**	2.65 **a**
	1	ND	1.77 **b**	1.93 **b**	2.14 **b**
	2	ND	1.55 **b**	0.96 **c**	2.04 **b**
	3	ND	1.27 **bc**	0.96 **c**	1.58 **c**
	4	ND	0.69 **d**	0.64 **c**	1.05 **d**
	5	ND	0.96 **cd**	0.64 **c**	1.05 **d**
	6	ND	0.96 **cd**	0.00 **d**	0.96 **d**
	LSD	-	0.5386	0.5677	0.2955
	***P* value**	-	<0.0001	<0.0001	<0.0001

[w]: Values followed by the same letter in bold type means that the two values are not significantly different according to the Fischer Test (P<0.05); [x]: LSD is the least significant difference within one temperature condition; [y]: *P* value is significant if the value is less than 0.5;[z]: ND means that the specific value is not available because it was not included in the present study.

Table 11 Growth dynamics of *Salmonella* Typhimurium under varying temperature conditions in a nutrient-rich and nutrient-poor broth and on a nutrient-free tile

Nutrient Conditions	Day	Temperature Conditions			
		Fluctuating	**0.5°C**	**4°C**	**21°C**
Nutrient Rich	**0**	5.02 **c** [w]	5.32 **a**	5.00 **c**	5.00 **c**
	1	8.74 **b**	5.26 **a**	5.61 **ab**	7.88 **b**
	2	8.93 **a**	4.88 **a**	5.90 **a**	9.12 **a**
	3	8.74 **b**	5.08 **a**	5.54 **ab**	9.02 **ab**
	4	8.74 **b**	4.93 **a**	5.31 **bc**	8.98 **ab**
	5	9.07 **a**	5.13 **a**	5.69 **ab**	9.09 **ab**
	6	9.04 **a**	5.09 **a**	5.86 **a**	9.16 **a**
	LSD[x]	0.1788	1.0013	0.4284	1.2200
	***P* value**[y]	<0.0001	0.9525	0.0075	<0.0001
Nutrient Poor	**0**	5.02 **c**	5.02 **a**	5.02 **a**	5.02 **d**
	1	8.12 **b**	4.83 **ab**	4.61 **b**	8.01 **c**
	2	8.09 **b**	4.54 **bc**	3.78 **e**	8.12 **bc**
	3	8.13 **b**	4.39 **cd**	4.48 **bc**	8.25 **ab**
	4	8.23 **ab**	4.11 **de**	4.21 **c**	8.37 **a**
	5	8.31 **a**	4.34 **cd**	4.17 **cd**	8.27 **ab**
	6	8.12 **b**	3.97 **e**	3.84 **de**	8.19 **abc**
	LSD	0.1739	0.3721	0.3721	0.2140
	***P* value**	<0.0001	0.0005	0.0005	<0.0001
Nutrient Free	**0**	ND[z]	2.40 **a**	2.40 **a**	2.40 **a**
	1	ND	2.23 **a**	1.13 **b**	2.09 **a**
	2	ND	2.47 **a**	1.23 **b**	1.87 **a**
	3	ND	1.11 **b**	0.96 **bc**	1.20 **b**
	4	ND	1.28 **b**	1.05 **bc**	1.05 **b**
	5	ND	0.48 **b**	0.96 **bc**	1.26 **b**
	6	ND	1.28 **b**	0.64 **c**	1.15 **b**
	LSD	-	0.4206	0.4651	0.5776
	***P* value**	-	<0.0001	<0.0001	0.0011

[w]: Values followed by the same letter in bold type means that the two values are not significantly different according to the Fischer Test (P<0.05); [x]: LSD is the least significant difference within one temperature condition; [y]: *P* value is significant if the value is less than 0.5; [z]: ND means that the specific value is not available because it was not included in the present study.

Table 12 Growth dynamics of *Staphylococcus aureus* under varying temperature conditions in a nutrient-rich and nutrient-poor broth and on a nutrient-free tile

Nutrient Conditions	Day	Temperature Conditions			
		Fluctuating	0.5°C	4°C	21°C
Nutrient Rich	0	4.96 **d** [w]	4.95 **abc**	4.92 **b**	5.05 **d**
	1	8.52 **a**	5.26 **a**	5.14 **ab**	8.12 **c**
	2	7.73 **ab**	4.98 **ab**	5.93 **ab**	8.26 **c**
	3	7.29 **bc**	4.83 **bc**	5.09 **ab**	8.84 **b**
	4	6.76 **c**	4.71 **bc**	4.47 **b**	9.06 **ab**
	5	7.40 **bc**	4.73 **bc**	4.63 **b**	9.16 **a**
	6	7.05 **bc**	4.66 **c**	4.91 **b**	9.13 **ab**
	LSD[x]	0.9476	0.3093	0.9435	0.3083
	***P* value**[y]	0.0001	0.0153	0.0971	<0.0001
Nutrient Poor	0	5.01 **d**	5.01 **ab**	5.01 **a**	5.01 **d**
	1	7.14 **c**	4.91 **abc**	4.98 **ab**	7.16 **c**
	2	7.02 **c**	4.94 **abc**	4.82 **ab**	8.23 **b**
	3	6.98 **c**	5.03 **ab**	4.86 **ab**	8.59 **a**
	4	8.30 **b**	5.12 **a**	4.92 **ab**	8.75 **a**
	5	9.26 **a**	4.83 **bc**	4.66 **bc**	8.77 **a**
	6	9.38 **a**	4.74 **c**	4.45 **c**	8.74 **a**
	LSD	0.2508	0.2392	0.3360	0.2025
	***P* value**	<0.0001	0.0704	0.0379	<0.0001
Nutrient Free	0	ND [z]	3.22 **a**	3.22 **a**	3.22 **a**
	1	ND	2.86 **bc**	2.52 **b**	2.77 **b**
	2	ND	2.97 **b**	1.54 **c**	2.89 **b**
	3	ND	2.72 **cd**	1.20 **cd**	2.28 **c**
	4	ND	2.75 **cd**	1.08 **d**	2.22 **c**
	5	ND	2.74 **cd**	0.37 **e**	1.59 **d**
	6	ND	2.55 **d**	0.96 **d**	1.28 **e**
	LSD	-	0.2010	0.4420	0.2030
	***P* value**	-	0.0003	<0.0001	<0.0001

[w]: Values followed by the same letter in bold type means that the two values are not significantly different according to the Fischer Test (P<0.05); [x]: LSD is the least significant difference within one temperature condition; [y]: *P* value is significant if the value is less than 0.5;[z]: ND means that the specific value is not available because it was not included in the present study.

Growth dynamics of foodborne pathogens under constant temperatures

When *E. coli* O157:H7 were incubated at 0.5^{o}C, the highest titres of the six day trial was determined on day 0, in both the nutrient-rich and -poor broths (P<0.001) (Table 9). When incubated at 0.5^{o}C in nutrient-rich broth day 1 and day 0 titres did not differ significantly (P<0.001) (Table 9). Titres on day 0 did not differ significantly from those on days 3 or 4 (P<0.001). Days 2, 5 and 6 did not differ significantly and these titres were significantly lower than the titres on day 0 (P<0.001) (Table 9). Therefore there was an overall decrease in *E. coli* O157:H7 titres from day 0 to day 6 incubated in both a nutrient-rich and -poor broth at 0.5 ^{o}C (P<0.001) (Table 9). In the nutrient-poor broth, the titres of *E. coli* O157:H7 followed a decreasing trend with the highest titre determined on day 0. Following day 0 there was a significant decrease in titre to day 1 (P<0.001). Titres recorded on day 2 were significantly higher than titres on day 1 but significantly lower than titres on day 0 (P<0.001). Following day 2 titres significantly decreased on day 3 and remained unchanged until day 6 (P<0.0001) (Table 9).

When incubated at 4^{o}C, *Escherichia coli* O157:H7 was able to grow in nutrient-rich conditions but not in nutrient-poor conditions (Table 9). Titres recorded on day 1 to day 6 were not significantly different (*P=0.1864*) (Table 9). Day 0 and day 4 were not significantly different (P = 0.1864) (Table 9). But titres recorded on day 0 were significantly lower than on days 1, 2, 3, 5 and 6 (P = 0.1864) (Table 9). Conversely in nutrient-poor broth, *E. coli* O157:H7 exhibited the highest titres on day 0 (P = 0.0112) (Table 9). Titres on days 1, 2, 3, 5 and 6 significantly lower than titres on day 0 (P = 0.0112) (Table 9). Titres determined on day 4 were significantly lower than titres recorded on day 1 (*P=0.0112*) (Table 9).

As expected *E. coli* O157:H7 was able to grow in both nutrient conditions at 21^{o}C, with both nutrient conditions exhibiting the lowest titres on day 0 and the highest on day 6 (*P<0.001)* (Table 9). The only difference between nutrient conditions is that the titres reached in nutrient-rich conditions were higher than those in nutrient-poor conditions (Table 9). *E. coli* O157:H7 incubated in the nutrient-rich broth at 21^{o}C increased from day 0 to day 1, which then remained constant throughout until day 6 (*P<0.001)*. Titres of *E. coli* O157:H7 growing in nutrient-poor broth increased from day 0 to day 6. Titres recorded on day 1 and day 2 were significantly higher than titres on day 0 (P<0.001). Titres on day 3 and day 4 were significantly higher than day 1 and day 2 (P<0.001). Titres on days 4, 5 and 6 were not significantly different and were the highest recorded for *E. coli* O157:H7 incubated at 21^{o}C in a nutrient-poor broth (P<0.001) (Table 9).

L. monocytogenes titres demonstrated an overall increase at all constant temperatures but it occurred faster and remained more stable at 21^{o}C, than at 0.5^{o}C and 4^{o}C (Table 10).

During growth in the nutrient-rich broth at 0.5^{o}C, *L. monocytogenes* titres increased systematically. Titres of *L. monocytogenes* were the lowest on day 0 and day 1, with a subsequent increase on day 2 (*P=0.0002)*. Days 2, 3, 4 and 5 were not significantly different and days 3, 4, 5 and 6 were not significantly different (*P=0.0002)* (Table 10). Day 6 was however significantly higher than day 2 (*P=0.0002).* Therefore there was an overall increase

in *L. monocytogenes* titres from day 0 to day 6 in nutrient-rich broth. *L. monocytogenes* titres were maintained in nutrient-poor broth when growing at 0.5^{o}C, with some fluctuation in titres observed (P<0.0001).Days 0, 1, 2, 3, 4 and 5 were not significantly different, and days 1, 4, 5 and 6 were not significantly different (P<0.0001) (Table 10).

L. monocytogenes increased in titres at 4^{o}C both in the nutrient-rich and nutrient-poor broths (Table 10). Titres of *L. monocytogenes* were the lowest on day 0 for both nutrient conditions. Growth of *L. monocytogenes* in nutrient-rich broth increased significantly from day 0 to day 1 (P=0.0002). Titres on day 1, day 2 and 3 were not significantly different (P=0.0002) (Table 10). Titres recorded on day 2, day 3 and day 4 were not significantly different and titres recorded on day 4 and day 5 were not significantly different (P=0.0002). Titres of *L. monocytogenes* incubated at 4^{o}C on day 5 and day 6 were not significantly different (P=0.0002). An overall growth was determined for *L. monocytogenes* growing in a nutrient-rich broth from day 0 to day 6 (P=0.0002) (Table 10). Following the incubation at 4^{o}C in nutrient-poor broth an overall increase was recorded for *L. monocytogenes* from day 0 to day 6 (P>0.0001). Titres recorded on day 0 and day 1 were not significantly different (P>0.0001). Titres on day 2 were significantly higher than titres recorded on day 0 and day 1 (P>0.0001). Titres recorded on day 2 were significantly higher than titres recorded on day 3 (P>0.0001) (Table 10). On day 3 the lowest titres of *L. monocytogenes* in the nutrient-poor broth incubated at 4^{o}C were recorded (P>0.0001).On day 4 there was a significant increase in titres from day 3 but titres on day 4 were not significantly different to titres on day 5 and to day 2 (P>0.0001) (Table 10). Following day 5 there was a final increase in *L. monocytogenes* titres to the highest recorded *L. monocytogenes* titre in nutrient-poor broth over the six day period (P>0.0001) (Table 10).

At 21^{o}C, *L. monocytogenes* titres fluctuated when incubated in the nutrient-rich broth, but an overall increase was recorded (Table 10). The lowest *L. monocytogenes* titres for the six day trial were recorded at the time of inoculation and the highest titres on day 1 and day 2 (P>0.0001). On day 3 the titres were significantly lower than on day 2 but not significantly different from those on day 1 (P>0.0001). Following day 3, a significant decrease in titres was recorded on day 4 (P>0.0001). Titres of *L. monocytogenes* achieved on day 4 when incubated at 21^{o}C in a nutrient-rich broth, were maintained until day 6 (P>0.0001) (Table 10). *L. monocytogenes* titres increased in nutrient-rich broths under all constant temperature conditions, but the increase at 21^{o}C occurred faster than at 0.5^{o}C and 4^{o}C. In the nutrient-poor broth there was a significant increase in *L. monocytogenes* titres following incubation at 21^{o}C from day 0 to day 1 (P>0.0001). Following day 1 all titres were maintained until day 6 (P>0.0001) (Table 10).

S. Typhimurium titres in a nutrient-rich broth at a constant temperature of 0.5^{o}C did not significantly differ from the titres upon inoculation throughout the study (P=0.9525) (Table 11). In contrast *S.* Typhimurium titres in a nutrient-poor broth incubated at 0.5^{o}C decreased (P=0.0005) (Table 11). The highest titre in the study was determined on the day of inoculation (day 0). Titres on day 1 were not significantly different from titres on day 0 (P=0.0005*)* (Table 11). Titres recorded on day 1 and day 2 were not significantly different,

but titres recorded on day 2 were significantly lower than titres on day 0 (P=0.0005). Titres recorded on day 2, day 3 and day 5 were not significantly different (P=0.0005) (Table 11). *L. monocytogenes* titre recorded day 4 and day 6 were not significantly different (P<0.0001. There was an overall decrease in *S.* Typhimurium titres observed when incubated at 0.5^{o}C in a nutrient-poor broth from day 0 to day 6 (Table 11).

S. Typhimurium was observed to grow when incubated in a nutrient-rich broth at 4^{o}C, the lowest titre recorded was on day 0 (Table 11). Titres recorded on day 1 were significantly higher than titres on day 0, titres achieved on day 1 were maintained until day 6 with slight fluctuations (P=0.0075. Titres recorded on days 1, 2, 3, 5 and 6 were not significantly different and titres recorded on days 1, 3, 4 and 5 were not significantly different (P=0.0075). In a nutrient-poor broth, *S.* Typhimurium demonstrated an overall decrease in titre from day 0 to day 6. Day 0 exhibited the highest *S.* Typhimurium titres in the study and the *S.* Typhimurium titres on day 1 were significantly lower than day 0 (P=0.0005). Titres recorded on day 1 and day 3 were not significantly different and titres recorded on days 3, 4 and 5 were not significantly different (P=0.0005. Titres recorded on day 5 and day 6 were not significantly different (P=0.0005. Titres recorded for *S.* Typhimurium on day 6 was not significantly different from day 2 (P=0.0005). Titres recorded on day 2 and 6 were the lowest titres of all six days (P=0.0005) (Table 11).

S. Typhimurium in a nutrient-rich and nutrient-poor broth is able to increase systematically over a period of 6 days at 21^{o}C (Table11). In the nutrient-rich broth the highest titres are achieved following 2 days, where in a nutrient-poor broth the highest titres are achieved following 3 days. *S.* Typhimurium titres in a nutrient-rich broth incubated at 21^{o}C were the lowest on the day of inoculation (P<0.0001). Titres on day 1 were significantly higher than titres on day 0 (P<0.0001). Titres again significantly increased from day 1 to day 2 (P<0.0001. Titres recorded on day 1 and day 2 were not significantly different to titres recorded on days 3, 4 and 5 (P<0.0001) (Table 11). Titres recorded on day 6 were not significantly different to titres recorded on days 2, 3, 4 and 5 (P<0.0001). *S.* Typhimurium exhibited an overall growth in nutrient-rich broth at 21^{o}C. Similarly, there was an overall growth pattern at 21^{o}C in a nutrient-poor broth (P<0.0001). Titres on day 0 were the lowest *S.* Typhimurium titres in the six day trial (P<0.0001). Titres on day 1 were significantly higher than titres recorded on day 0 (P<0.0001). Recorded titres of *S.* Typhimurium on day 1, day 2 and day 6 were not significantly different (P<0.0001). Titres on days 3, 4, 5 and 6 were not significantly different and were the highest recorded titres of *S.* Typhimurium during the six day trial (P<0.0001). Titres on days 3, 5 and 6 were not significantly different (P<0.0001) (Table 11).

S. aureus titres remained relatively constant when incubated in a nutrient-rich broth at 0.5^{o}C, with titres recorded on day 0 and day 6 not significantly different (P=0.0153) (Table12). Titres recorded on days 1, 2 and 3 were not significantly different and were the highest recorded for *S. aureus* titres when incubated at 0.5^{o}C in nutrient-rich broth (P=0.0153*)* (Table 12). Titres recorded on days 1, 3, 4 and 5 were not significantly different and titres on days 3, 4, 5 and 6 were not significantly different (P=0.0153) (Table 12). Similarly in a nutrient-poor

broth the titres remained relatively constant but there was an overall significant decrease in *S. aureus* titres (*P*=0.0704) (Table 12). Titres recorded on days 0, 1, 2, 3 and 4 were not significantly different and were the highest recorded titres for *S. aureus* incubated at 0.5^{o}C in a nutrient-poor broth (*P*=0.0704). Titres recorded on days 0, 1, 2, 3 and 5 were not significantly different and titres recorded on days 1, 2, 5 and 6 were not significantly different (*P*=0.0704) (Table 12). Titres recorded on day 0 and day 6 were significantly different and therefore there was an overall decrease in *S. aureus* titres when incubated at 0.5^{o}C in a nutrient-poor broth.

When incubated at 4^{o}C in nutrient-rich broth, *S. aureus* titres did not increase or decrease overall (*P*=0.0971) (Table 12). Titres on days 0, 1, 3, 4, 5 and 6 were not significantly different and titres on days 1, 2 and 3 were not significantly different (*P*=0.0971) (Table 12). When *S. aureus* is incubated in a nutrient-poor broth at 4^{o}C there was an overall and systematic decrease in titres (*P*=0.0379) (Table 12). Titres on days 0, 1, 2, 3 and 4 were not significantly different but titres on days 1, 2, 3, 4, 5 and 6 were not significantly different and titres recorded on days 5 and 6 were not significantly different (*P*=0.0379) (Table 12). Titres on day 6 were however significantly lower than titres on days 0, 1, 2, 3 and 4, therefore demonstrating the evident decrease in titres when incubated in nutrient-poor broth at 4^{o}C (*P*=0.0379) (Table 12).

At 21^{o}C, *S. aureus* titres both in the nutrient-rich and poor broth all increased systematically; although in the nutrient-poor broth the increase to the highest titres took more time. In the nutrient-rich broth the lowest titre recorded was on day 0. Titres recorded on day 1 were significantly higher than titres on day 0 (*P*<0.0001) (Table 12). Titres recorded on day 1 were maintained until day 2 (*P*<0.0001). Titres on day 3 were significantly higher than titres on day 2 (*P*<0.0001). Titres recorded on day 3 were not significantly different from titres recorded on day 4 or day 6 (*P*<0.0001) (Table 12). Titres recorded on day 4 were maintained to the completion of the study on day 6 (*P*<0.0001). In the nutrient-poor broth, *S. aureus* titres increased significantly from day 0 to day 1 (*P*<0.0001). Titres again increased from day 1 to day 2 and then titres increased again from day 2 to day 3 (*P*<0.0001). *Staphylococcus aureus* titres recorded on day 3 were maintained until day 6 (*P*<0.0001). Titres of *S. aureus* on days 3, 4, 5 and 6 were the highest recorded of all six days (*P*<0.0001) (Table 12).

- **Growth dynamics of foodborne pathogens on tiles at constant temperatures**

Titres of *E. coli* O157:H7 incubated on tiles at 0.5^{o}C, 4^{o}C and 21^{o}C did not demonstrate a significant increase or decrease (*P*=0.8818, *P*=0.5661, *P*=0.3063, respectively) (Table 9). Titres of *E. coli* O157:H7, even though there was no significant difference from the day of inoculation, were not detected from day 4 at 4^{o}C, nor on day 6 at 21^{o}C but were detected from tiles on day 6 at 0.5^{o}C (*P*=0.8818, *P*=0.5661, *P*=0.3063, respectively) (Table 9).

At 0.5^{o}C, *L. monocytogenes* titres recorded were the highest on day 0 and then titres significantly decreased on day 1 (*P*<0.0001) (Table 10). *Listeria monocytogenes* titres were not significantly different on day 1, day 2 and day 3 (*P*<0.0001) (Table 10). *Listeria*

monocytogenes titres recorded on day 4 were significantly lower than titres on days 1, 2 and 3 ($P<0.0001$). The titre observed on day 4 remained constant to the completion of the study ($P<0.0001$). *L. monocytogenes* titres, when incubated at 0.5°C, were not significantly different on days 3, 5 and 6 ($P<0.0001$) (Table 10). When incubated at 4°C, the *L. monocytogenes* titres decreased systematically with day 0 having the highest titre followed by day 1 ($P<0.0001$). Titres on day 2 were significantly higher than titres on day 1 ($P<0.0001$) (Table 10). Titres recorded on day 2 were not significantly different from days 3, 4 and 5 ($P<0.0001$) (Table 10). Titres recorded on day 6 were the lowest for all six days of the experiment ($P<0.0001$) (Table 10). The titre of *L. monocytogenes* when incubated at 21°C also systematically decreased throughout the 6 day study ($P<0.0001$) (Table 10). Tiles with *L. monocytogenes* incubated at 21°C demonstrated highest titre at the time of inoculation ($P<0.0001$) (Table 10). Day 0 was significantly higher than day 1 and day 1 was not significantly different from day 2 ($P<0.0001$) (Table 10). Titres determined on day 3 were significantly less than titres on day 1 and day 2 ($P<0.0001$) (Table 10). Titres recorded on days 4, 5 and 6 were significantly less than on day 3 ($P<0.0001$). *L. monocytogenes* survived on the nutrient-free tiles at 0.5°C and 21°C, but not at 4°C ($P<0.0001$) (Table 10).

S. Typhimurium titres decreased when incubated at all three temperatures on the tile surface but were detected on day 6 at all three temperatures, therefore demonstrating the ability of the organism to survive even with the lack of nutrients (Table 11). Titres on day 0 were the highest for all three temperatures (Table 11). *S.* Typhimurium titres were maintained at the same level as day 0 until day 2 when incubated at 0.5°C ($P<0.0001$) or 21 °C ($P=0.0011$) (Table 11). Titres on day 3 were significantly lower than titres on day 2 ($P<0.0001$) (Table 3). *S.* Typhimurium titres on day 3 were maintained until day 6 when incubated at 0.5°C ($P<0.0001$) or 21°C ($P=0.0011$) (Table 11). At 4°C, *S.* Typhimurium titres decreased significantly on day 1 when compared to day 0 ($P<0.0001$). Titres recorded on day 1 were maintained until day 5 ($P<0.0001$) (Table 11). Titres recorded on day 3 were not significantly different to days 4 and 5 ($P<0.0001$) (Table 11).

An overall decrease in *S. aureus* titres was observed on a nutrient-free tile at all three temperatures and *S. aureus* was still detected on tiles from all three temperatures on day 6 (Table 12). When incubated at 0.5°C the highest titre was recorded on day 0, followed by a significant decrease on day 1 which was maintained to day 2 ($P=0.0003$) (Table 12). Titres recorded on day 3 were significantly lower than titres on day 2 ($P=0.0003$). Titres recorded on day 3 were maintained until day 6 ($P=0.0003$). Titres on days 1, 3, 4 and 5 were also not significantly different ($P=0.0003$). Interestingly, *S. aureus* titres on tiles following incubation at 0.5°C at the end of the study were higher than those on day 6 incubated at 4°C or 21°C. A significant decrease in *S. aureus* titres were observed following day 1 of 4°C incubation. A significant decrease was recorded in titres from day 1 to day 2 ($P<0.0001$). Titres recorded on day 2 were maintained until day 3 ($P<0.0001$) (Table 12). Titres recorded for day 3 and day 4 were not significantly different. However, titres recorded on day 2 and day 4 were significantly different ($P<0.0001$) (Table 12). Titres recorded on day 5 were significantly lower than titres recorded for day 4 ($P<0.0001$). Titres recorded on day 6 were significantly higher than titres recorded on day 5 ($P<0.0001$) (Table 12). At 21°C *S. aureus* titres on the

nutrient-free tiles followed a more systematic decrease (P<0.0001) (Table 12). The highest *S. aureus* titre was recorded on day 0 (P<0.0001). Titres recorded on day 1 were significantly lower than titres recorded on day 0 (P<0.0001). Titres recorded on day 1 were maintained until day 2 (P<0.0001) (Table 12). Titres recorded on day 3 were significantly lower than titres on day 2 and these titres were maintained until day 4 (P<0.0001). Titres recorded on day 5 were significantly lower than titres on day 4 (P<0.0001). Titres recorded on day 6 were significantly lower than titres on day 5 (P<0.0001) (Table 12).

- **Growth dynamics of foodborne pathogens on fruit at fluctuating temperatures**

All pathogens were able to survive on peaches and plums from day 0 to day 6 under fluctuating temperature regimes (Table 13).

No significant difference in *S. aureus* titres was observed on peaches (P=0.3999), neither was there a significant difference in *E. coli* O157:H7 titres (P=0.0063) on peaches, except following storage at 0.5^{o}C where titres decreased significantly (Table 13). *L. monocytogenes* had the highest titres on peaches on day 0. Titres of *L. monocytogenes* only decreased significantly after day 4 and remained constant for the duration of the study *(*P=0.0441) (Table 13). *S.* Typhimurium titres on peaches were significantly lower for day 0 and 1 until after -0.5^{o}C and 21^{o}C storage where titres were seen to increase and were then maintained at the same level as day 0, day 1 and day 4 *(*P=0.0273) (Table 13).

E. coli O157:H7 titres on plums were the highest on day 0, followed by a significant decrease on day 1, followed by a further significant decrease on day 4 after which titres remained constant (Table 13). *S. aureus* (P=0.0008) and *S.* Typhimurium (P<0.0001*)* also followed the same trend on plums with a significant decrease following day 1, with stabilisation in titres from days 4 to 6 (Table 13). *L. monocytogenes* (P<0.0001) titres increased significantly following day 0 with a significant decrease to day 4, which then remained constant until day 6 (Table 13).

Table 13 Summary of the pathogen titres on stone fruit following artificial inoculation

Fruit	Day	*Escherichia coli*	*Listeria monocytogenes*	*Salmonella* Typhimurium	*Staphylococcus aureus*
Peach Fruit	**Day 0**	3.21 **a** [x]	3.74 **a**	1.43 **b**	4.55 **a**
	Day 1	2.02 **b**	3.16 **ab**	0.88 **b**	4.19 **a**
	Day 4	4.12 **a**	2.83 **b**	2.74 **a**	4.15 **a**
	Day 6	3.43 **a**	3.48 **ab**	1.59 **ab**	4.38 **a**
	LSD[y]	1.1271	0.6506	1.1989	0.5314
	***P* value**[z]	0.0063	0.0441	0.0273	0.3999
Plum Fruit	**Day 0**	2.88 **a**	3.23 **b**	2.40 **a**	4.73 **a**
	Day 1	2.01 **b**	4.19 **a**	2.81 **a**	4.97 **a**
	Day 4	0.11 **c**	0.80 **c**	0.38 **b**	3.90 **b**
	Day 6	0.39 **c**	1.18 **c**	0.53 **b**	3.87 **b**
	LSD	0.8264	0.7922	<0.0001	0.5999
	***P* value**	<0.0001	<0.0001	0.7248	0.0008

[x]: Values followed by the same letter in bold type means that the two values are not significantly different according to the Fischer Test (P<0.05); [y]: LSD is the least significant difference within one temperature condition; [z]: *P* value is significant if the value is less than 0.5.

Discussion and conclusion

All four pathogens exhibited an overall increase in titres when grown in a nutrient-rich or poor broth at fluctuating temperatures. Therefore it can be concluded that available nutrients do not play a role in the growth of these pathogens under such temperature conditions. However, on peaches the titre of all four pathogens remained constant compared to the initial viable concentration, while an overall decrease was noted on plums. Temperature alone is therefore not a limiting factor in the growth of these organisms. Other factors that can influence the growth of these four pathogens is the pH and water activity of the substrate (Adams and Moss, 2000). Plums are more acidic (pH 2.8-3.0) than peaches (pH 3.4 to 4.1). *E. coli* O157:H7 has different behaviour patterns depending on the temperature, pH and water activity (Rocelle *et al.,* 1996). Rocelle *et al.* (1996) determined that at pH 4.8, *E. coli* O157:H7 was not able to grow as vigorously as at pH 5.4 and 6.0 at 4, 20 and 30^{o}C. Therefore with an increase in acidity, *E. coli* O157:H7's growth decreases. Audia *et al.* (2001) reported however that *E. coli* O157:H7 was able to tolerate pHs as low as 2. The authors also demonstrated that water activity only had a major effect on the growth of *E. coli* O157:H7 at 30^{o}C (Rocelle *et al.,* 1996), higher temperatures than were used in this study. The pH of the substrate (plums and peaches) therefore plays an important role in the growth of *E. coli* O157:H7. Tienungoon *et al.* (2000) determined that *L. monocytogenes* is able to grow at a pH of 6 at 5 and 20^{o}C but no growth was recorded when the pH was decreased to 4, similarly in this study there was no growth on peaches (pH 3.4 to 4.1) and a decrease in titre on plums (pH 2.8-3.0) due to the different pH levels. Again for *S.* Typhimurium incubated under fluctuating temperatures, this was not the limiting factor as growth was observed in the broths but not on the peaches or plums. Gordon and Small (1993) demonstrated that *S.* Typhimurium

was unable to survive below a pH of 3. Waterman and Small (1998) demonstrated that the acidity of a substance is a critical factor in determining the survival of enteric pathogens. *Staphylococcus aureus* was unable to grow or survive at a pH of 3 at 5 or 20^{o}C for longer than one day and at a pH of 4, *S. aureus* was only able to survive for approximately 2.5 days (Whiting *et al*., 1996). For all four foodborne pathogens the acidity of the peaches and plums was the limiting factor of growth because at a pH of 7 all four pathogens grew under both nutrient conditions.

According to the authors' knowledge, this is the first study determining the growth of *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* at 0.5^{o}C in different media/environments. It was found that *L. monocytogenes* was the only one of the four foodborne pathogens tested able to grow at 0.5^{o}C. *L. monocytogenes*, a psychrotroph, is able to grow at 2^{o}C and can survive freezing conditions (-18^{o}C). This study demonstrates that *L. monocytogenes* is able to grow at 0.5^{o}C irrespective of the amount of nutrients present when at a neutral pH and optimum water activity. When no nutrients or water are present, such as on a floor tile environment, *L. monocytogenes* is not able to grow at these low temperatures and the combined effect of all three limiting factors (temperature, nutrient content and water activity) play a synergistic role in suppressing growth and causing a decrease in titres. *S.* Typhimurium and *S. aureus* only exhibited an overall decrease in titres when the nutrients were not present at a high concentration. When nutrients were limited or not present there was an overall decrease in titre when incubated at 0.5^{o}C. Neither *S. aureus* nor *S.* Typhimurium are psychrotrophic organisms. Therefore when nutrients, pH and water activity are optimum *S.* Typhimurium and *S. aureus* are able to survive without decrease at 0.5^{o}C, but if the nutrients are lowered then there is a decrease in pathogen titre. *E. coli* O157:H7 titres decrease at 0.5^{o}C irrelevant of the nutrient concentration.

Rocelle *et al.* (1996) demonstrated that *E. coli* O157:H7 was not able to substantially grow when incubated for two days at 5^{o}C. In the present study this was the case for the nutrient-poor broth but there was a 1 log increase in titre following two days incubation at 4^{o}C in a nutrient-rich broth. Overall *E. coli* O157:H7 and *S.* Typhimurium incubated at 4^{o}C showed a general increase in growth in a nutrient-rich broth and a decrease in a nutrient-poor broth, therefore the combination of the low temperature and the reduced nutrients synergistically limit the growth of *E. coli* O157:H7 and *S.* Typhimurium. Some *Salmonella* strains have been shown to grow at 4^{o}C, however growth is slower than at an optimum temperature (Russell and Gould, 2002 as cited by Russell, 2002). Mattick *et al.* (2003) also demonstrated that *S.* Typhimurium is able to grow at 8^{o}C. Pintar *et al.* (2007) however demonstrated that *S.* Typhimurium was unable to grow, but sustained the original titre, on chicken breast with the addition of a nutrient broth. As with incubation at 0.5^{o}C, *L. monocytogenes* is able to grow when in a nutrient-rich broth but also in a nutrient-poor broth. Tienungoon *et al.* (2000) demonstrated that *L. monocytogenes* was able to grow at 4^{o}C at a pH of 6. *L. monocytogenes* is not limited by nutrients at 4^{o}C because the organism can grow in a range from 2^{o}C. Some strains of *S. aureus* have also been recorded to grow at refrigeration temperatures (Russell and Gould, 2002 as cited by Russell, 2002). In this study *S. aureus* was unable to grow in the nutrient-rich broth and titres decreased with limited nutrients. Whiting *et al.* (1996)

demonstrated that *S. aureus* had a D_4 of 2500 hours, therefore following approximately 100 days, 10% of the initial inoculum was still present when incubated at 4°C. As expected all four pathogens were able to grow at 21°C in a nutrient-rich or -poor broth, irrespective of the level of available nutrients. The findings of this study are in agreement with previous studies on the growth of *E. coli* O157:H7 (Rocelle *et al*., 1996), *L. monocytogenes* (Tienungoon *et al.*, 2000), *S.* Typhimurium (D'Aoust, 1991) and *S. aureus* (Whiting *et al.*, 1996) at an optimum temperature of 21°C when nutrients are available.

Even though these pathogens were able to grow in liquid broth containing nutrients within their optimum range of temperature, these organisms were not able to grow on a nutrient-free tile surface. All the pathogens were able to survive on the surface with an overall decreasing trend except *E. coli* O157:H7 that was unable to be detected following six days at 4 or 21°C nor was *L. monocytogenes* detected at 4°C. Kusumaningrum *et al.* (2003) made the same observation that *S. aureus* and *Salmonella* Enteritidis exhibited a significant decrease in titres following 96 hours (or 4 days) at room temperature on a dry stainless steel surface to only just detectable when the initial inoculums was similar to this study. The same trend was observed when *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* were inoculated onto the nutrient-free tiles and stored at 0.5°C, 4°C and room temperature (21°C), indicating that the temperature was not the major factor in limiting growth but the lack of nutrients and water were synergistically limiting the growth of these pathogens. Kusumaningrum *et al.* (2003) also recorded that with the introduction of liquid food residues which resulted in an increase in nutrients and water available, the survival of the pathogens increased. It is important to note that on the tiled surface all the pathogens were able to survive following 6 days, except *E. coli* O157:H7 at 4 or 21°C and *L. monocytogenes* at 4°C. Pathogens that were able to survive could therefore lead to cross-contamination of food products stored or moving through the specific temperatures.

In conclusion, a number of factors influence the growth or survival of foodborne pathogens on contact surfaces and on food products. This study shows that if *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* contaminate peaches or plums at a high dosage, these pathogens have the ability to survive on the fruit. The decrease in titre under cold storage conditions cannot be attributed to the limiting factor of temperature only, but possibly the synergistic effect of temperature, pH and water activity which all influence the growth of these pathogens on a fruit surface. Further research should focus on determining the synergistic effect of these effects factors on the growth of foodborne pathogens under cold storage conditions. On tiles, the tested pathogens are able to survive at temperatures that simulate working environments and therefore could potentially lead to the contamination of food products coming into contact with such surfaces.

3.2.5 THE EFFECT OF CHLORINE, BLANCHING AND FREEZING ON *CRYPTOSPORIDIUM* ASSOCIATED WITH BROCCOLI

Specific aim

To investigate how processing methods (chlorine, blanching and freezing) affect *Cryptosporidium*, implicated in a number of foodborne disease outbreaks world-wide, on vegetable surfaces, i.e. broccoli.

Materials and Methods

Oocysts inoculation onto green pepper

Live *Cryptosporidium* oocysts were obtained from Waterborn Inc. (USA) $5x10^6$ oocysts in 8 mℓ PBS. Eighty micro litres of the stock inoculum were inoculated onto a 2 g green pepper piece previously washed with ethanol and placed inside a 5 mℓ sterile tube. Each inoculated piece was left to dry inside its tube at 4˚C for 1 hour before being treated. Six pieces of green pepper were inoculated for each treatment in order to have six replicates of each treatment.

Blanching treatment of oocysts

The temperature/time combination for the blanching treatment used was chosen as an average temperature and time used to blanch vegetables in industry (Puupponen-Pimia *et al*., 2003). Six tubes containing inoculated green pepper were placed in a 96˚C water bath for 3 min and then cooled in 4ºC water for 3 min.

Blast freezing treatment of oocysts

The parameters used for the freezing treatments were chosen to mimic the initial blast freezing treatment used in the industry during the processing of frozen green pepper strips. Six tubes containing inoculated green pepper were placed in a blast freezer at -20˚C for 4 min and then thawed in 4ºC water for 3 min.

Chlorine treatment of oocysts

The period of exposure used for the chlorination treatments were chosen to mimic the washing treatments used in the industry during the processing of frozen green pepper strips. The effect of chlorine at two different concentrations was studied.

All glassware used was washed in chlorine-free distilled water. Chlorine solutions, 100 ppm and 200 ppm were prepared from 3.5% hypochlorite.

1 mℓ 100 ppm chlorine was added to each of six tubes containing inoculated green pepper. One mℓ 200 ppm chlorine was added to another 6 tubes for 40 min. Subsequently, 50 µℓ of a 10% $Na_2S_2O_3$ solution was added to each tube, to neutralize the free chlorine (Korich *et al*., 1990). Complete neutralization of chlorine was tested with potassium iodide starch paper (Macherey-Nagel GmbH and Co KG, Germany).

Microwave treatment of oocysts

The microwave treatment used in this study was chosen to imitate the cooking instructions provided on the packaging of fresh vegetables in the retail market in South Africa.

Six tubes containing inoculated green pepper pieces were microwaved at 900 Watts for five minutes (Daewoo model KOR 145Q, 900 Watts, 245 MHz). The samples were then cooled down in 4ºC water for 3 minutes.

Successive treatments

- **Chlorination and blast freezing treatment of oocysts**

Six tubes containing inoculated green pepper pieces were first treated with 200ppm chlorine and then immediately frozen,

- **Chlorination and microwaving treatment of oocysts**

Six tubes containing inoculated green pepper pieces were first treated with 200ppm chlorine as described and then immediately microwaved and cooled down.

Positive control

Positive control was used to determine the PI baseline fluorescence emitted by live stained *Cryptosporidium* oocysts. Six green peppers were inoculated at the same time as the others and kept at 4˚C at all times and then recovered and stained the same way as the other samples.

Recovery of oocysts after treatments

Four millilitres buffer made of peptone buffer saline and 0.01% Tween 20 was added to each tube. The tubes were mixed using a vortex for 30 seconds to wash the oocysts off the green pepper pieces. The content of each tube was then transferred to a 5 mℓ centrifuge tube through a 35µm pore size nylon filter placed in a funnel. The tubes were centrifuged at 2500 x g for 10 min brake set at 025. The supernatant was aspirated with a micropipette to leave 1 mℓ pellet in each tube. The tubes were kept at 4˚C until staining.

Staining

Three microliters *Cryptosporidium* labelled Fluorescein-iso-thiocyanate (FITC) (Davies Diagnostic, South Africa) were added to each tube one hour prior to analysis, vortexed and kept in a cooler box.

Five minutes before flow cytometry analysis, 500 mℓ Propidium Iodide (PI) (Beckman Coulter Miami, Florida, USA) was added to each tube.

Flow cytometry analysis

The viability of oocysts after treatment was measured by determining the percentage cells positive for the nucleic acid dye propidium iodide with a flow cytometer. A strong correlation has been found between oocysts infectivity and nucleic acid staining intensity (Newmann *et al.*, 2000).

Analysis was performed on an Epic Ultra flow cytometer (Beckman Coulter, Miami Florida) equipped with water cooled enterprise laser and using the Expo 32 MultiCOMP software (Beckman Coulter). Cells were identified by FITC fluorescence and forward scatter. The percentage FITC positive cells positive for propidium iodide (PI) was determined. The positive control was used to determine the baseline PI fluorescence. Any fluorescence below the positive control percentage PI fluorescence was seen as negative.

Statistical analysis

Single factor ANOVA analyses were performed to compare groups of treatments. T-test, independent by group, was performed to compare each treatment against the control or against another treatment. Analyses were done using Statistica software for Windows Version 7 (Tulsa, Oklahoma, USA, 2003).

Frozen vegetables (e.g. broccoli) undergo various pre-processing treatments prior to freezing. Pre-processing steps include washing in chlorinated water and blanching (Fig. 24). The effect of each individual treatment involved in the manufacture of this product as well as the combined effect of the different treatments were examined. The viability of oocysts after treatment was measured by flow cytometry by staining with fluorogenic dye (Neumann *et al*., 2000). Viable *Cryptosporidium parvum* oocysts were obtained from Waterborne™, Inc. USA.

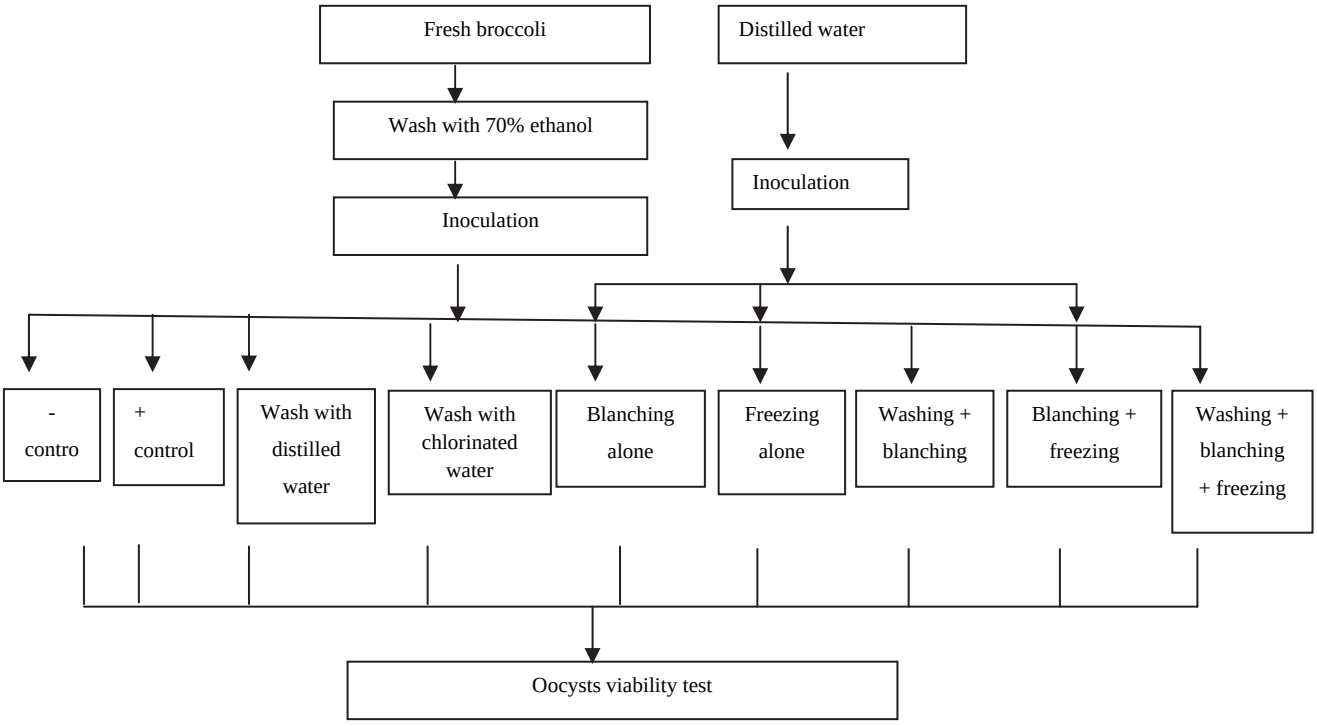


Figure 24 Vegetable processing experimental design

Results and Discussion

Of the control samples 5.7% oocysts included PI while 7% of the oocysts chlorinated with 200ppm included PI and had thus been affected by the treatment. However, this difference was not significant ($p=0.39$) (Table 14; Fig. 25). Blanching and microwaving were effective ($p<0.01$) to inactivate oocysts as 93.2% and 93.5% of oocysts included PI after each respective treatment. Both blanching and microwaving were significantly more effective

(p<0.01) than blast freezing. After blast freezing, only 20.1% of oocysts included PI. However, a significant percentage (p<0.01) of oocysts were affected by blast freezing.

Table 14 t-test p-value of each treatment compared to the control

Treatment	p-value
Chlorination with 200ppm	0.387
Blanching	<0.0001
Blast freezing	<0.0001
Microwaving	<0.0001

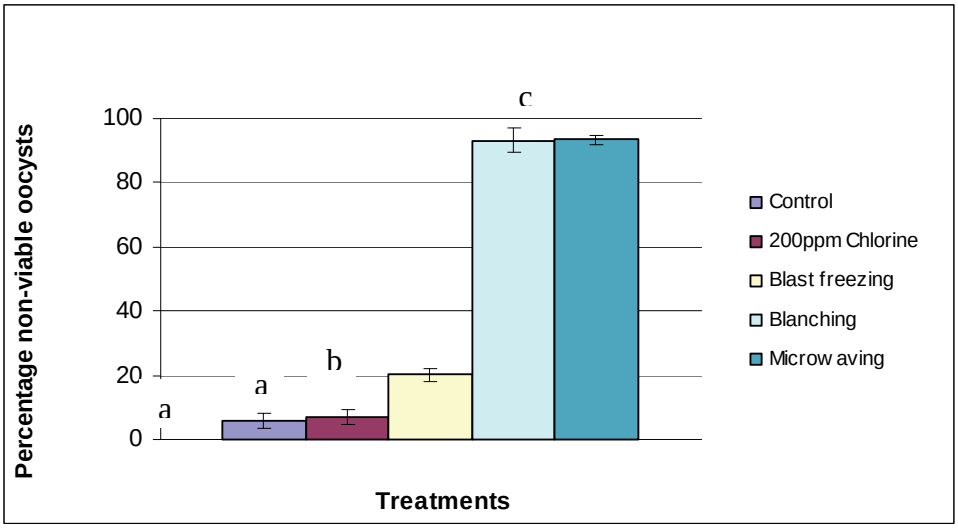


Figure 25 Percentage affected oocysts after chlorination, blanching, microwaving and blast freezing treatment. Error bars on columns represent standard deviations. Average values with different superscripts letters differ significantly (p<0.01)

(1) (2)

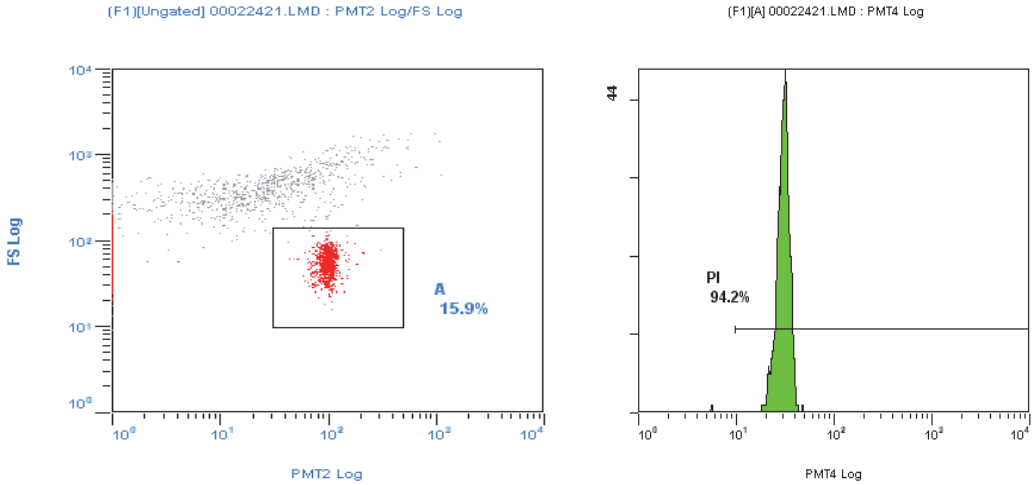


Figure 26 (1) Flow cytometric analysis of *Cryptosporidium* on bivariate dotplots of size (FS) versus green fluorescence (PMT2). The gate (A) was defined to include all FITC fluorescent cells. (2) Percentage *Cryptosporidium* oocysts positive for propidium iodide fluorescence after blanching.

As the consumption of vegetable increases so does the number of foodborne associated outbreaks. As this and previous studies have shown, reduction in oocyst load on vegetables during processing is difficult to achieve due to the protozoa's resistance to disinfectants. Only heat treatment seems to be effective at killing *Cryptosporidium* oocysts during the processing of fresh produce. It is thus very important to minimize contamination of fresh produce from the field, through processing and up to consumption to ensure safety to the consumer, especially if the produce is to be eaten raw without any subsequent heat treatment.

3.2.6 EFFECT OF CHLORINE, BLANCHING, FREEZING, AND MICROWAVE HEATING ON *CRYPTOSPORIDIUM PARVUM* VIABILITY INOCULATED ON GREEN PEPPERS

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Cryptosporidium parvum oocysts have been found on the surface of vegetables in both developed and developing countries. *C. parvum* can contaminate vegetables via various routes, including irrigation water. This study investigated the effect of individual treatments of chlorine, blanching, blast freezing, and microwave heating, as well as combined treatments of chlorine and freezing, and chlorine and microwave heating on the viability of *C. parvum* oocysts inoculated on green peppers. The viability of the oocysts after the treatments was assessed using propidium iodide and a flow cytometer. Based on the propidium iodide staining, the chlorine treatments did not affect the viability of the oocysts. Blast freezing significantly inactivated 20% of the oocysts. Microwave heating and blanching significantly inactivated 93% of oocysts. Treatment with chlorine followed by blast freezing did not affect the viability of the oocysts significantly. Treatment with chlorine and microwave heating was significantly more effective than microwave heating alone and inactivated 98% of the oocysts. The study indicates that *C. parvum* oocysts are sensitive to heat and, to some extent, to blast freezing, but are resistant to chlorine. Therefore, the use of chlorine during vegetable processing is not a critical control point for *C. parvum* oocysts, and the consumption of raw or minimally processed vegetables may constitute a health risk as *C. parvum* oocysts can still be found viable on ready-to-eat, minimally processed vegetables.

3.2.7 THE EFFECT OF MINIMAL PROCESSING AND COOKING ON THE SURVIVAL OF *LISTERIA INNOCUA* (SURROGATE FOR *LISTERIA MONOCYTOGENES*) ON SPRAY IRRIGATED BROCCOLI

Specific aim

The aim of this study was to determine the effect of minimal processing and subsequent cooking on the survival of *L. monocytogenes* on broccoli.

Experimental procedures

Growth, isolation and maintenance of *Listeria innocua* culture

Bacterial inoculum preparation

A *L. innocua* culture (ATCC 33090), LM surrogate, was obtained from Microbiologics (Minnesota, USA) in the form of a "Kwik-stick". The culture was streaked onto Tryptic Soy Agar and single colonies were isolated after 48 hours of incubation at 37°C. From this plate, a single colony was inoculated into Tryptic Soy Broth (Biolab, Merck, South Africa), which was incubated in a shaking water bath (166 rpm) at 37°C for 18 to 20 hours, during which time the culture was in a state of transition between the late logarithmic and early stationary phase of growth (Taormina and Beuchat, 2001). The bacterial cells were harvested by dispensing into 2 mℓ Eppendorf tubes, which were centrifuged for 10 min at 4000 *g* (6600 rpm, RT150, Brake: 30) (Digicen20 centrifuge, Orto-Alresa). The supernatant was removed and the harvested cell pellets were washed with three volumes of sterile saline solution (0.9% NaCl) before repeating centrifugation. The supernatant was removed again and the cells resuspended in saline at a cell density of 10^8 CFU/mℓ, by means of comparison with the MacFarland standard (0.5) (Bhagwat, 2003). The solution was diluted further in sterile distilled water to a cell density of 10^6 CFU/mℓ. These cell numbers were confirmed by enumerating on *Listeria* selective media (Oxford formulation) (Oxoid, Basingstoke, UK).

Determination of contamination level for inoculation water:

The exact *L. innocua* inoculation level of the contaminated water used to inoculate the broccoli before each treatment was determined by enumeration before inoculating the broccoli crops.

Maintenance of culture:

The culture was maintained by subculturing on Tryptone Soy Agar (Merck) at intervals of a minimum of 7 days and growth at 37°C (Bhagwat, 2003).

Broccoli samples

The broccoli samples for the processing phase of the experimental study were obtained from a local greengrocer, Pretoria, South Africa, as untreated broccoli heads enumerated for *L. innocua* and total plate counts.

Contamination of broccoli plants

Broccoli heads were cut into florets to weigh 10 g and the florets were then individually dipped into the inoculated water at a cell density of 10^6 CFU/mℓ, held for 30s. In addition, 3 mℓ of water was dripped onto the floret by means of a sterile syringe to ensure extended contact time of the organisms with the floret surface and to simulate contact conditions during actual processing. The florets were dried in a laminar flow cabinet for 30 min (Fig. 27).

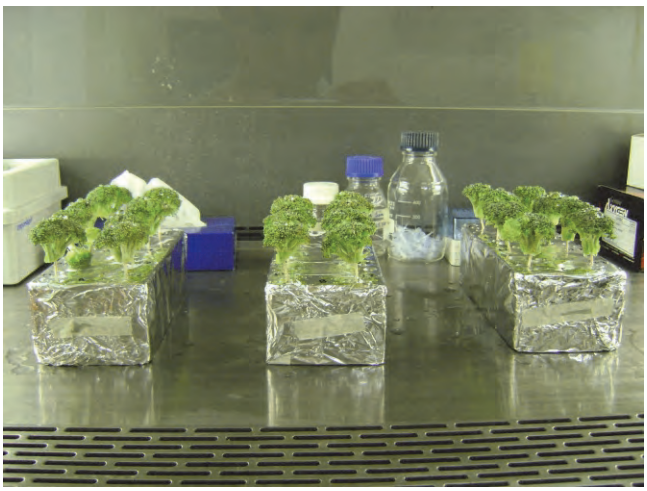


Figure 27 Minimal processing of the broccoli; a) contamination of the florets; b) broccoli in individual packaging

Processing

Minimal Processing

1) Washing

The experimental design consisted of three washing treatments to which the contaminated broccoli florets were subjected. The first treatment (A) contained 8 florets which were contaminated with *L. innocua*, dried and then washed with sterile distilled water, by pouring 100 mℓ water into sterile stomacher bags, individually adding the florets, followed by gentle shaking for 30s and resuspension of the florets in an upright position for drying. For Treatment B, the florets were contaminated, dried and then washed with sterile, distilled water containing 200 ppm chlorine. The chlorine solution was prepared from a 3.5% m/v Jik solution and after preparation, protected from light, held at 21 ± 2°C, and used within 1 hour of preparation. The same washing method was followed as for Treatment A. Treatment C served as control for the wash process and these florets were also contaminated and dried, but not rinsed after contamination. After washing, the florets were dried in a laminar flow cabinet for 30 min. After washing, one floret from each wash treatment was processed for enumeration to determine the effect of the washing procedure on the prevalence of *L. innocua* on the broccoli floret.

2) Packaging

The florets were packaged by assigning the florets to two different packaging conditions. Two florets from each wash treatment were packaged on polystyrene plates and covered with PVC cling wrap. The remaining florets were packaged under modified atmosphere conditions in polypropylene bags of 20 cm x 20 cm size, suitable for vegetable modified atmosphere packaging, obtained from Packaging World, Pinetown, South Africa. The film was non-perforated, with characteristics as in Table 15.

Table 15 Characteristics of the film used for modified atmosphere packaging

Thickness (µm)	Permeability O_2 ($m\ell.m^{-2}.d^{-1}.atm^{-1}$)	Permeability CO_2 ($m\ell.m^{-2}.d^{-1}.atm^{-1}$)
20	1600	3600

*Serrano *et al*., 2006

The modified atmosphere was obtained by filling the packages with a gas mixture containing 5% CO_2, 5% O_2 and 90% NO_2 and sealing the packages with heat (Multivac A300 vacuum sealer). One package from each of the three wash treatments was kept unrefrigerated for 30 min after sealing. The concentration of oxygen and carbon dioxide inside the packages was monitored by using a Gaspace 2 gas analyser (Systech Instruments Ltd., Thame, Oxfordshire, UK). A syringe was inserted into the package through a rubber seal placed on the film. These florets were processed for enumeration and the remaining packages were then stored under refrigeration at 4°C for 48 hours.

3) Cooking treatment:

After completion of the 48 hour refrigerated storage period, the florets were submitted to different cooking treatments. The first treatment served as the control and the florets were not cooked. For the second treatment, the florets were submitted to cooking with boiling water and for the third treatment the florets were heated in the microwave. The water cooking procedure was carried out by placing the florets in their sealed packaging into a boiling water bath at 100°C, facilitating a steam blanching effect at 95±2°C, for 3 min. Thereafter they were enumerated. For the microwave cooking procedure the packages were cut open on the upper left corner by cutting off a 3cm by 3cm triangle. The packages were then placed in the centre of a domestic microwave oven at 2450 MHz with a maximum output power of 850 W and exposed to microwaves at full power for 30s before they were removed and processed for enumeration.

Enumeration repetition

Broccoli samples (10 g) were stomached for 2 min in 90 mℓ Buffered Peptone Water (0.1%), from which serial dilutions were prepared and spread plated onto *Listeria* Selective media (Oxford formulation) (Oxoid). The plates were incubated at 37°C for 24 to 48h to allow enumeration of *L. innocua* colonies.

Statistical analysis

Three replicate experiments were conducted for each trial. All the samples used for colony enumeration, including controls were prepared in duplicate. The mean values triplicate plate counts of duplicate samples were calculated and reported with 99% confidence interval. Data were subjected to analysis of variance (ANOVA) (Han *et al*., 2000) and mean comparisons were performed to examine differences of variables were significant ($P< 0.05$), so as to assess the effect on the survival of *L. innocua* on the broccoli surface and therefore the efficacy of

the individual treatments on the reduction of food safety risk during the minimal processing procedure.

Results

Wash treatment water and chlorine:

The water wash treatment, as determined directly after treatment on day 0, reduced the *L. innocua* numbers on broccoli by 1 log unit to 4.23 log CFU/g and the chlorine wash treatment reduced the numbers a further 1 log unit to 3.11 log CFU/g, as determined 30 minutes after exposure of the contaminated broccoli to the treatment (Table 16). The differences between the numbers before and after treatment were, however, not significant ($P > 0.01$). Treatment variances were not homogeneous; therefore significance testing was done at the 1% level method TTC.

Table 16 The effect of washing treatment on microbial counts on broccoli (D0)

Treatment	*L. innocua* (log CFU/g) (±SD)	TCC define (log CFU/g) (±SD)
Wash: water	4.23 (±0.26)	6.02 (±0.30)
Wash: chlorine (200ppm)	3.11 (±0.24)	6.69 (±0.51)
No wash	5.13 (±0.91)	6.95 (±0.40)

Wash treatment: Main effect of washing

The main effect of washing (as measured on day 2) includes the effect of storage and cooking on the washed samples. Washing with water facilitated a 1 log reduction, to a level of 2.37 log CFU/g, in *L. innocua* numbers compared to the unwashed sample (control) (Fig. 28). Washing with 200 ppm chlorine induced a further 1 log reduction, to a level of 1.46 log CFU/g, in cell numbers when compared to the sample washed with water. The differences between the *L. innocua* numbers as well as between total colony counts (TCC) after the treatments were, however, not statistically significant ($P > 0.01$). When only the trend in cell number changes were studied, it was deduced that the broccoli sample washed with water showed a slight increase in microbial load in comparison to the unwashed sample, whereas the total colony count on broccoli washed with chlorine was somewhat lower (less than 1 log unit) (Fig. 28).

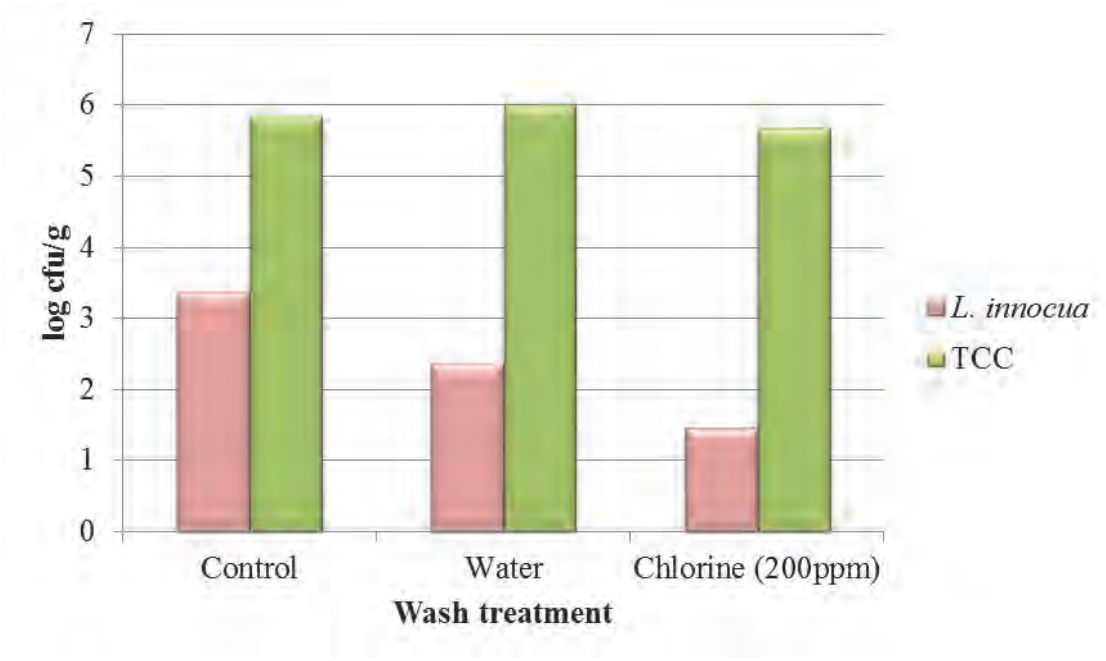


Figure 28 The effect of washing with water and chlorine, compared to a control on *L. innocua* and total colony counts (TCC) on broccoli

Cooking treatment

Microwave cooking had a lethal effect on *L. innocua* and the numbers after this treatment were not only significantly lower than the uncooked sample, but also significantly lower than the resulting *L. innocua* numbers from cooking treatment ($P < 0.001$) (Fig. 29). Total colony counts were affected by microwaving in this same way. Microwaving samples (in-bag) for 30s resulted in total destruction of *L. innocua* on broccoli, as no viable organisms were detected on the sample after treatment, causing a reduction of 3.37 log units. Cooking treatment resulted in *L. innocua* counts that were lower in numerical value than those that remained uncooked, even though these differences were not statistically significant ($P > 0.01$). Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units and total colony counts by between 1 and 2 log units (Fig. 29).

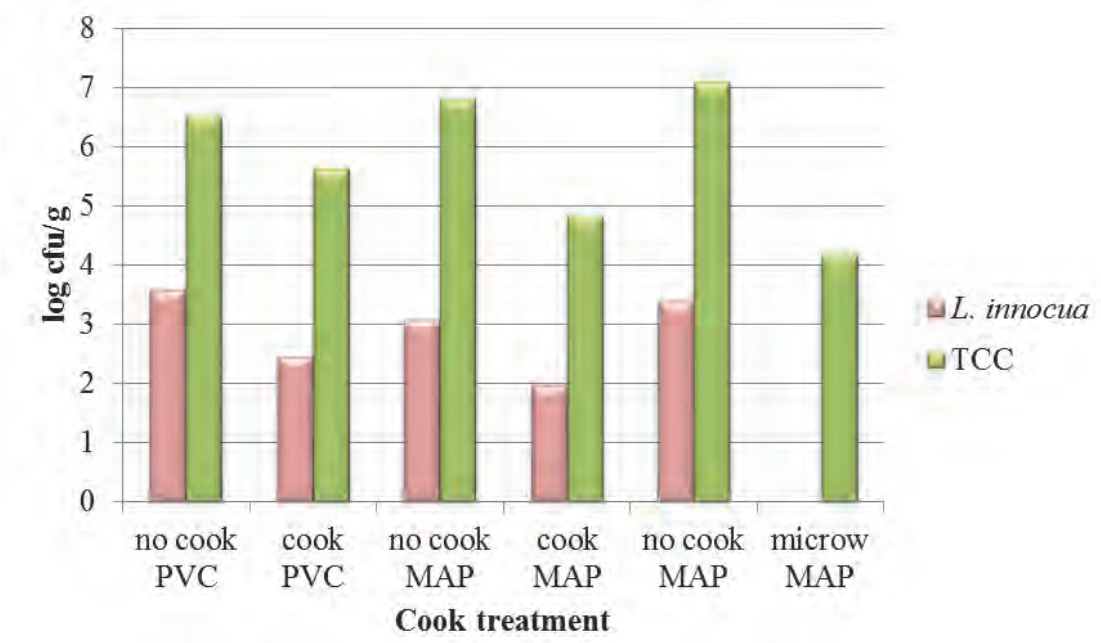


Figure 29 The effect of cooking on *L. innocua* numbers and total colony counts on broccoli; Same coloured columns with the same letter are not significantly different from each other (P > 0.01)

Total effect of minimal processing and cooking on *L. innocua*

Results from the ANOVA indicated that the combination of wash and cook treatment was not significant (P > 0.01). Comparing the same storage and cooking conditions, but different wash treatments, provided insight into the main effect of washing and how this effect is influenced by changing the other processing parameters, as illustrated in Fig. 30. For samples stored under atmospheric conditions, not cooked after storage, *L. innocua* numbers after chlorine wash (log 2.13 CFU/g) were less than after water wash (log 2.71 CFU/g), and also substantially less than the numbers on the unwashed sample (log 4.86 CFU/g) (Fig. 30). Cooking samples after atmospheric storage also resulted in cell numbers for samples washed with chlorine (1.66 log CFU/g) to be lower than those washed with water (2.31 log CFU/g) and substantially lower than the unwashed sample (2.37 log CFU/g). Samples stored under MAP conditions after wash treatment without final cooking, followed the same trend for the wash effects, with the cell numbers on the sample washed with chlorinated water (1.74 log CFU/g) being lower than on the water washed sample (2.34 log CFU/g) and both of these were substantially less than the numbers on the unwashed sample (5.11 log CFU/g). Samples that were stored under MAP and then cooked, displayed cell numbers for chlorine treatment (1.63 log CFU/g) that were not less than on water washed samples (1.72 log CFU/g), but as for the uncooked samples, both washed samples resulted in lower numbers than the unwashed sample (2.52 log CFU/g). Submitting the samples to microwave treatment had a lethal effect on *L. innocua* cells (Fig. 30).

After water wash treatment the *L. innocua* numbers on the uncooked sample stored under MAP displayed a high level of variability, making the difference from the PVC sample insignificant (also due to the large standard deviations within the treatments). MAP combined

with cooking (log 1.32 CFU/g) resulted in less cells than a combination of PVC and cooking (log 2.31 CFU/g).

The results after the chlorine wash treatment represented the attached cells as affected by chlorination. The different storage and cooking conditions within this wash treatment did not differ significantly, with only the numbers on uncooked chlorinated samples packaged in PVC (log 2.13 CFU/g) being slightly higher than those receiving other additional treatments. The chlorine treated, PVC stored, cooked samples, had lower *L. innocua* values (numerically) (log 1.66 CFU/g) than those not washed (log 3.37 CFU/g) or only washed with water (log 2.31 CFU/g) before cooking.

Numbers on the unwashed, uncooked samples were not significantly different from each other (log 4.86 CFU/g, log 5.11 CFU/g and log 4.33 CFU/g; $P > 0.01$) (Fig. 31). The samples that received cooking treatments were different from those that did not receive cooking treatment after both storage conditions (log 4.86 CFU/g vs log 3.37 CFU/g for PVC storage; log 5.11 CFU/g vs log 2.52 CFU/g for MAP storage and log 4.33 CFU/g vs 0 for MAP with microwave cooking). Combining MAP storage and cooking afterwards (log 2.52 CFU/g), did result in lower numbers than the combination of PVC storage and subsequent cooking (log 3.37 CFU/g).

Total effect of minimal processing and cooking on total colony counts

TCC numbers on uncooked samples were similar for unwashed and for washed samples ($P > 0.01$). Microbial counts on broccoli were reduced by cooking without wash treatment as well as after water and chlorine wash treatments and also after being packaged in PVC and stored under modified atmosphere. Cell numbers were decreased substantially after combined treatments of chlorine wash, MAP storage and cooking, as well as after chlorine wash, MAP storage and microwave cooking.

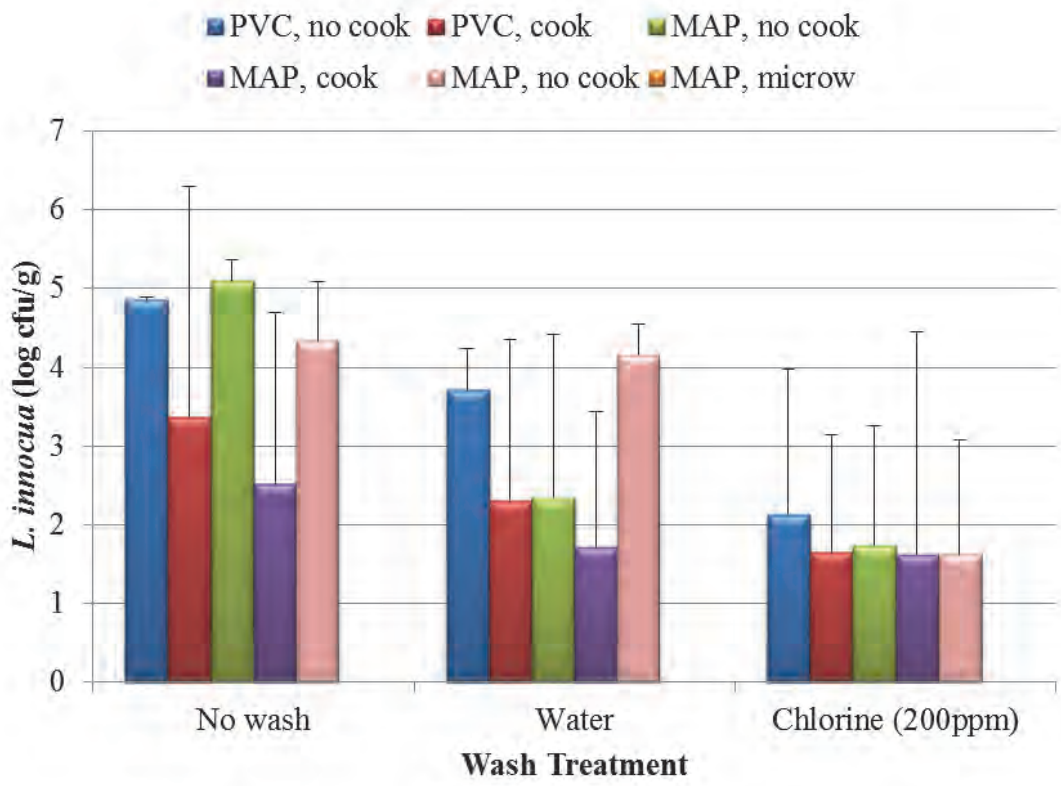


Figure 30 The effect of minimal processing, (washing with water and chlorine and control, followed by refrigerated storage) and cooking on *L. innocua* on broccoli.

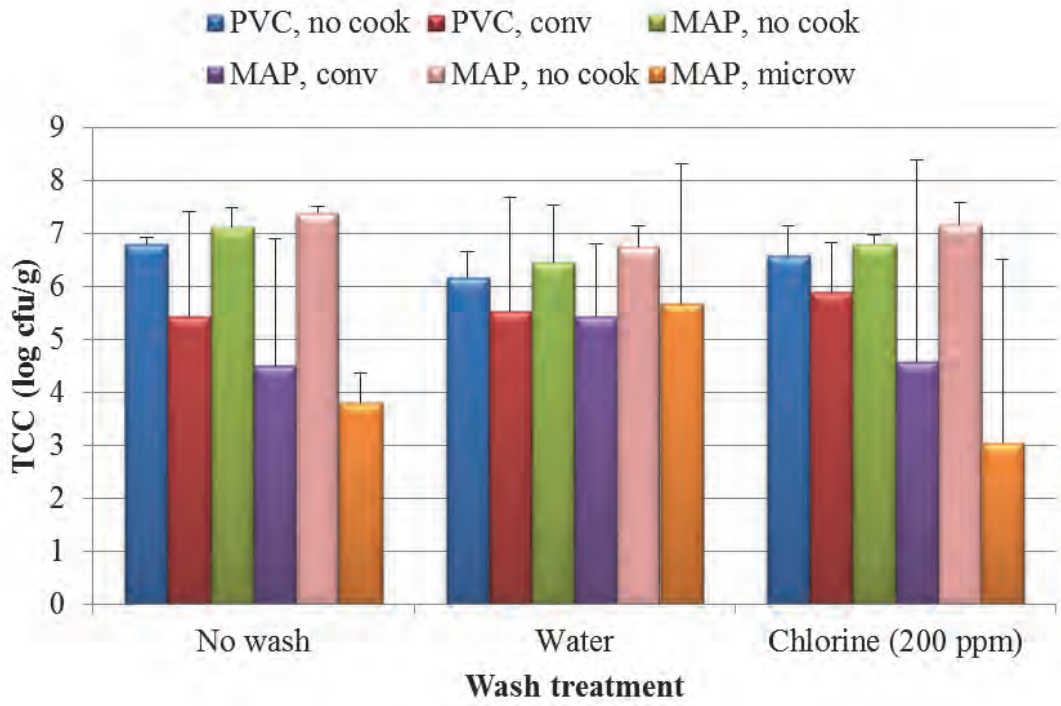


Figure 31 The effect of minimal processing (washing with water and chlorine and control sample, followed by refrigerated storage) and cooking on Total Colony Counts (TCC) on broccoli.

Table 17 Percentage reduction in cell numbers by minimal processing and cooking

Wash	Pack	Cook treatment	Final counts (log CFU/g)		% Reduction*	
			Total Colony Counts (Initial: 6.95)	*L. innocua* (Initial: 5.13)	TCC	Li
No wash	PVC	cook	5.44	3.37	22	34
	MAP	cook	4.51	2.52	35	51
	MAP	microwave	3.81	0	45	100
Water	PVC	cook	5.54	2.31	20	55
	MAP	cook	5.43	1.72	22	66
	MAP	microwave	5.67	0	18	100
Chlorine 200 ppm	PVC	cook	5.91	1.66	15	68
	MAP	cook	4.58	1.63	34	68
	MAP	microwave	3.04	0	56	100

* %Reduction = [(Initial count – Final counts)/Initial counts)] x 100

Washing with water in combination with storage and cooking caused a reduction of around 60% in *L. innocua* numbers and only an average reduction of 20% in total microbial counts. The smallest reduction in *L. innocua* after storage and cooking, occurred on samples that did not receive washing, compared to those that were washed as the first step of minimal processing. Washing with water prior to storage and cooking did not induce a greater reduction in total cell counts than stored, cooked samples that were not washed. This was also observed for washed and unwashed samples that were microwave cooked. A treatment combination of chlorinated washing, storage under modified atmosphere and final microwave cooking induced the greatest reduction of total cell counts (56%) and *L. innocua* counts (100%) on broccoli.

Discussion and conclusion

Washing

The trends in cell numbers as influenced by the wash treatments were as expected, with washing with chlorinated water having the greatest effect on cell numbers and washing with water having a smaller effect in comparison with the unwashed sample. This is in agreement with results from Brackett (1987), who reported that counts of *L. monocytogenes* were reduced in the order of 100 times after treating with chlorinated water at a chlorine concentration of 200 ppm, a factor of 10 times higher than for those washed with water (Francis *et al*., 1999). Zhang and Farber (1996) also observed a reduction in *L. monocytogenes* after chlorination on freshly cut lettuce and cabbage (Francis *et al*., 1999). Ells and Hansen (2006) and Gorski *et al*. (2003) reported that while washing with water removes unattached organisms, chlorine further kills or injures cells, as was seen by Taormina and Beuchat (2001). These researchers also noted that subpopulations of chlorine-treated cells that remained showed signs of being injured to an extent (Taormina and Beuchat, 2001).

The difference between the total colony counts on the broccoli were insignificant ($P > 0.01$) and in addition to that, not corresponding with the trend observed for the *L. innocua* on the broccoli. This suggested that the autochthonous flora was not affected by the wash treatments. As Schoeller *et al*. (2002) suggested, vegetable organic matter could have decreased the active chlorine in wash solutions, causing the chlorine rinse employed to be less effective. Soriano *et al*. (2000) also found that leaving lettuce samples unwashed or even washing them with distilled water incurred no significant decreases in aerobic microorganisms, but they did observe a significant effect of chlorine. Ukuku and Fett (2002) established that washing with water did not cause a significant reduction of native microflora, but chlorine treatment (1000 ppm) did. The reason that this was not in agreement with our results, was possibly because of the higher chlorine concentration that the researchers used in their experiment.

The levels of *L. innocua* present on the broccoli as affected by washing could be better evaluated by looking at the cell numbers on day 0, directly after wash treatment and before packaged storage and cooking treatment so as to assess the individual effect of washing as a single step in minimal processing. The *L. innocua* levels on the broccoli as determined directly after water as well as after chlorine wash treatment (day 0), are above the accepted values as stipulated by regulatory authorities (Francis *et al*., 1999). After minimal processing (day 2), *L. innocua* numbers as affected by washing were also still above the values proposed for *L. monocytogenes* on vegetables, whereas chlorine treatment facilitated a reduction to below this level. Due to the high initial level inoculated onto the broccoli for practical research purposes, these values are relative and may not reflect microbial levels actually encountered in the field (Flessa *et al*., 2005); this has to be kept in mind when comparing with standards.

Soriano *et al*. (2000) suggested a diminished activity of chlorine, which could explain the lack of significance in the differences between the wash treatments. Chlorine affectivity efficacy could be affected by various factors, such as the presence of hydrophobic pockets on the broccoli surface or crevices in the broccoli surface structure, possibly creating an isolated habitat for the microorganisms and allowing them to escape the effect of the chlorine (Adams *et al*., 1989) or the reduced effect of chlorine could be attributed to resistance of the bacteria to the chemical (Lisle *et al*., 1998).

Cooking treatment

Cooking in boiling water resulted in a reduction in the numbers of *L. innocua* on broccoli and the total colony counts as affected by cooking, followed the same trend. These results are in agreement with the results of Mazzotta (2001), who established that submitting broccoli florets to heat at 75°C for 10s had an anti-listerial effect, causing up to a 10^6-fold reduction in *L. monocytogenes* (Lund *et al*., 1989). Microwave treatment (30s) had the greatest effect on *L. innocua* numbers on broccoli. Rodriguez-Marval *et al*. (2009) also reported that heat treatment by means of microwaving at high power for 75s inactivated up to 3.7 log CFU/cm^2 (on meat) of *L. monocytogenes*. Woo *et al*. (2000) established that microwave heating dramatically diminished viable cell counts by causing Gram positive organisms to suffer membrane damage. While the total colony counts on the cooked samples did not differ

significantly from their uncooked samples, the microwaved sample did differ significantly from the samples that did not receive heat treatment, indicating that microwaving could indeed have a more prominent destructive effect on the microbial population on the vegetable. Regarding the total reduction of the processing treatments, this was seen to be the case, with microwave processing repeatedly causing the greatest reduction in cell numbers.

Total effect of minimal processing and cooking on *L. innocua*

Even though analysis of variance revealed the wash-cook treatment combinations not to be significantly different from each other statistically, the numerical differences did still suggest influences of the treatments and treatment combinations on the *L. innocua* survival on the broccoli post-harvest, as each individual combination of treatments could be analysed and compared to a different combination separately so as to assess the effect and efficacy thereof in the management of pathogen contamination of vegetables. Chlorine treatment (200 ppm) had the greatest diminishing effect on cell counts, whilst water washing did seem to either facilitate detachment or wash off unattached cells as a lowering of cell numbers in comparison to the unwashed samples was observed. Ukuku and Fett (2002) also found that washing with water did not cause a significant reduction of *L. monocytogenes* on fresh produce surface (melon), but chlorine treatment did.

Results from the water and chlorine washed samples, cooked after atmospheric storage indicate that the chlorine treatment could have sensitised cells to heating, as was suggested by Taormina and Beuchat (2001). It is however also possible that after chlorine treatment, less cells were present than on the water washed sample due to the effect of chlorine in addition to the rinsing effect of the water and so subsequent cooking resulted in lower final numbers on the chlorinated sample.

MAP storage without other minimal processing steps (such as washing) did not have an effect different from that of storage under atmospheric conditions, but in combination with water or chlorine wash, the numbers for MAP are lower than for PVC (Zeitoun and Debevere, 1991). Cooking did not display a synergist effect with MAP, as the numbers were similar to the PVC samples and to the water washed samples, which agrees with the findings of Kakiomenou *et al*. (1998) with regards to the hurdle effect of MAP. *L. innocua* numbers on the broccoli did not increase during the 2 days refrigerated storage, as was also found by Schoeller *et al*. (2002) and Flessa *et al*. (2005) after 7 days refrigerated storage. Duh and Schaffner (1993) did however conclude that refrigerated storage alone cannot ensure that the growth of *L. monocytogenes* will be inhibited.

The effect of water wash treatment signifies the behaviour of the attached organisms, as washing should have removed any unattached cells. Comparing the water washed samples with each other, the analysis of the packaging method in this wash treatment was inconclusive. This does not signify that the packaging method does not have an influence, as values for MAP stored, uncooked samples and the large standard deviations within treatments varied. The MAP, cooked samples were a log unit lower than the PVC cooked sample, but

this could rather be ascribed to the effect of cooking, rather than synergism between MAP and heat treatment.

The lack of differentiation between the samples exposed to chlorinated wash, suggests that the effect of the chlorine is the main effect in the hurdle system. Chlorine alone is effective, but the effect is greater in combination with other hurdles, as was seen by the fact that if subsequent storage in PVC and no cooking treatment followed, the numbers were slightly higher than MAP stored and cooked samples. Zeitoun and Debevere (1991) found that *L. monocytogenes* numbers (on chicken legs) stored under MAP (90% CO_2, 10% O_2) increased during storage, but that the MAP did have synergistic effect with a decontaminant. This CO_2 concentration is, however, much higher than MAP conditions for broccoli storage, which would therefore cause a different bacteriostatic effect, which explains the absence of this prominent synergism. Chlorine treated, cooked samples had lower values than those not chlorinated, which indicates a possible sensitisation of cells by chlorine to heat. Taormina and Beuchat (2001) proposed a theory that two subpopulations of *L. monocytogenes* cells are created as a result of chlorine stress. This involves the weaker cells dying, as they are less resistant, with only the more stable cells, which are perhaps approaching stationary phase, remaining and succeeding in surviving thermal treatment. Within the chlorine treatment, the cooked values did however not differ significantly from uncooked values, which might be because the cells surviving chlorination are more resistant, rather than being more sensitive or that the effect of chlorine was already so large that the cooking effect was not significant in terms of the numbers of cells that remained after washing. Sub-lethal injury by one treatment could also induce a stress response in the pathogen, making it more resistant to subsequent treatment. Bunduki *et al*. (1995) observed that chlorine seemed to cause a large amount of damage, as an extended time was needed for cell repair.

The unwashed, uncooked samples show the effect of the refrigerated storage only. Storing the broccoli under MAP conditions does not have a different effect on *L. innocua* growth or survival than storage under atmospheric conditions in PVC. Kakiomenou *et al*. (1998) also indicated that packaging in modified atmospheres is not necessarily of greater value as an additional hurdle for growth of *L. monocytogenes* in comparison to conventional packaging in aerobic conditions. The main effect of heat treatment on microbial safety during broccoli preparation was evident from the reduced numbers after cooking. Microwave treatment of the *L. innocua* on broccoli proved to be lethal, possibly due to the damaging effect of microwave radiation on the membrane of the organism (Woo *et al*., 2000).

Total effect of minimal processing and cooking on Total Colony Counts

The effect of cooking on the total microbial load is evident, with samples from all wash treatments and packaging conditions exhibiting lower numbers after receiving heat treatment. As the effect of cooking is, however, small, this could suggest the presence of spores within the microbial population, as these are more resistant to the effect of heat (Haas *et al*., 1999). Samples exposed to chlorine and stored under MAP did not have substantially lower numbers than those that received a water wash treatment, compared to the unwashed samples. This

suggests that the effect of chlorine on the total colony counts was not the main influencing factor in the diminishing of cells, which is supported by the findings of Soriano *et al*. (2000).

The uncooked samples within the different wash treatments did not differ significantly from each other, therefore the autochthonous flora is less affected by chlorine, than observed with *L. innocua*, suggesting that the background flora contain organisms that might better attach to the surface and some may also survive well under modified atmosphere. The possibility exists that the organisms might have a good recovery mechanism, but a sensitising effect to heat by chlorine could still be a factor, especially in combination with MAP. Nguyen and Carlin (1994) also stated that the composition of mesophilic microflora was not significantly altered in end products, indicating that processing did greatly affect the population.

The combination of chlorine washing, MAP storage and microwave cooking seemed to be the best methods for reduction of total microbial load on broccoli, although even in the absence of a wash treatment, reduction was also achieved. The reduction calculated from these initial numbers to the final numbers after treatments, was therefore greater than when comparing final numbers between cooked and uncooked samples, signifying the entire minimal processing procedure and heat treatment combination. The initial numbers represent those for day 0, before any treatments were carried out. Combining chlorine wash and MAP storage did not pose to be a greater hurdle than water wash and MAP, facilitating the same reduction in *L. innocua* numbers and thereby suggesting that a combination of water wash, MAP storage and cooking is just as effective a hurdle system as chlorine wash with MAP and cooking. The hurdle effect of washing is evident (both with chlorine and water), as a much lower (20% less) reduction was observed for the unwashed samples.

3.2.8 EFFECT OF WASHING, PRESERVATIVE TREATMENTS AND STORAGE ON THE SURVIVAL OF MICROORGANISMS, INCLUDING FOODBORNE PATHOGENS, ON FRESH PRODUCE

Specific aim

This study investigated the effect of the different washing methods and storage at refrigeration temperature after washing, on the microbial load of fresh produce.

Experimental procedures

Sample collection

Four different varieties of fresh produce (broccoli, cabbage, Crisphead lettuce and spinach) were collected from a farm in Camperdown, KZN. This produce was collected in sterile plastic bags and transported in Styrofoam boxes containing icepacks (this was performed in triplicate). The samples were stored at 4°C until required for processing (Mukherjee *et al.*, 2004). Initial experiments were performed within 24 hours of sample collection.

Treatments and analysis of produce

Approximately 250 g of the samples were treated with 5 ℓ of solution using household methods (i, ii) as well as commercial methods (iii, iv, v), except for the non-aqueous treatment (vi) where 250 g of the samples were exposed to Ultra-Violet (UV) light as indicated below:

i) Samples were washed in tap water for 120s (Vina *et al*., 2007; Hassenberg and Idler, 2005);
ii) Samples were subjected to a household treatment by adding a handful of salt (5 g) to 1000 mℓ of water, which is equal to 25 g of NaCl per 5 ℓ water;
iii) The chlorine solution for treatment was prepared using commercial sodium hypochlorite (6.15%), which was adjusted to pH 6 using HCl, the concentration that was used was (40 µℓ/ ℓ), the samples were dipped into this solution for 3 min (Fonseca and Rushing, 2006);
iv) Blanching was performed by submerging the samples for 1 min in a water bath at (100°C) followed by quick submersion for 1 min in water at 4 °C (Vina *et al*., 2007). Despite this method is not being applied industrially to cabbage, lettuce and spinach, it was included in this study in order to compare the effectiveness of all the treatment methods on different types of produce;
v) The samples were washed with 5% H_2O_2 (which was prepared from a 30% stock solution by dilution with sterilized tap water). This solution was used to wash the produce by agitation for 5 min (Ukuku, 2004);
vi) A non-aqueous treatment, UV light, was used for 3 min using a fluorescent lamp with UV emission at 254 nm. The bulb was placed 15 cm above the samples (Fonseca and Rushing, 2006).

An unwashed control was also included in this experiment.

The treated and control samples were analysed for microbial contaminant; also 15 g of the samples were placed in sterile polyethylene bags and were sealed and stored at 4 °C for 6 days to determine the effect of storage on the microbial load.

Microbiological analysis

The plant samples were prepared for analyses according to Islam *et al*. (2004). Approximately 10 g of each plant sample was homogenized with 90 mℓ of 0.1% peptone water using a blender. This served as the 10^{-1} dilution which 10-fold serial dilutions were made from, using 0.1% peptone water. A 100 µl of appropriate dilutions were spread plated onto different selective media (Table 18). Appropriate dilutions were also spread plated onto nutrient agar, which was incubated at 25 °C for 72 hours (Hassenberg and Idler, 2005), in order to enumerate total bacterial counts. The different bacterial populations as well as total counts were enumerated by counting the number of colonies per plate. These values were expressed as colony forming units per gram (CFU/g) of the sample.

Table 18 Selective media for the growth of specific organisms and required growth conditions

Specific organisms	Medium	Growth conditions	Appearance on medium
E. coli and Coliforms	coliform-chromo agar	37 °C, 24 h	*E. coli* – blue to dark violet Coliforms – salmon to red
Listeria monocytogenes	Agar Listeria Ottaviani and Agosti (ALOA)	30 °C, 24 h	Appear surrounded with a distinct opaque halo-like precipitation zone
Salmonella spp. and *Shigella* spp.	Salmonella-Shigella agar (SS agar)	37 °C	*Salmonella* spp. – colourless colonies with black centers, *Shigella* spp. – colourless colonies

Results

Microbiological analysis

It was evident that *Campylobacter jejuni* (Fig. 32) was present in the control and in all the produce samples tested after treatment at day 0 and 6. However, this organism was not detected in all the produce samples tested after blanching (method iv) and exposure to UV-light (method vi). After refrigeration, *C. jejuni* counts had increased in the control as well as in the treated samples. A similar trend of the presence of *C. jejuni* in the treated samples as well as the control, were seen in all the fresh produce tested.

Coliforms (Fig. 33a) were not detected in broccoli after treatment with NaCl at day 0 and after refrigeration at day 6. These organisms were only detected on broccoli at day 0 in the untreated control and in the produce washed with tap water. Coliforms were detected, however, at day 6 in chlorine, blanching, hydrogen peroxide and UV treated samples at a concentration of approximately 3.5 log CFU/g. It was interesting to note however, that at day 6 the control and the tap water treated broccoli sample had the highest bacterial counts at 4.75 and 5.24 log CFU/g. Coliforms (Fig. 33b) were not detected in cabbage samples tested, including the unwashed control before and after refrigeration. However, after the 6 day refrigeration of the tap water treated samples, coliforms were detected at 3.53 log CFU/g. Coliforms (Fig. 33c) were not detected in lettuce after treatment with chlorine and blanching at day 0 and day 6, and not detected in the hydrogen peroxide and UV treated samples at day 0. However, at day 6 an increase of about 3.83 and 3.49 log CFU/g was observed in these treated samples. Coliforms (Fig. 33d) were not detected in blanched spinach at day 0 and 6, and in hydrogen peroxide treated spinach at day 0, however, coliforms were detected at day 6 in this treated sample. The presence of coliforms in the control as well as the treated samples (i, ii, ii, vi) were observed at day 0 and 6 with the UV treated sample (vi) showing the lowest counts of coliforms at day 6 of 3.58 log CFU/g.

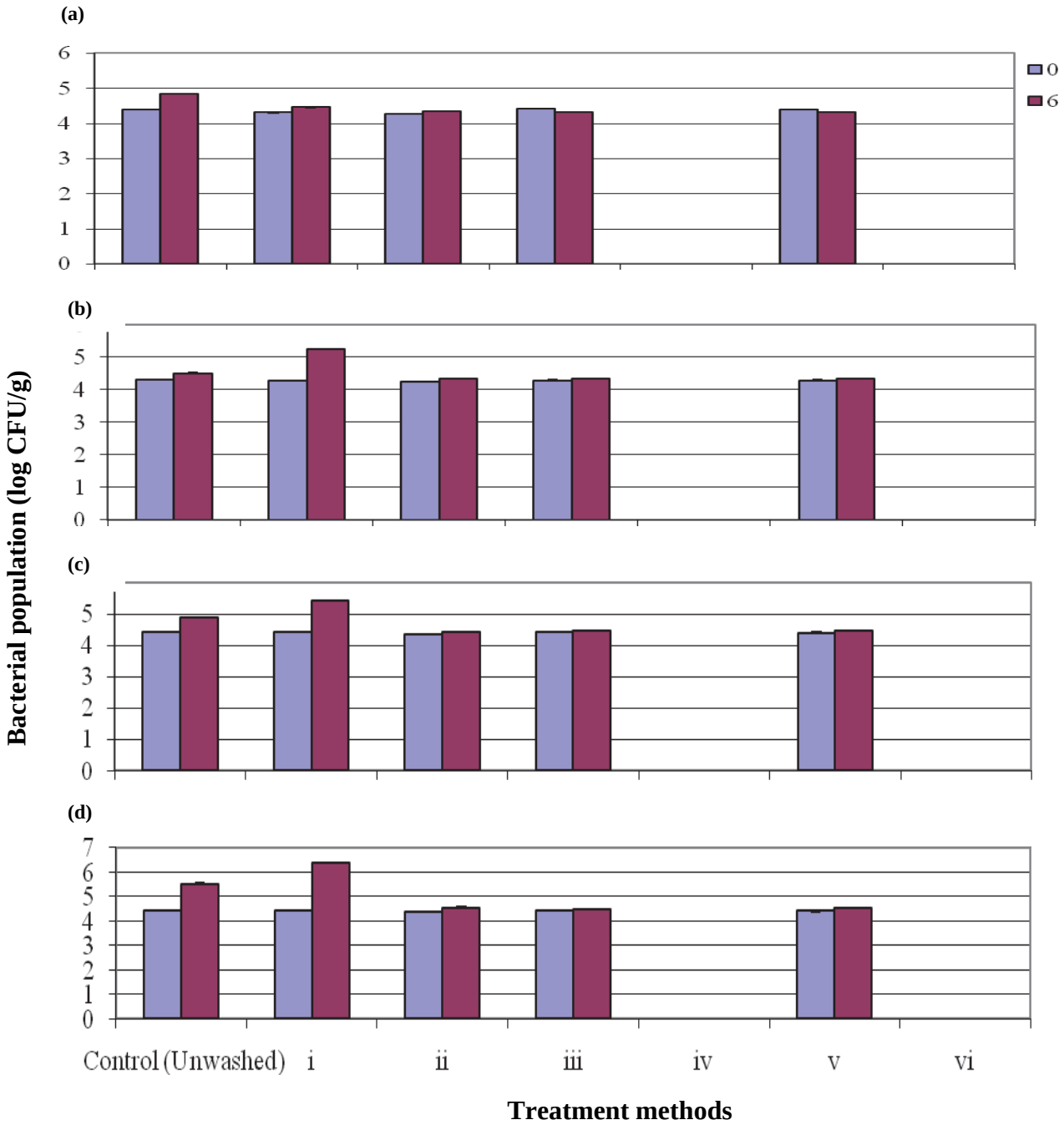


Figure 32 Population of *C. jejuni* in the four produce tested (a = broccoli, b = cabbage, c = lettuce, d = spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

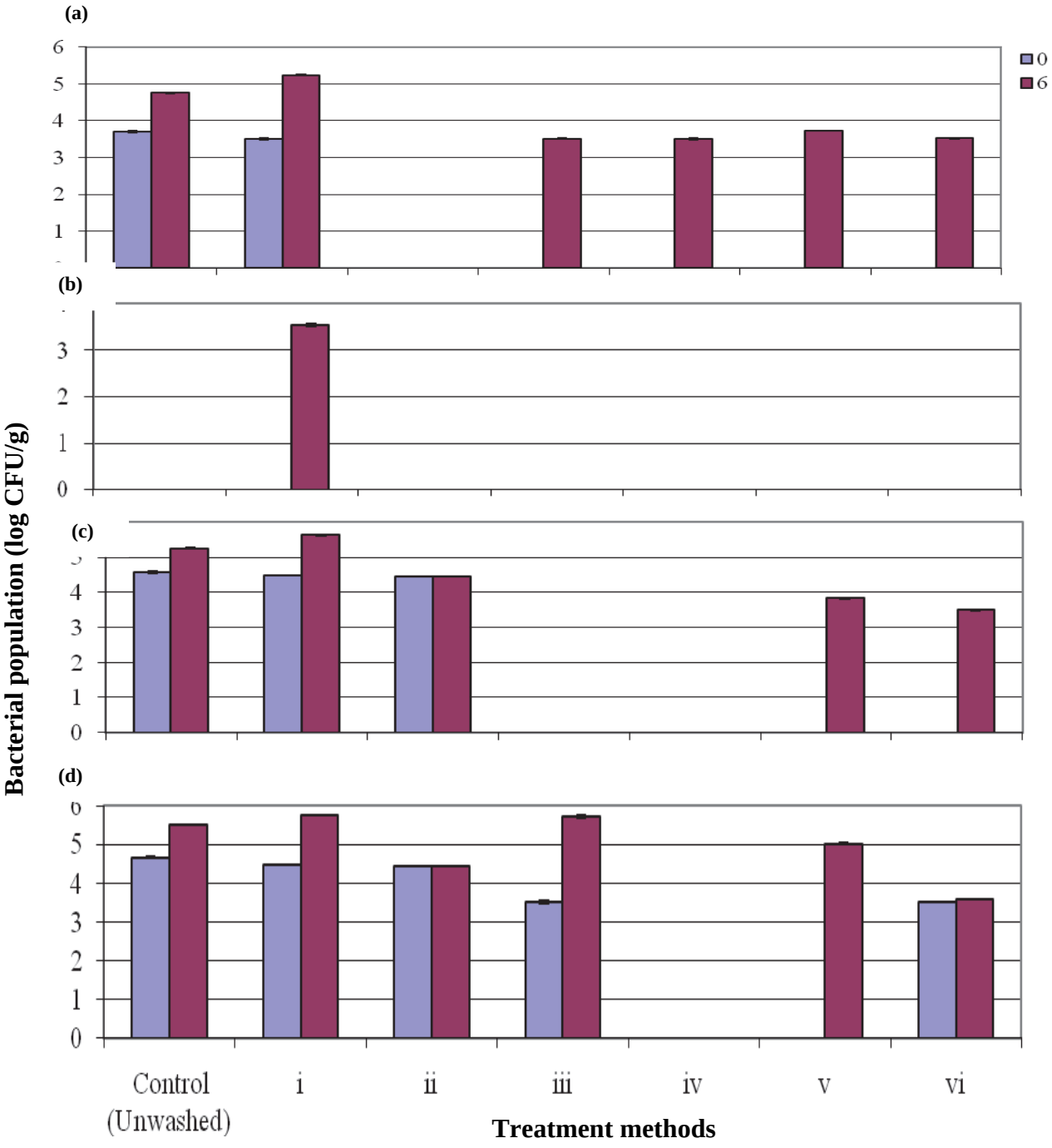


Figure 33 Population of Coliforms in the four produce tested (a = broccoli, b = cabbage, c = lettuce, d=spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

Escherichia coli was neither detected in the unwashed broccoli control nor in the treated broccoli samples on day 0 and on day 6, after refrigeration. *E. coli* was not detected in any of the blanched and UV treated cabbage samples at day 0 and 6. At day 0 (Fig. 34a) *E. coli* was not detected in any of the cabbage samples tested. However, at day 6 it was detected in the control as well as the treated samples (i, ii, iii, v). The highest *E. coli* population increase after refrigeration was observed in the NaCl treated cabbage at 4.88 log CFU/g. *E. coli* was

observed in the lettuce control (Fig. 34b) as well as in the tap water and NaCl treated samples at day 0, with a slight increase in the *E. coli* population after refrigeration. However, in the chlorine and hydrogen peroxide (iii, v) treated lettuce, *E. coli* was only evident after refrigeration of the treated produce. A similar trend to that of lettuce was observed in the spinach samples tested (Fig. 34c). However, the presence of *E. coli* in the chlorine treated sample was detected at day 0 and day 6 at 3.54 and 4.50 log CFU/g respectively.

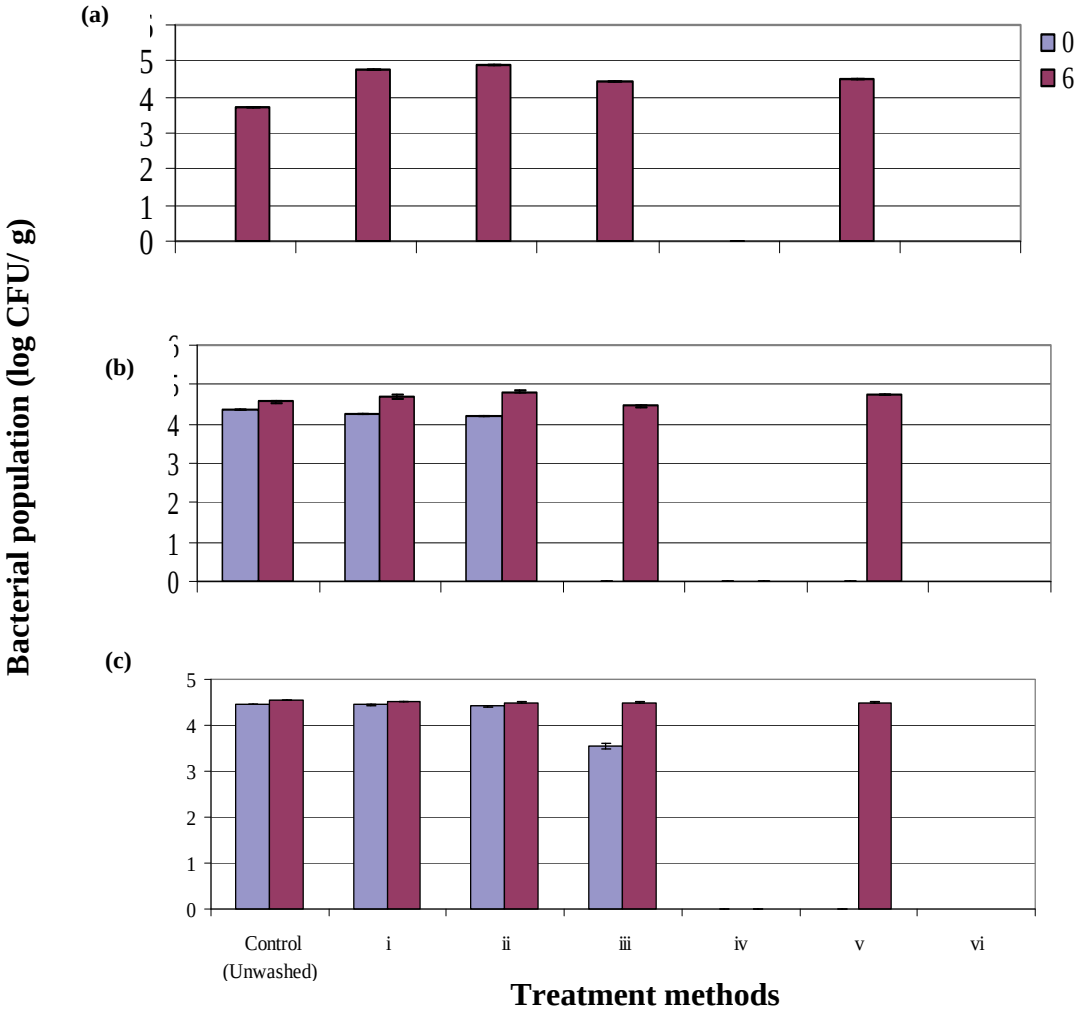


Figure 34 Population of *E. coli* in the four produce tested (a = cabbage, b = lettuce, c = spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

L. monocytogenes was not detected on all the broccoli samples tested before (day 0) and after refrigeration (day 6). This organism was only detected after refrigeration of the chlorine treated (iii) and blanched (iv) cabbage samples at 3.51 and 3.53 log CFU/g. A similar trend was observed in the lettuce treated samples as *L. monocytogenes* was only detected in the chlorine treated (iii) and blanched (iv) lettuce samples at 3.51 and 3.79 log CFU/g. Comparing the control spinach (Fig. 35) with the other treated spinach samples, no differences were observed and a slight increase in all the samples were observed after refrigeration. The blanched lettuce sample differed from the control as this organism was not

detected in the blanched sample at day 0 but an increase of 3.74 log CFU/g was observed after refrigeration which was 0.12 log CFU/g higher than the control.

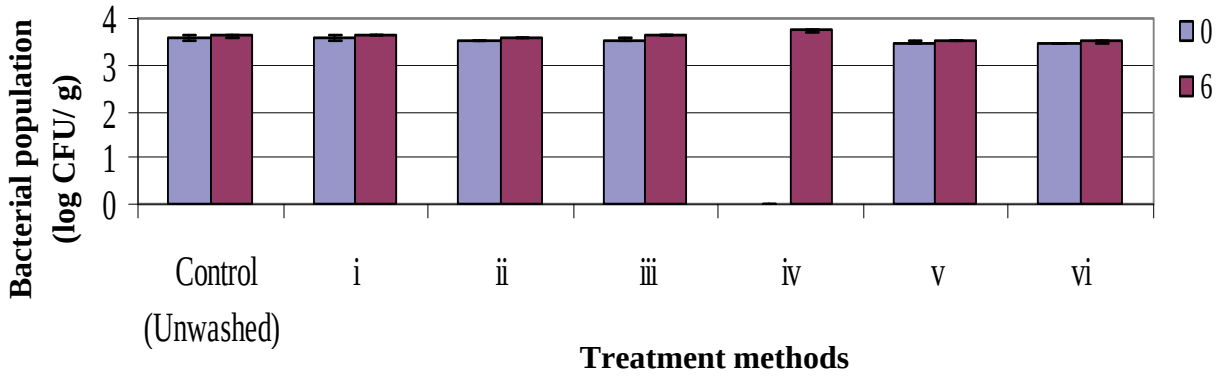


Figure 35 Population of *L. monocytogenes* in spinach after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

Salmonella spp. was not detected in any of the broccoli samples tested before and after refrigeration. Of all the cabbage samples tested *Salmonella* spp. were only detected in the blanched cabbage sample after refrigeration at 3.5 log CFU/g. *Salmonella* spp. were detected in control lettuce sample (Fig. 36a) before and after refrigeration with a slight decrease observed at day 6. The tap water and NaCl (i, ii) treated samples were similar to the control before and after washing. However, a slight increase in the counts of *Salmonell*a spp. was observed in the NaCl treated sample. S*almonella* spp. was not detected in the hydrogen peroxide and UV treated lettuce samples and this organism was detected in the blanched lettuce sample after refrigeration. Colony counts of *Salmonella* spp. were detected in the chlorine treated sample at day 0 at 3.5 log CFU/g. *Salmonella* spp. was not detected in the UV treated spinach (Fig. 36b) sample throughout the experiment, even after refrigeration. A similar pattern to that of *Salmonella* spp. in the control, tap water and NaCl (i, ii) treated lettuce sample, was observed with the spinach sample, except that the presence of *Salmonella* spp. was approximately, 1 log CFU/g higher in the spinach sample. *Salmonella* spp. was detected in the chlorine (iii) treated spinach sample only after refrigeration at 4.34 log CFU/g. *Salmonella* spp. was not detected in the UV (vi) treated spinach after treatment and during refrigeration. This organism was present in the blanched spinach at day 6 at 3.97 log CFU/g. Also the largest reduction (compared to the control) of *Salmonella* spp. in spinach was seen with the hydrogen peroxide wash method at 1.3 log CFU/g after the 6 day refrigeration period.

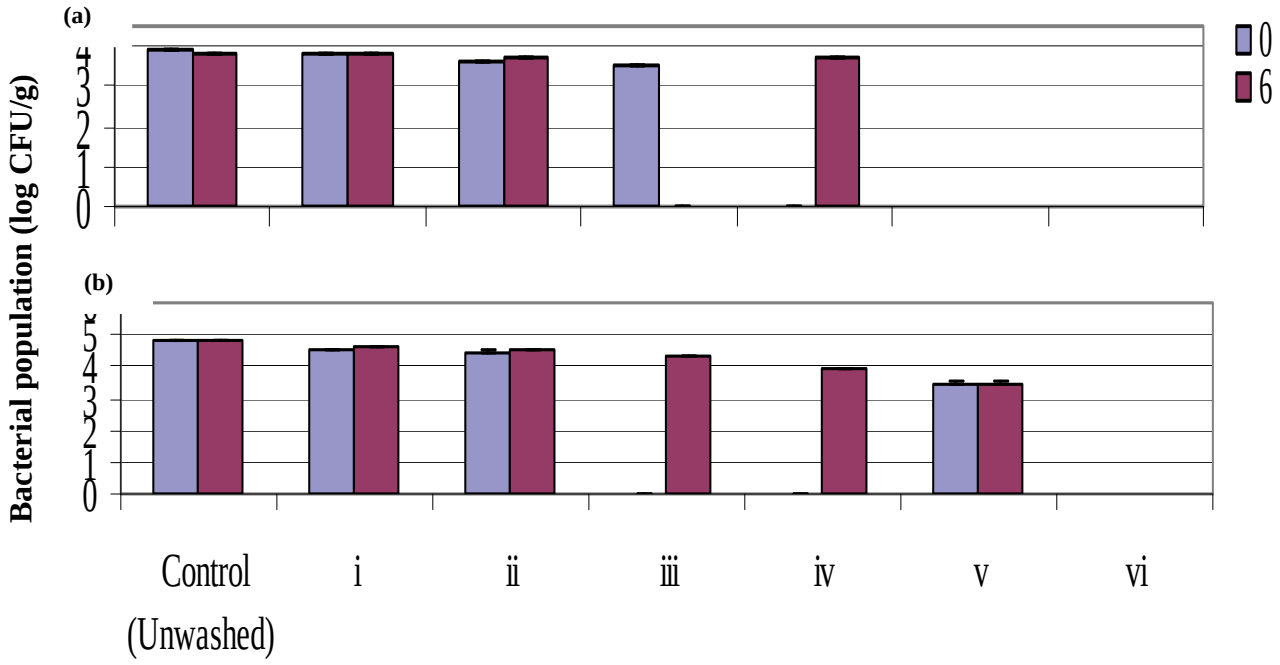


Figure 36 Population of *Salmonella* spp. in the fresh produce tested (a = lettuce, b = spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = H_2O_2, v = blanching, vi = UV) at day 0 and after refrigeration at day 6.

Shigella spp. were only detected in the broccoli control on day 0 at 3.75 log CFU/g (Fig. 37a). At day 6, an increase of *Shigella* spp. in the control of approximately 0.65 log CFU/g was noted. *Shigella* spp. was not detected in the blanched and hydrogen peroxide treated broccoli samples at day 6. However, the concentration of *Shigella* spp. in the control at day 6 was similar to that found in the tap water and NaCl (ii) treated broccoli samples. The highest reduction in counts of *Shigella* spp. in broccoli was due to the chlorine and UV (vi) treatment methods. A similar trend of the broccoli control was observed with the cabbage control (Fig. 37b) at day 0 and 6. *Shigella* spp. were not detected in the blanched and UV treated cabbage samples. *Shigella* spp. were detected in the control, tap water and NaCl treated cabbage samples at day 0 at approximately 4.5 log CFU/g. These counts were maintained after refrigeration of the tap water and NaCl treated samples. However, the control had increased by 0.93 log CFU/g by day 6 of refrigeration. *Shigella* spp. were detected in the chlorine and hydrogen peroxide treated samples 4.13 and 3.53 log CFU/g, respectively, at day 6. In the lettuce samples (Fig. 37c), *Shigella* spp. were present at day 0 in the control, tap water, NaCl and in the UV treated samples, however, at day 6 this organism was not detected in the UV treated sample. UV was shown to cause the highest reduction of *Shigella* spp. in lettuce. *Shigella* spp. was found to be abundant in the control spinach as well as all the treated spinach samples at day 0, except the blanched spinach sample in which this organism was not detected. At day 6, *Shigella* spp. were not detected in the UV treated samples and an increase in the counts of this organism was evident in the control and the chlorine, blanched and UV treated spinach.

For the broccoli samples (Fig. 37a), an overall increase in all THB counts were observed after refrigeration for 6 days. However, no bacteria were detected in blanched broccoli samples at day 0, however, at day 6 the THB were detected at 3.59 log CFU/g. At day 0, the highest and lowest THB counts were present in the control and the chlorine (iii) treated sample at 5.53 and 3.54 log CFU/g, respectively. After refrigeration, at day 6, the highest and lowest THB counts were present in the tap water treated and the blanched broccoli sample at 7.49 and 3.59 log CFU/g, respectively. A very similar trend was observed with the cabbage and lettuce samples tested (Figs. 38b and c). THB counts were evident at day 0 in all the spinach (Fig. 38d) samples tested except the blanched spinach sample, however, THB counts were detected at 6.41 log CFU/g after refrigeration. Before refrigeration, the chlorine (iii) and hydrogen peroxide (v) treated samples had the highest reduction of THB counts as compared to the control. The THB in all the treated samples as well as the control had increased after refrigeration, except for the NaCl treated spinach samples which displayed decreased THB counts by 1.64 log CFU/g after refrigeration.

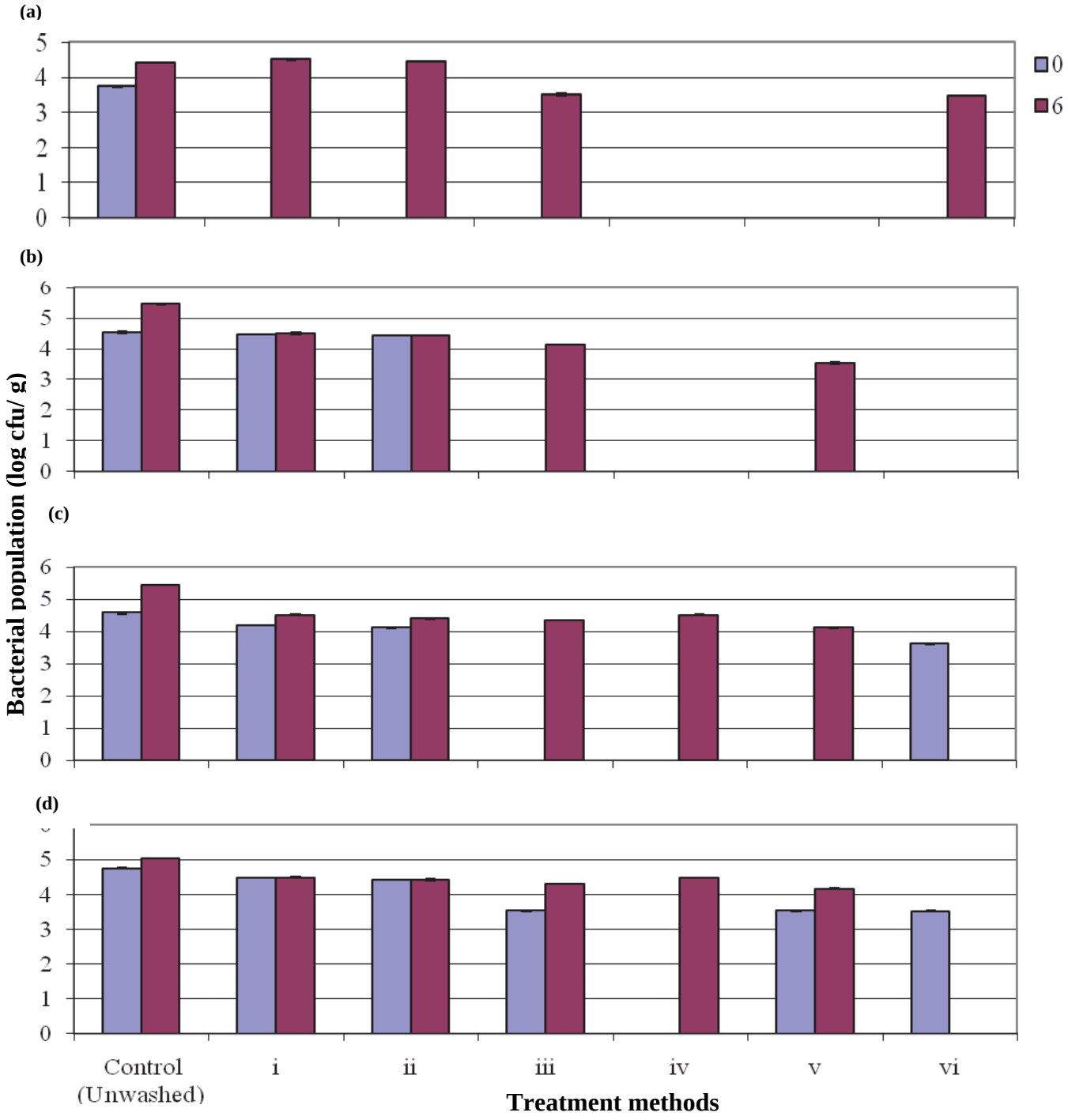


Figure 37 Population of *Shigella* spp. in the four produce tested (a = broccoli, b = cabbage, c = lettuce, d = spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

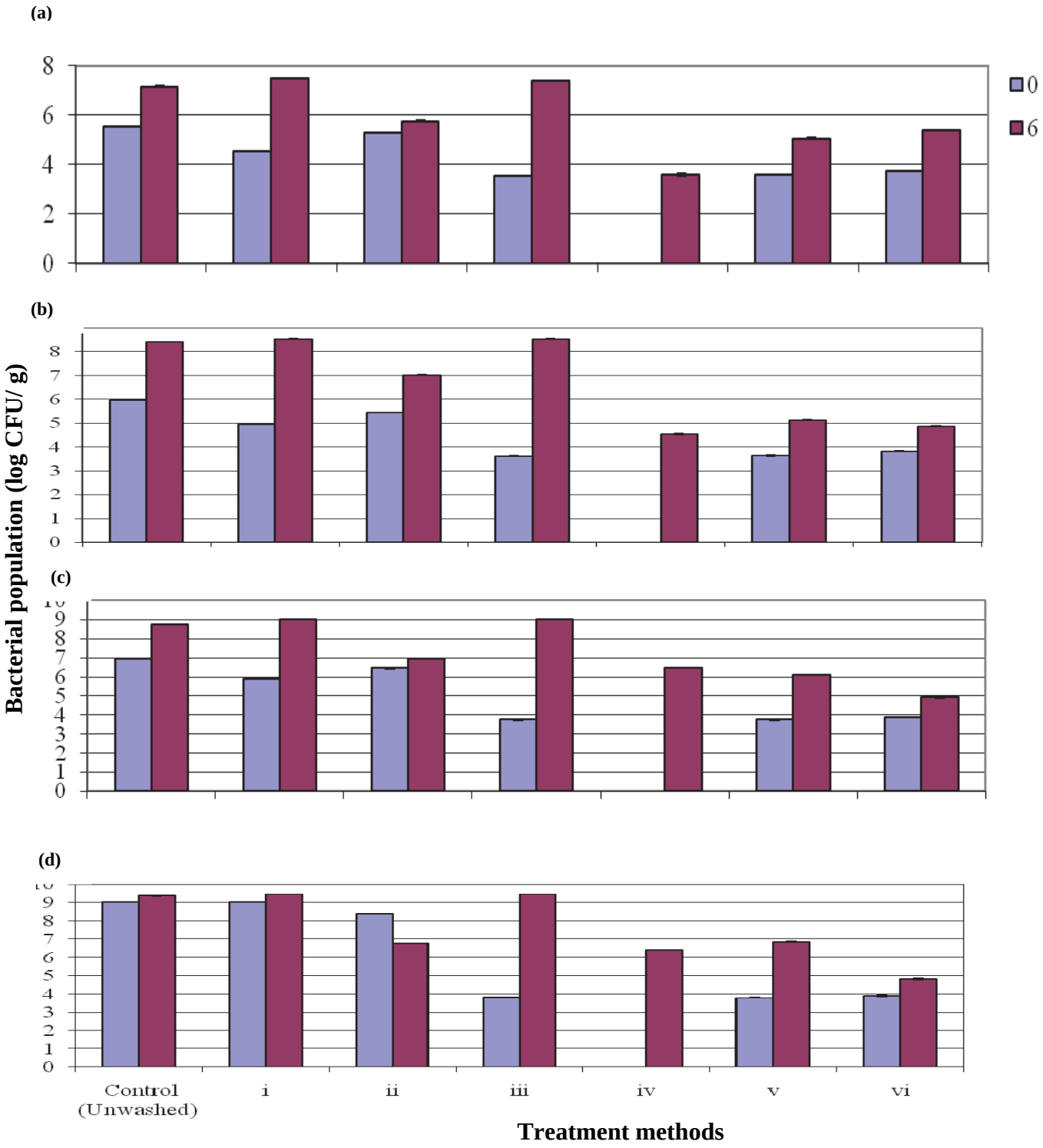


Figure 38 Population of Total heterotrophic bacteria (THB) spp. in the four produce tested (a = broccoli, b = cabbage, c = lettuce, d=spinach) after washing using various treatment methods (i = tap water, ii = NaCl, iii = chlorine, iv = blanching, v = H_2O_2, vi = UV) at day 0 and after refrigeration at day 6.

Discussion and conclusion

Fresh produce has been known to contribute significantly to the healthy lifestyle of individuals, but several outbreaks have proven that even though this produce is very important to consumers, it can harbour pathogens that can be extremely hazardous (Core, 2005). Therefore the key goal of washing and sanitizing treatments is the removal or the inactivation of pathogens (Sapers, 2001). The response of microbes to washing and sanitizing treatments depends partly on the conditions of contamination that affect the attachment and survival of these microbes on produce surfaces (Sapers, 2001).

It is evident from this study that removal of *C. jejuni* from fresh produce was achieved by the blanching and UV treatment methods (Fig. 32). The most effective method in the removal of coliforms from fresh produce was the blanching method (Fig. 33). However, this method did not completely remove coliforms from the broccoli treated sample as these bacteria were detected only after refrigeration. *E. coli*, *L. monocytogenes* and *Salmonella* spp. were not detected in the broccoli samples tested before and after refrigeration. The most effective method in reducing the concentrations of *E. coli* (Fig. 34) from fresh produce has been proven to be blanching and UV treatment method. *L. monocytogenes* (Fig. 35) still remained abundant after day 6, in all the spinach samples tested with no differences compared to the control after refrigeration. *L. monocytogenes* was also detected in the blanched cabbage and lettuce samples after refrigeration. Refrigeration has always been used as a means of controlling the spoilage of produce, by retarding the growth of microorganisms (Cameron *et al.*, 1994). This study has, however, proven that in the majority of the samples tested, the bacterial counts had increased after refrigeration.

The most effective method of reducing the microbial counts of *Salmonella* spp. (Fig. 36) was UV light. Researchers have shown that *Salmonella* spp. cells are able to attach to fresh produce and form biofilms, thus, protecting them from harsh washing conditions during post-harvest treatment (Core, 2005). *Shigella* spp. (Fig. 37) was found abundant in all fresh produce tested. UV treatment was found to be the most effective in reducing *Shigella* spp. but only after refrigeration of the lettuce and spinach samples. This treatment was the most effective method for the reduction of *Shigella* spp. from cabbage. However, this method was not effective against this organism in the broccoli sample. In terms of the THB (Fig. 38) counts of lettuce and spinach produce, the most effective method for the reduction of the THB is UV treatment. For cabbage samples the most effective method for the reduction of THB was blanching followed by UV treatment. For the reduction of THB from broccoli samples the most effective method was blanching. Overall, it can be seen that UV treatment has been the most effective in reducing the microbial content of fresh produce. Fonseca and Rushing (2006) demonstrated the effect of chlorine, ozone and UV-light on fresh cut watermelon cubes, with the latter treatment method being non-aqueous. It was evident in this study that the aqueous treatment methods used were not effective in reducing microbial counts as compared to the non-aqueous method, UV-light, with the quality of the cubes also being lower when the aqueous methods were used (Fonseca and Rushing, 2006). UV-C treatments have previously been shown to reduce the microbial populations in fresh processed vegetables (Allende and Artes, 2003; Lemoine *et al*., 2007).

The hydrogen peroxide treatment method did cause a reduction in the microbial counts after treatment. Hydrogen peroxide in this case was shown to be more effective in reducing microbial pathogens than the chlorine treatment method. Chlorinated water is currently being used industrially in packing-houses for the purpose of sanitizing fresh produce in order to reduce the post-harvest decay of fresh produce. Hypochlorite treatments (pH 6), significantly reduced the microbial counts of fresh cut muskmelons stored at 2ºC. However, chlorine solutions have been found to be less effective or completely ineffective towards the bacterial pathogen, *L. monocytogenes* (Ayhan *et al*., 1998; Beuchat and Brackett, 1990). In this study it was found that the effectiveness of this chlorine treatment was limited. However, this treatment was effective against Coliforms in the cabbage and lettuce treated samples. Even though chlorine treatment is widely known, the potential hazards that have been associated with chlorine reaction by-products as well as issues regarding the disposal of wastewater, have led to evaluation of other possible methods for fresh produce disinfection (Suslow, 2000). Tap water was shown to be less effective than chlorine. Adams *et al*. (1989) reported a 92.4% reduction of the lettuce leaf microflora after washing with tap water. Han *et al*. (2000) suggested that water washing alone is not sufficient to remove bacteria that are tightly attached to injured surfaces of vegetables, in this case green peppers. In this study, it was also evident that washing produce with tap water alone was not sufficient for the removal of microbial pathogens. Hassenberg and Idler (2005) found that after six days of storage at 4°C, a four log increase in the microbial counts had been observed after the initial treatment of washing with tap water. Similarly, an increase in the majority of the tap water washed samples had been observed after refrigeration. The NaCl treatment method showed similar patterns to the tap water treated samples throughout this study.

Sapers *et al*. (2000, 2001) found that the effectiveness of the washing method depends on the time interval between the contamination event and the time of washing. Also, when bacteria attach to the surfaces of fresh produce they are likely to move into pores, indentations or irregularities found on the surface of produce, thus limiting their exposure to the washing treatment (Seo and Frank, 1999). Han *et al.* (2000) noted that *E. coli* O157:H7 appeared to not have penetrated the intact surface of the vegetable (green pepper), however, this bacterium seemed to attach to coarse, porous and injured surfaces.

Sapers (2001) noted that it must be realised that the conventional washing technology was developed in order to remove soil from the fresh produce and not microorganisms, and even with the use of newer sanitizing agents “improvements in efficacy have been incremental”. However, UV treatments in this study have caused a large decrease in microbial counts, but using this method, bacterial colonies were still detected, in some cases, as being “too few to count” (less than 30), but this may not be enough to ensure the safety of the product (Fonseca and Rushing, 2006).

3.2.9 VALIDATION OF AN OPTIMISED VIRUS RECOVERY METHOD AND MULTIPLEX REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION OF SELECTED ENTERIC VIRUSES ON "MOCK IRRIGATED" STRAWBERRY SAMPLES

Specific aim

Optimisation of a virus recovery method for the isolation of viruses from fresh produce (strawberries) and multiplex real-time reverse transcriptase polymerase chain reaction assays for the detection of selected enteric viruses.

Experimental procedures

Strawberry samples

Strawberries were purchased from a commercial outlet and three strawberries were randomly selected for each experiment, with an additional three strawberries used for each control. Prior to seeding and viral recovery the stem and leaves were carefully removed from the base of the strawberry.

Virus stock

Hepatitis A virus **(HAV):** The cell culture-adapted strain HM-175 43c (1 x 10^8 $TCID_{50}$/mℓ) used was supplied by Prof A Bosch of the Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain and further propagated and titrated in FRHK-4 cell cultures.

Human astrovirus **(HAstV):** The virus stock (Lab number: 1918761) was obtained from a clinical specimen referred to the Diagnostic Laboratory, NHLS Tshwane Academic Division for the analysis of gastroenteritis viruses.

Mengovirus**:** The viral stock culture (passage 4; 1.5 x 10^7 $TCID_{50}$/mℓ) was supplied by Prof A Bosch of the Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain and further propagated and titrated in the Vero African green monkey kidney cell line and stored at -70º.

Norovirus GI **(NoV GI):** Two different stocks of virus are available. Both stocks originated from clinical specimens, the first from an outbreak of waterborne gastroenteritis and the second was obtained from a stool specimen referred to the Diagnostic Laboratory, NHLS Tshwane Academic Division for the analysis of gastroenteritis viruses.

Norovirus GII **(NoV GII):** The viral stock originated from a clinical strain detected in the stool specimen from a patient after an outbreak of gastroenteritis on a cruise ship. The virus was typed by Dr M Wolfaardt from the Enteric Virus and Environmental Research Group, Department of Medical Virology, University of Pretoria and quantified by Prof A Bosch and co-workers, Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain.

Sapovirus **(SaV):** The virus stock (Lab number: 1383767) was obtained from a clinical specimen referred to the Diagnostic Laboratory, NHLS Tshwane Academic Division for the analysis of gastroenteritis viruses.

Simian rotavirus **(SA11):** A simian rotavirus strain (ATCCVR-899) that was propagated and titrated in an African green monkey kidney cell line MA104, was used as a surrogate for human rotavirus (HRV).

Positive control: A 1 mℓ viral suspension of the abovementioned viruses was prepared by mixing a 100 µℓ of the stock solution of each virus, which was then made up to a final volume of 1 mℓ with nuclease free water (Promega Corp., Madison WI). The concentrations of the respective viruses, as determined using *rt* RT-PCR, are presented in Table 19.

Table 19 Concentrations of the selected enteric viruses used for the artificial contamination of strawberries

Virus	Concentration: copies per mℓ	Concentration: copies per 20 µℓ
Hepatitis A virus	1.0×10^7	2.0×10^5
Human astrovirus	6.7×10^8	1.3×10^7
Norovirus GI	4.9×10^8	9.8×10^6
Norovirus GII	2.9×10^8	5.7×10^6
Sapovirus	2.8×10^9	5.6×10^7
Simian rotavirus	1.9×10^8	3.9×10^6
Mengovirus	2.0×10^6	4.0×10^4

"Mock irrigation" water

Stored (at 4°C) surface water samples from the Rietspruit River (RV), Gauteng, referred for testing over a four month period and which were shown to contain one or more enteric viruses, namely HAV, HAstV, NoV GI, NoV GII, HRV and SaV, using commercial singleplex *rt* RT-PCR assays as "mock" irrigation water samples. The stored water sample was shaken thoroughly and 100 mℓ was dispensed into a container for use. A 100 mℓ volume of distilled water was dispensed and served as a negative "mock irrigation" water control.

"Mock irrigation" of strawberries with river water

The strawberries were impaled, at their base, onto a disposable inoculation loop (Thermo Fisher Scientific, Rochester, NY). Impaled strawberries were immersed ten times into the 100 mℓ container filled with the "mock irrigation" river water sample. As a negative control three strawberries were immersed ten times into a container filled with sterile distilled water. As a positive control three strawberries were seeded with 20 µℓ of the virus stock positive control. The inoculation loops were firmly placed into a polystyrene base with the "irrigated" strawberries in the air. To prevent cross contamination the negative and positive control strawberries were placed onto a separate polystyrene base to the experimental strawberries. The experimental and positive control strawberries were seeded with 10 µℓ of the mengovirus (process control) and dried at room temperature (~25˚C) in a dead box until all the water drops had evaporated (~ 1-2 h).

Viral recovery and concentration

The individual berries were immersed in 30 mℓ elution buffer (50 mM glycine-3% beef extract (GBE) buffer; pH 7.2) in a 50 mℓ centrifuge tube (BD Biosciences, Franklin Lakes, NJ) and shaken gently at room temperature for 20 minutes. After elution the berries were removed from the buffer and the pH of the eluate was adjusted to pH 7 using 1 M hydrochloric acid (Merck, Darmstadt, Germany) or 1 M sodium hydroxide (Merck) as

required. Pectinase (Pectinex® Ultra SPL, Sigma, Buchs Switzerland) was added to a final concentration of 5 µℓ/mℓ and the solution was mixed by inverting the tube several times and gently shaken at 60 rpm (Labnet 211DS Shaker Incubator: Labnet International Inc.) for 30 min at room temperature (25˚C). The eluted viruses were then concentrated to a final volume of 5 mℓ in phosphate buffered saline pH 7.2 (Sigma-Aldrich Inc., St. Louis MO), by polyethylene glycol $_{8000}$ (Amresco, Solon, OH)/sodium chloride (Merck) precipitation using the method recommended by the European Committee of Standardisation (CEN) Technical Committee (2010).

Virus detection

Nucleic acid extraction: Nucleic acid was extracted from the recovered virus concentrates using two different technologies. Genomic viral nucleic acid was extracted from 1 mℓ of the recovered virus concentrate using the Nuclisens® easyMAG™ automated magnetic extraction platform (Boom method) (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's instructions and from a second 1 mℓ aliquot using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC Robotic instrument (Roche), following the manufacturer's instructions. Nucleic acid was eluted in 100 µℓ, aliquoted and stored at -20°C.

Viral amplification and detection: Optimised qualitative multiplex *rt* RT-PCR assays based on TaqMan technology and published primers and probes (Table 20) were applied for the detection of the selected enteric viruses.

Briefly complementary DNA (cDNA) was prepared using RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences, Burlington, Ontario) according to the manufacturer's instructions. Amplification was performed using the EXPRESS qPCR Universal Mix (Invitrogen, Carlsbad, CA), primers (4 µM), probe (2 µM) and 5 µℓ cDNA in a final volume of 20 µℓ. The reactions were carried out in MicroAmp™ Optical 96-well reaction plates (Applied Biosystem, Foster City, CA) in a 7300 Real Time PCR System (Applied Biosystem). An extraction negative control (nuclease-free water; Promega Corp), a *rt* RT-PCR negative control and a *rt* RT-PCR positive control, nucleic acid extracted from the positive control, were included in each assay. The samples were initially screened with Reaction C (HAstV, process control [mengovirus] and IAC) to control for efficient recovery and to validate amplification reactions. Dependent on these results ten-fold dilutions of the nucleic acid were prepared to dilute and minimise the effect of inhibitory substances and then re-tested. Thereafter samples were screened with Reactions A and B at the appropriate nucleic acid concentration.

Table 20 Primers, probes, fluorophores and virus combinations used in the three optimised multiplex real-time reverse transcriptase polymerase chain reactions

Multiplex reactions/ viruses	Forward primer	Reverse primer	Probe	Fluorophore	Reference
REACTION A					
Norovirus GII	QNIF2	COG2R	QNIFS	FAM	Loisy *et al*. (2005) Kageyama *et al*. (2003)
Hepatitis A virus	HAV 68	HAV 240	HAV 150	NED	Costafreda *et al*. (2006)
IAC*	QNIF2	COG2R	FP6	JOE/VIC	This study
REACTION B					
Norovirus GI	QNIF4	NVILCR	NVILCpr	VIC	Svraka *et al*. (2007) Da Silva *et al*. (2007)
Human rotavirus	RotaF	RotaR	Rota pr	FAM	Zeng *et al*. (2008)
Sapovirus	CU-SV-F1 CU-SV-F2	CU-SV-R	CU-SV-Pr	NED	Chan *et al*. (2006)
REACTION C					
Human astrovirus	AV2	AV1	AVs	NED	Le Cann *et al*. (2004)
IAC	QNIF2	COG2R	FP6	JOE/VIC	This study
Mengovirus	Mengo 110	Mengo 209	Mengo 147	FAM	Pintó *et al*. (2006)

* Internal Amplification Control

Results and discussion

Initial results (not shown) indicated that the process control and IAC were not detected by the Reaction A multiplex *rt* RT-PCR assay when nucleic acid had been extracted using the Nuclisens® easyMAG™ automated magnetic extraction platform (bioMérieux SA). The reason for this observation is unclear and needs further investigation. Consequently results were only available for nucleic acid extracted using the MagNA Pure LC Robotic instrument (Roche). The virus detection results using the three multiplex *rt* RT-PCR assays are presented in Table 21. The detection of mengovirus (process control) from all the strawberries, except Berry B irrigated with water dated 2012-04-23, demonstrated that the viral recovery and nucleic acid extraction processes were optimal and successful. The detection of the IAC and mengovirus in reactions where no other viruses were detected indicates that the amplification reaction was not inhibited and the detection results for the enteric viruses are not false negative. As no viruses were detected in the negative control, no cross contamination occurred during the analytical process. The results using water samples 2012-05-21 and 2012-07-23 for “mock irrigation” clearly indicate that enteric viruses such as RV (SA11) and NoV GI present in irrigation water can be transferred to and detected on soft fruits. It is also evident from the data obtained from strawberries “mock irrigated” with water

sample dated 2012-06-18 that enteric viruses may be present at undetectable levels in the water sample but could still result in contamination of irrigated soft fruits.

Table 21 Summary of the virus detection results from the "mock irrigated" strawberries and the river water samples used to "mock irrigate" the strawberries using the optimised multiplex real-time reverse transcription-polymerase chain reaction assays.

Water Sample	Berry	Mengo	IAC**	HAstV	HAV	SA11	NoV GI	NoV GII	SaV
RV 2012-04-23 Control	A	+	+	-	-	-	-	-	-
	B	-	+	-	-	-	-	-	-
	C	+	+	-	-	-	-	-	-
		nd*	+	+	-	-	-	-	-
RV 2012-05-21 Control	A	+	+	-	-	-	-	-	-
	B	+	+	-	-	-	-	-	-
	C	+	+	-	-	-	+	-	-
		nd	+	+	-	-	+	-	-
RV 2012-06-18 Control	A	+	+	-	-	-	-	-	-
	B	+	+	-	-	-	-	-	-
	C	+	+	+	-	+	+	-	-
		nd	+	-	-	-	-	-	-
RV 2012-07-23 Control	A	+	+	-	-	+	+	-	-
	B	+	+	-	-	+	+	-	-
	C	+	+	-	-	+	+	-	-
		nd	+	+	-	+	+	-	-
Positive control	A/B/C	+	+	+	+	+	+	+	+
Negative control	A/B/C	nd	+	-	-	-	-	-	-

***** nd = No process control added

****** IAC = Internal amplification control

From these experiments, where optimised virus recovery methods and multiplex *rt* RT-PCR assays were applied, it is evident that faecally contaminated surface water used for irrigation purposes has the potential to contaminate soft fruit. NoV GI was detected more frequently than NoV GII which is in agreement with reports and was relevant as NoV GI is more frequently associated with food and water outbreaks (Mattison *et al*., 2010). The methods applied to demonstrate this contamination used appropriate quality control procedures and were therefore validated.

3.2.10 ATTACHMENT AND SURVIVAL OF *SALMONELLA* TYPHIMURIUM ON CITRUS FRUIT

Specific aim

The objectives of this study were to determine whether *Salmonella* Typhimurium can attach to and colonize lemon fruit surfaces and to assess the survival and/growth of the pathogen under a simulated export chain.

Experimental procedures

Test organism and inoculum preparation

American Type Culture Collection (ATCC) culture *Salmonella enterica* subsp. *enterica* serovar Typhimurium was used as a reference culture in this study. A stock culture maintained lyophilized and stored at -70^{o}C was activated by streaking on standard 1 medium (Merck, Johannesburg, South Africa) and incubated at 37^{o}C for 24 hours. One isolated colony was transferred to 100 mℓ tryptone soy broth supplemented with 0.5% yeast extract (TSBYE) and incubated at 37^{o}C for 18 hours in a shake incubator (150 rpm) to achieve 9 log CFU/mℓ. Cells were harvested by centrifugation (9 000rpm, 5 min), washed twice with 0.1% (w/v) buffered peptone water (BPW) and pellets were re-suspended in 1 mℓ of sterile PBW. A 10 fold serial dilution in 0.1% PBW was performed to achieve the desired initial inoculum level of approximately 7 log CFU/mℓ verified by enumeration on Standard-1 agar.

Lemon fruit

Lemon fruit freshly harvested from a commercial orchard in the Eastern Cape Province of South Africa were shipped overnight to Plant Pathology Laboratories and stored in a cold room at 4^{o}C. Fruit was transferred to room temperature the night before experimental use. Waxed fruit was also collected at the packhouse on the same farm and used for export simulation. Unwaxed fruit was used to determine attachment and colonization of *Salmonella* onto lemon fruit surfaces using scanning electron microscopy (SEM). This set of fruit was surface sterilized with a 30-s dip in 70% ethanol followed by air drying in a biohazard safety cabinet. Waxed fruit was used to study the survival of *Salmonella* under a simulated export chain and was surface sterilised by washing in 0.05% (v/v) sodium hypochlorite for 30s, rinsed twice with sterile distilled water and air dried in a biohazard safety cabinet.

Inoculation procedure for SEM

Lemon fruit were spot-inoculated (ca. 7 Log CFU/mℓ) on a surface area (5 by 5 mm) that was marked with a felt pen. A 50µl cell suspension was distributed in drops on the surface of the fruit at room temperature. For attachment studies, the culture was aspirated at respective time intervals (1, 5, 20 min and 1, 4 and 24h). The inoculated area was then rinsed with 100µℓ of sterile distilled water, excised with a sterile blade and immediately processed for SEM. Fruit inoculated with sterile distilled water was used as negative controls.

Preparation of samples for SEM examination

Samples were prepared for SEM according to the procedure by Getz *et al*. (1983) with some modifications. Inoculated sections of the lemon fruit were fixed overnight in 1 mℓ of fixing solution consisting of 25% glutaraldehyde in 0.075 M phosphate buffer mixed according to

Coetzee and Van der Merwe (1994). Samples were rinsed three times in 0.075 M phosphate buffer for 15 min each, followed by successive 15 min dehydration rinses in 50, 70 and 90% ethanol. Finally samples were dehydrated for 15 min in100% ethanol with the last rinse left overnight before critical-point drying in a Biorad dryer under liquid carbon-dioxide. Following drying, samples were mounted with nonconductive tape, carbon coated and examined under a Zeiss scanning electron microscope operating 1 kV.

Survival of Salmonella under a simulated export chain

To simulate survival potential of *Salmonella* under simulated export conditions, 36 waxed lemon fruits were inoculated as described for attachment studies. Inoculated fruit was distributed equally into citrus carton boxes sterilised by spraying with 70% ethanol and stored at room temperature (24±1^{o}C) for one week to simulate transport conditions from packing house to port. Lemons were prevented from rolling over by securing with adhesive tape at the stylar end. The remaining fruit (27) was transferred to a cold room and stored at 4±2^{o}C for two weeks to simulate export conditions. After 14 days of cold storage, the remaining fruit was placed at room temperature (24±1^{o}C) to simulate market conditions.

Enumeration of Salmonella

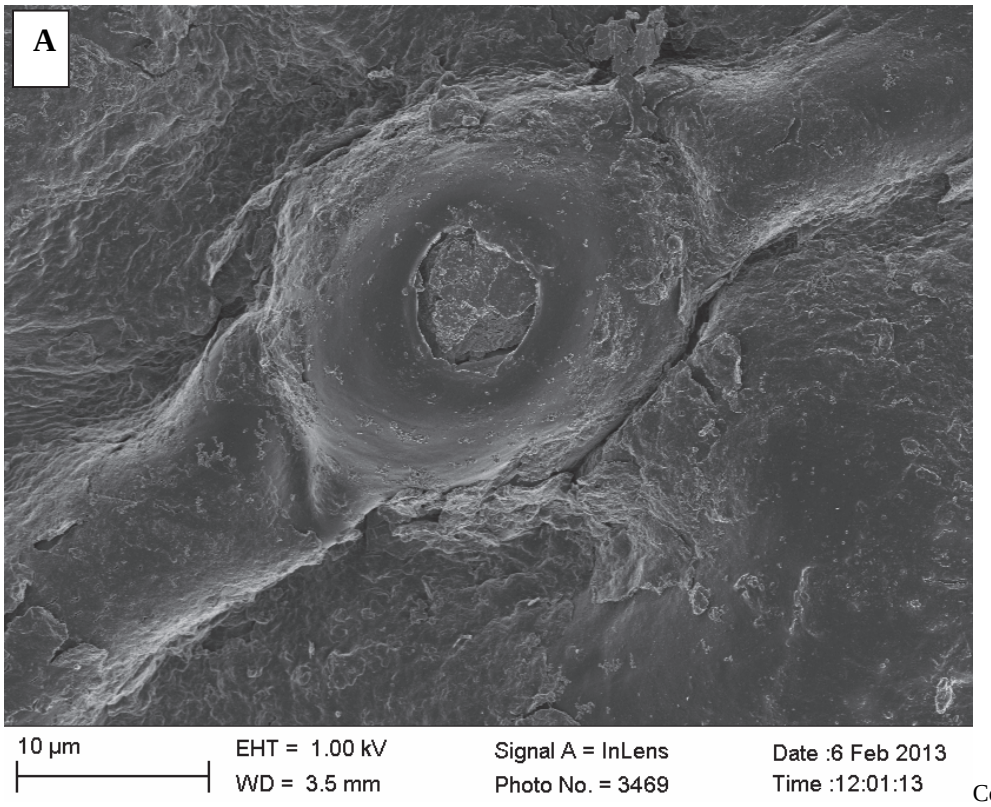
Nine lemons (three fruit per replication) were randomly removed from the boxes at weekly intervals and used to quantify *Salmonella* survivors under each time/temperature regime. Using an ultrasonic bath (Labotec, Johannesburg, South Africa), fruit were washed for five minutes in sterile 2ℓ beakers containing 1ℓ quarter-strength Ringer's solution supplemented with 0.02% Tween 80 (v/v). Washings were filtered through a 0.45µm nitrocellulose membrane using a vacuum filtration system. The whole filter paper was transferred to a 9 mℓ tryptone soy broth (TSB) tube and thoroughly mixed by vortexing to dislodge any microorganism from the filter paper. Ten-fold serial dilutions were then conducted in 0.1% peptone buffered water followed by spread plating 0.1 mℓ aliquots in duplicate onto xylose lysine desoxycholate (XLD) agar for the enumeration and isolation of *Salmonella*. Plates were incubated for 48 hours at 37^{o}C after which numbers of colonies were counted and reported as (CFU)/cm^{2}. Volume displaced (vd) for each fruit was recorded and used to calculate fruit surface area using the following formula: $A = 4.84[(vd)^{1/3}]^{2}$. To determine whether the treatments (time/temperature regimes) had caused a bacteriostatic or bactericidal effect on the pathogen during storage, TSB tubes for each time interval were incubated overnight for enrichment of possible survivors at 37^{o}C. One millimetre of the enriched TSB was inoculated into 9 mℓ of Rappaport broth for the selective enrichment of *Salmonella*, incubated at 42^{o}C for 24h followed by streaking on XLD agar. Confirmation of bacterial colonies was performed by matrix assisted laser desorption ionisation-time of flight (MALDI-TOF).

Results and discussion

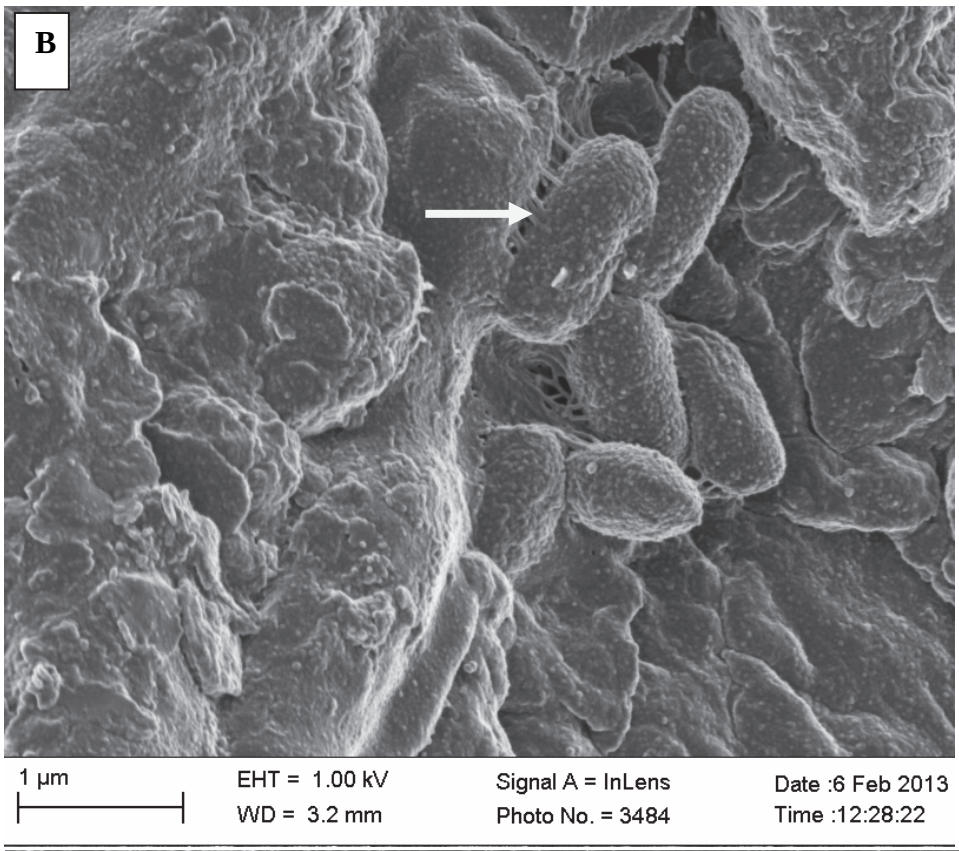
Attachment and colonization studies

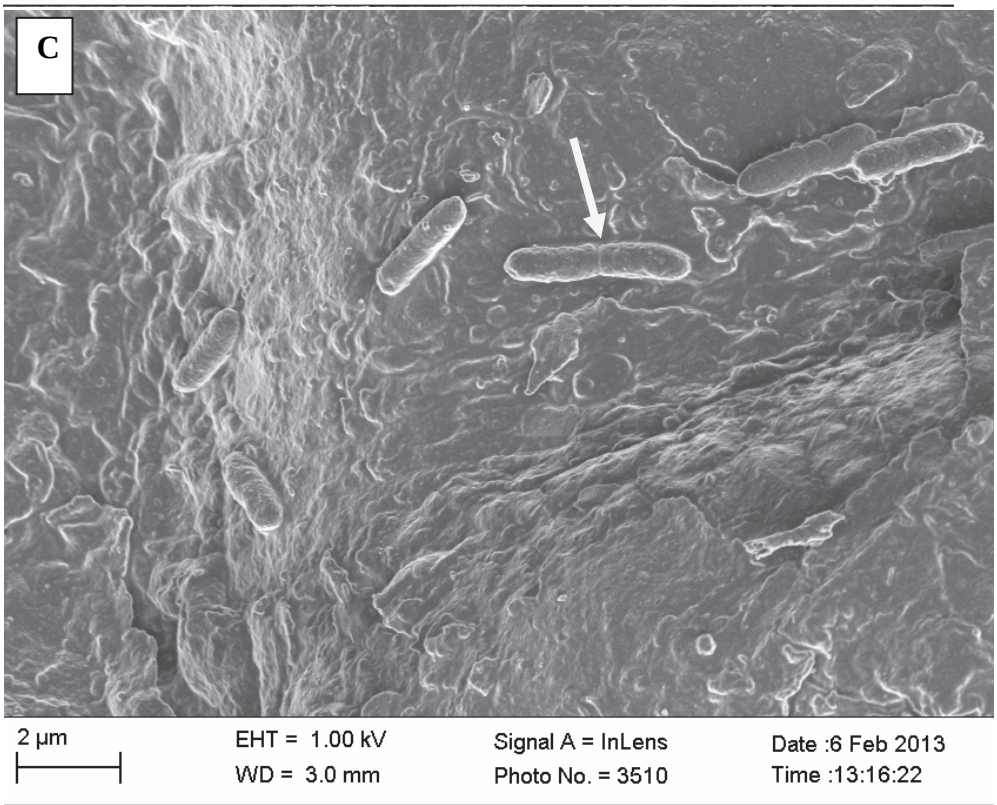
After only one minute exposure of *Salmonella* cells on lemon surfaces, thin fibres that are generally associated with the production of extracellular polymers (Iturriaga *et al.*, 2007), were observed (Fig. 39B). Similar results were observed on *Salmonella* Typhimurium inoculated on orange surfaces (unpublished data). *Salmonella* cells were observed dividing after 20 minutes exposure on lemon surfaces suggesting that it took only a few minutes for the

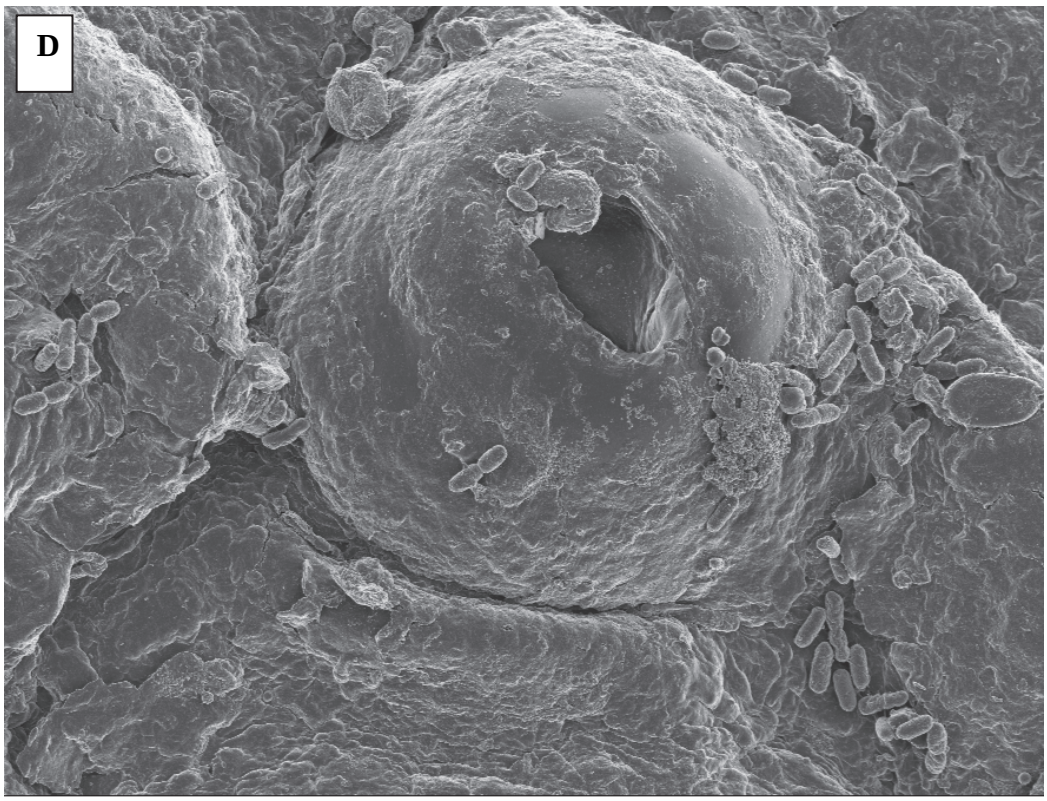
bacteria to adapt to the new environment before starting to grow (Fig. 39C). A lower growth rate of *Salmonella* Montevideo on red, ripe tomatoes was reported by Iturriaga *et al.* (2007) as the bacteria started multiplication only after 90 minutes. On the other hand, *Salmonella* Typhimurium significantly increased on peaches and plums 30s post-inoculation (Collignon and Korsten, 2010). Differences in the behaviour of *Salmonella* could be due to several factors, including the plant environments and the strains used. Scanning electron micrographs revealed an obvious direct proportion between cell densities and exposure time, although populations were not enumerated. Higher cell densities were observed on lemon surfaces exposed for 4 hours compared with those exposed for 1 hour (Fig. 39D, E). Duffy *et al.* (2005) observed similar trends with mixed *Salmonella* strains (Anatum, Javiana and Rubislaw) on parsley surfaces. Biofilm development could be seen as early as 4 hours and at 24 hours, a well-defined biofilm containing numerous bacteria was present. As reported by Iturriaga *et al*. (2007), *Salmonella* Montevideo only formed well-defined biofilms after 4 days on red, ripe tomatoes. Studies have shown that *Salmonella* normally forms better biofilms in nutrient-limited environments than in nutrient-rich environments (Stepanovic *et al*., 2004). Cells were observed near the oil glands of the lemon skin and within cracks of the waxy cuticle (Fig. 39 E, F).

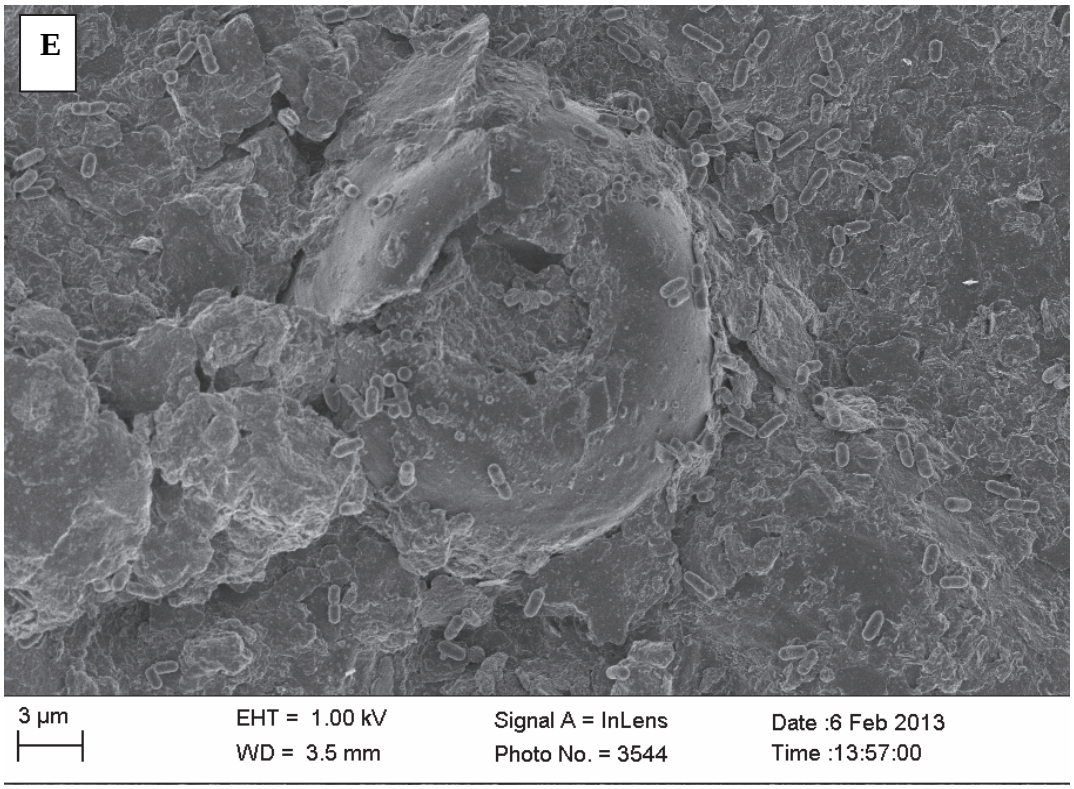


Control


B
1 µm
EHT = 1.00 kV
WD = 3.2 mm
Signal A = InLens
Photo No. = 3484
Date :6 Feb 2013
Time :12:28:22


C
2 µm
EHT = 1.00 kV
WD = 3.0 mm
Signal A = InLens
Photo No. = 3510
Date :6 Feb 2013
Time :13:16:22


D
2 µm
EHT = 1.00 kV
WD = 3.2 mm
Signal A = InLens
Photo No. = 3526
Date :6 Feb 2013
Time :13:36:17


E
3 µm
EHT = 1.00 kV
WD = 3.5 mm
Signal A = InLens
Photo No. = 3544
Date :6 Feb 2013
Time :13:57:00

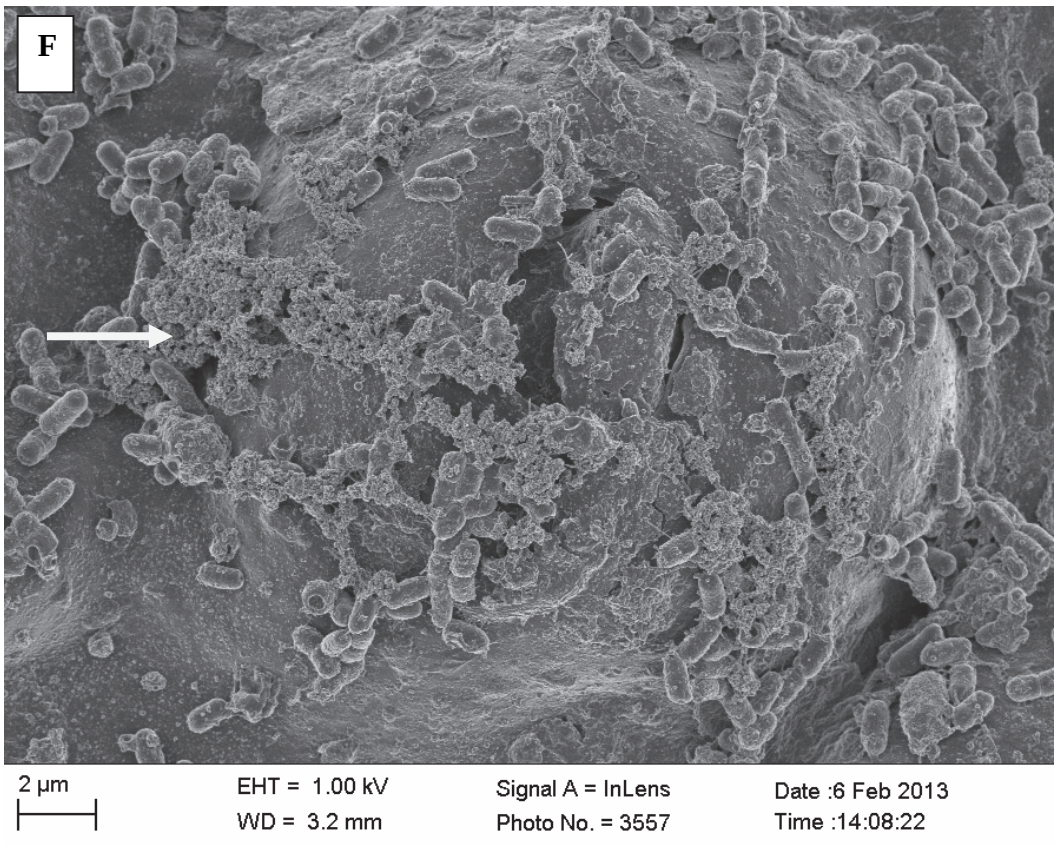


Figure 39 (A-F). Scanning electron micrographs of *Salmonella enterica* sv. Typhimurium cells on lemon surfaces at different time exposures. (A) Control – lemon surface. (B) 1 min, arrow indicating attachment structures. (C) 20 min, arrow indicating dividing cell. (D) 1 h. (E) 4h, extensive cell division and cell density. (E) 24h, arrow indicating biofilm formation.

Survival of Salmonella Typhimurium *under a simulated export chain*

Salmonella Typhimurium was not detected on lemon fruit stored under a simulated export chain at all time intervals under a simulated export chain. However, the bacteria were isolated following an enrichment step in TSB and Rappaport at 37^{o}C for 24 hours up to week 3, indicating that the cold storage conditions in this study did not result in 100% reduction of *Salmonella* cells. This result indicates that given the right conditions, even very low numbers of *Salmonella* Typhimurium could represent a significant health risk. On the other hand, *Salmonella* Typhimurium was detected at week 4 either directly or after enrichment in Rappaport selective broth for *Salmonella*.

Conclusion

Deposition of *Salmonella* Typhimurium on the surface of lemon fruit could result in attachment and subsequent colonization under suitable conditions. It can be concluded that under favourable environmental conditions, biofilm formation by *Salmonella* Typhimurium on lemon surfaces cannot be ruled out. These biofilms can provide a protective environment for pathogens and reduce the effectiveness of decontamination strategies. Although *Salmonella* Typhimurium was able to attach and colonize lemon surfaces, this pathogen was not able to survive a simulated export chain.

3.3 SUMMARY OF RESEARCH FINDINGS

Impact of environmental and nutritional parameters on the survival and growth kinetics of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium

This study revealed that the survival of both *E. coli* O157:H7 and *S.* Typhimurium decreased proportionally with an increase in the concentration of NaCl (1%, 3% and 5%). At a concentration of 1% NaCl no inhibition of growth was detected for both organisms and *S.* Typhimurium survived better than *E. coli* O157:H7 at all 3 NaCl concentrations. Both *E. coli* O157:H7 and *S.* Typhimurium survived best at pH 7 and an alkaline pH (pH 9) favoured the survival of both bacteria over that of an acidic pH (pH 4).

Effect of iron on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water was tested at three different iron concentrations, i.e. 10 mg/ℓ, 20 mg/ℓ and 30 mg/ℓ. There was a progressive increase in the *E. coli* O157:H7 and *S.* Typhimurium numbers under all iron concentrations with 30 mg/ℓ iron resulting in the highest increase from 4.7 log CFU/mℓ to 5.1 log CFU/mℓ in one day. These findings are of major concern since it has been reported that an increase in the iron concentration markedly stimulates the toxigenicity of some strains of *E. coli* O157:H7.

Effect of nitrate on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water was tested at three different nitrate concentrations, i.e. 10 mg/ℓ, 20 mg/ℓ and 30 mg/ℓ. In this study *E. coli* O157:H7 survived best at low concentrations of nitrate (10 mg/ℓ); however, there was a decrease in growth after day 5. At higher nitrate concentrations, survival decreased from day 4 and day 2 at 20 and 30 mg/ℓ, respectively. In the control irrigation water which contained 6 mg/ℓ nitrate, *E. coli* O157:H7 survival was found to be most favourable with a gradual increase in growth throughout the survival study. This correlates with the findings of Whiting and Golden (2003), who reported that an increase in nitrate concentration decreases the survival time of *E. coli* O157:H7 and *S.* Typhimurium.

S. Typhimurium survived equally well when the irrigation water was supplemented with low concentrations of nitrate as compared to the higher nitrate concentrations.

Additionally the effect of the combination of pH, NaCl and iron on the growth and survival of *E. coli* O157:H7 and *S.* Typhimurium in irrigation water was determined. The combination of a high salt concentration (5%) and low pH (pH 4) produced a synergistic effect on the survival of both organisms, as compared to the individual parameters. It was found that both *E. coli* O157:H7 and *S.* Typhimurium survived and increased in growth throughout the study period of 10 days. The second combination of physicochemical factors tested was high pH (pH 9) and low iron concentration (10 mg/ℓ). The results demonstrated that *E. coli* O157:H7 survived well under these conditions as compared to the individual conditions. This study also showed that *E. coli* cells survived at pH 9 for up to 11 days, but when iron was added, *E. coli* cells survived up to 30 days.

The effect of irrigation methods (drip, sprinkler and flood) on the surface contamination of fresh produce

The degree of contamination by *S.* Typhimurium and *L. monocytogenes* on the surface of lettuce irrigated via different irrigation methods was assessed over a period of three weeks. Flood irrigated lettuce leaves showed the highest levels of contamination with a maximum of 3.95 log CFU/g for *S.* Typhimurium and 4.33 log CFU /g for *L. monocytogenes* obtained by the third week. This was followed by sprinkler irrigation that resulted in a contamination level of 3.79 log CFU/g for *S.* Typhimurium and 4.04 log CFU/g for *L. monocytogenes* on lettuce leaves. By contrast, drip irrigated lettuce leaves showed a minimal amount of contamination, resulting in >19-fold reduction for *S.* Typhimurium and >53-fold reduction of *L. monocytogenes* as compared to flood irrigated lettuce leaves. The population of *L. monocytogenes* was two-fold greater than that observed for *S.* Typhimurium on lettuce leaves.

The degree of contamination by *S.* Typhimurium on the surface of tomatoes irrigated via different irrigation methods was assessed over the three week duration. From the results obtained, the highest numbers of *S.* Typhimurium (3.16 log CFU /g) and *L. monocytogenes* (3.21 log CFU/g) were observed on sprinkler irrigated tomatoes; followed by flood irrigated tomatoes with a contamination level of 2.87 log CFU/g of *S.* Typhimurium and 2.17 log CFU/g of *L. monocytogenes.* Drip irrigation caused minimal contamination of tomatoes and resulted in >14-fold reduction of *S.* Typhimurium compared to sprinkler irrigation, with no contamination by *L. monocytogenes* observed in weeks one and two, and minimal contamination of 2 log CFU/g at week three. Previous reports suggest that irrigation methods that do not allow for direct contact of the contaminated water with the edible regions of the plant, facilitate a lower risk of contamination. This is evident in the results of the current study. Drip irrigation allows for minimal contact between contaminated irrigation water and edible regions of the plant and is also water efficient. However, flood irrigated lettuce plants are more susceptible to surface contamination via irrigation because they are low-lying and have a large surface area and deep crevices.

Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* enterica subsp. enterica serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants

The internalisation potential of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. enterica* subsp. *enterica* serovar Typhimurium in lettuce was evaluated using seedlings grown in vermiculite in seedling trays as well as hydroponically grown lettuce. Sterile distilled water was spiked with either one of the four human pathogenic bacteria (10^5/mℓ) and used to irrigate the plants. The potential for pathogen internalisation was investigated over time using light microscopy, transmission electron microscopy and viable plate counts. Additionally, the identities of the pathogens isolated from internal lettuce plant tissues were confirmed, using PCR with pathogen specific oligonucleotides. Internalisation of each of the human pathogens was evident in both lettuce seedlings and hydroponically grown mature lettuce plants. To our knowledge this is the first report of *S. aureus* internalisation in lettuce plants. In addition, the levels of background microflora in the lettuce plants were determined by plate counting and

the isolates identified using Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF). Background microflora assessments confirmed the absence of the four pathogens evaluated in this study. A low titre of endophytes and soil inhabitants, i.e.: *Enterobacter cloacae, Enterococcus faecalis, Lysinibacillus fusiformis, Rhodococcus rhodochrous, Staphylococcus epidermidis* and *Staphylococcus hominis* were identified.

Growth dynamics of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* under different nutrient and temperature conditions

Foodborne pathogens potentially associated with fresh produce can cause a number of diseases that affect the well-being of consumers. An important control factor in food safety assurance systems is the use of temperature to regulate and control the growth of these microorganisms. In the stone fruit supply chain, the fruit and contact surfaces are exposed to three temperatures, 0.5^{o}C, 4^{o}C and 21^{o}C. If these foodborne pathogens are able to survive and grow at these temperatures, control strategies need to be improved to prevent contamination of fresh produce and contact surfaces. The aim of this study was to determine if temperature plays a role in the growth dynamics of *E. coli* O157:H7, *L. monocytogenes, S. enterica* subsp. *Enterica* serovar Typhimurium and *S. aureus* type cultures inoculated on fruit surfaces, under different simulated nutrient conditions and environments (on nutrient-free surfaces using tiles as a contact surface sample). Prepared cultures of *E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium and *S. aureus* were used to inoculate peaches, plums, nutrient-rich tryptone soy broth, nutrient-poor tryptone soy broth and tiles. Once inoculated, the fruit and broth were incubated at fluctuating temperatures and the broths and tiles were incubated at either 0.5^{o}C, 4^{o}C or 21^{o}C for six days to determine the growth dynamics of the four pathogens under different simulated commercial conditions in nutrient-rich media over a six day incubation period. On peaches the pathogen titres remained constant and on plums the titres decreased. All pathogens either remained constant or decreased in titre when incubated at 0.5^{o}C in the nutrient-rich and -poor broths, except *L. monocytogenes* which was able to grow at 0.5^{o}C increasing from 4.95 log /cm^{2} on day 0 to 6.33 log /cm^{2} on day 6. The decrease in titre under cold storage conditions cannot be attributed to the limiting factor of temperature only, but possibly to the synergistic effect of temperature, pH and water activity that all influence the growth of these pathogens on fruit surfaces. All pathogen titres remained constant or increased at 4^{o}C when the nutrient content of the broth was optimal. *E. coli* O157:H7 counts ranged from 4.86 log /cm^{2} to 5.57 log /cm^{2} (4^{o}C) and grew actively at 21^{o}C with bacterial numbers ranging between 4.85 log /cm^{2} to 9.16 log /cm^{2} 21^{o}C. *L. monocytogenes* counts varied 4.99 log /cm^{2} to 8.54 log /cm^{2} (4 ^{o}C) and 4.93 log /cm^{2} to 9.19 log /cm^{2} at21^{o}C. *S.* Typhimurium counts ranged from 5.00 log /cm^{2} to 5.90 log /cm^{2} (4^{o}C) and 5.00 log /cm^{2} to 9.16 log /cm^{2} 21^{o}C and *S. aureus* counts ranged from 5.00 log /cm^{2} to 5.90 log /cm^{2} (4 ^{o}C) and 5.00 log /cm^{2} to 9.16 /cm^{2} 21^{o}C. All four pathogens were unable to grow but were able to survive on the tile surfaces at temperatures that simulate working environments and therefore could potentially lead to the contamination of food products coming into contact with such surfaces. It is imperative that contamination of fruit and contact surfaces be prevented because

survival and growth is possible at simulated commercial temperatures and environmental conditions.

The effect of chlorine blanching and freezing on *Cryptosporidium* associated with vegetables (broccoli)

Blanching and microwaving had the greatest effect on oocyst viability, with 93.2% and 93.5% of the oocysts being non-viable. Both blanching and microwaving were significantly more effective than blast freezing. After blast freezing, only 20.1% of the oocysts were non-viable. Chlorine at 200ppm had little effect on the viability of oocysts.

As this and previous studies have shown, reduction in oocysts load on vegetables during processing is difficult to achieve due to the protozoa's resistance to disinfectant. Only heat treatments seem to be effective at killing *Cryptosporidium* oocysts during the processing of fresh produce. It is thus very important to minimize contamination of fresh produce from the field, through processing and up to consumption to ensure safety to the consumer, especially if the produce is to be eaten raw without any subsequent heat treatment.

***Cryptosporidium parvum* (known concentration) effect of blanching, chlorine, freezing, microwaving on green pepper**

Cryptosporidium parvum oocysts have been found on the surface of vegetables in both developed and developing countries. *C. parvum* can contaminate vegetables via various routes, including irrigation water. This study investigated the effect of individual treatments of chlorine, blanching, blast freezing, and microwave heating, as well as combined treatments of chlorine and freezing, and chlorine and microwave heating on the viability of *C. parvum* oocysts inoculated on green peppers. The viability of the oocysts after the treatments was assessed using propidium iodide and a flow cytometer. Based on the propidium iodide staining, the chlorine treatments did not affect the viability of the oocysts. Blast freezing significantly inactivated 20% of the oocysts. Microwave heating and blanching significantly inactivated 93% of oocysts. Treatment with chlorine followed by blast freezing did not affect the viability of the oocysts significantly. Treatment with chlorine and microwave heating was significantly more effective than microwave heating alone and inactivated 98% of the oocysts. The study indicates that *C. parvum* oocysts are sensitive to heat and, to some extent, to blast freezing, but are resistant to chlorine. Therefore, the use of chlorine during vegetable processing is not a critical control point for *C. parvum* oocysts, and the consumption of raw or minimally processed vegetables may constitute a health risk as *C. parvum* oocysts can still be found viable on ready-to-eat, minimally processed vegetables.

The effect of minimal processing and cooking on the survival of *Listeria monocytogenes* on spray irrigated broccoli

The aim of this study was to determine the effect of minimal processing and subsequent cooking on the survival of *L. innocua* (surrogate for *L. monocytogenes*) on broccoli. Washing with water caused a 1 log reduction of the *L. innocua* numbers on broccoli to 4.23 log CFU/

whilst washing with 200 ppm chlorinated water facilitated a further 1 log reduction to 3.11 log CFU/g, as determined 30 minutes after exposure of the contaminated broccoli to the treatment. Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units to 3.5 log CFU/g. Microwave cooking had a lethal effect on *L. innocua* on broccoli, as no viable organisms were detected on the sample after treatment, causing a reduction of 3.37 log units. Combining chlorinated wash treatment, PVC storage and cooking induced a 68% reduction in *L. innocua* and a combined treatment of washing with chlorine, storage in modified atmosphere (MAP) (5% CO_2, 5% O_2) for two days at 4°C and final microwave heating resulted in the lowest pathogen numbers, causing a 5.13 CFU/g log reduction. Therefore, even though chlorine is effective in reducing *L. monocytogenes* during minimal processing, it does not suffice to eliminate pathogens from vegetables. In the same manner, MAP storage is only effective as part of a hurdle procedure. A final cooking step is essential in destroying *L. monocytogenes* present on broccoli and to ensure vegetables that are safe for consumption.

Effect of washing, preservative treatments and storage on the level of microbial contamination of fresh produce

Broccoli, cabbage, Crisphead lettuce and spinach were collected from a farm in Camperdown, KZN and the effect of washing in tap water, addition of NaCl, chlorine solution, blanching, H_2O_2, UV light and refrigeration on the microbial load of the fresh produce determined.

Viable plate counts showed that washing of produce using tap water alone or with the addition of NaCl did not reduce the number of viable bacteria. Additionally, it was determined that the presumptive *Campylobacter jejuni*, *Salmonella*, coliform, *Escherichia coli*, *Listeria monocytogenes* and *Shigella* numbers were also not reduced using these treatments. The effectiveness of the chlorine treatment was limited. However, this treatment was effective against coliforms in the broccoli and lettuce samples reducing the coliform levels from 4.5 log /g to zero on day 0. Although chlorine treatment is widely used, the potential hazards that have been associated with chlorine reaction by-products as well as issues regarding the disposal of wastewater, have led to the evaluation of other possible methods for fresh produce disinfection (Suslow, 2000).

The most effective method for the removal of coliforms from the fresh produce was found to be the blanching method, reducing the coliform levels on broccoli, lettuce and spinach from 4 log /g to 0 log /g on day 0. It was evident from this study that removal of *Campylobacter jejuni* from the four products tested was achieved by blanching and UV treatment methods reducing the numbers from an average of 4 log /g to 0 log /g for the respective treatments. *L. monocytogenes* was not detected on the broccoli before or after refrigeration. This organism was only detected after refrigeration of the chlorine treated and blanched cabbage samples at 3.51 log and 3.79 log /g respectively.

The viable bacterial numbers were reduced by Hydrogen peroxide treatment (H_2O_2) from an average of 6.8 log /g to 4 log /g. Coliform and *E. coli* numbers were also reduced from an average of 4.5 log /g to 0 log /g on day 0. However, *C. jejuni*, *Shigella* and *L. monocytogenes* were not reduced on any of the fresh produce samples using H_2O_2. *Salmonella* numbers were

however reduced from an average of 5 log /g to 3.5 log /g for the spinach samples and from 4 log /g to 0 log /g on the lettuce samples.

Overall, the UV treatment was the most effective in reducing the microbial content of fresh produce reducing the viable number of bacteria from an average of 6.8 log/g to 4 log/g, similar to the hydrogen peroxide treatment. *Salmonella* and *C. jejuni* and *E. coli* were completely inhibited after UV treatment, however coliform and *Shigella* inhibition varied depending on the specific produce being analysed. *L. monocytogenes* numbers were not reduced at all on spinach remaining at 3.5 log/g. It was evident in this study that the aqueous treatment methods used were not as effective in reducing microbial counts as compared to the non-aqueous method (UV light).

Refrigeration, by retarding the growth of microorganisms, has invariably been used as a means of controlling the spoilage of produce. However, in this study the bacterial counts for the majority of the fresh produce samples tested increased after refrigeration (6 days).

Validation of an optimised virus recovery method and multiplex real-time reverse transcriptase polymerase chain reaction assays for the detection of selected enteric viruses on "mock irrigated" strawberry samples

These experiments, applying optimised virus recovery methods and multiplex *rt* RT-PCR assays, demonstrate that faecally contaminated surface water used for irrigation purposes has the potential to contaminate soft fruit. The detection of NoV GI more frequently that NoV GII was relevant as NoV GI is more frequently associated with food and water outbreaks (Mattison *et al.*, 2010). The methods applied to demonstrate this contamination used appropriate quality control procedures and were therefore validated.

Attachment and survival of *Salmonella* Typhimurium on citrus fruit

A study was carried out to determine if attachment onto lemon fruit spot inoculated with 10^6/mℓ of *S.* Typhimurium could be observed using a scanning electron microscope (SEM). Scanning electron micrographs confirmed that *S.* Typhimurium was able to attach to and colonize lemon fruit surfaces and also revealed an obvious direct proportion between cell densities and exposure time. Attachment structures were observed at one minute exposure and cell division at 20 minutes post inoculation. After 4 hours of exposure, biofilm formation was noticed and at 24 hours, a well-defined biofilm was apparent. Survival of the pathogen under a simulated export chain was evaluated by means of total viable counts. Although *S.* Typhimurium was able to attach to and colonize lemon fruit surfaces as confirmed by SEM observations, at no time was the pathogen directly isolated from fruit surfaces following a simulated export chain. Nevertheless, *S.* Typhimurium was detected following enrichment of fruit washings sampled after 1, 2 and 3 weeks. Conversely, *S.* Typhimurium was detected at week 4 either directly or after enrichment in Rappaport selective broth for *Salmonella*.

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CHAPTER 4

EXTENT OF MICROBIAL CONTAMINATION FOUND IN IRRIGATION AND AGRICULTURAL SPRAY WATER

4.1 INTRODUCTION

Most water requirements in South Africa for rivers, dams and streams are provided by surface water, with about 64% of the nation's groundwater used for irrigation purposes in the agricultural sector (CSIR, 2010). These sources are also used for domestic, recreational, mining and industrial purposes. The huge demand for fresh water in the fresh produce industry, together with other costs, force farmers to use all available water resources. In many parts of South Africa, river water is used without any treatment. This water also receives most of the nation's treated sewage and therefore may contain high concentrations of microorganisms (DWAF, 1996). This coupled with increased informal settlements in urban areas, pose a risk of contamination of surface water sources such as rivers with sewage (Britz *et al*., 2007; Chigor *et al.*, 2013; CSIR, 2010). Contamination of rivers can also be caused by illegal dumping of industrial waste, resulting in high concentrations of microorganisms and heavy metals. Therefore, the use of water for irrigation can be a major source of human pathogens that contaminate fresh produce.

Studies carried out on South African rivers have shown that they are contaminated with enteric bacterial pathogens which pose a risk to human health by causing gastroenteritis and related illnesses (Obi *et al*., 2002; Olaniran *et al*., 2009; Ijabadeniyi, 2010; Duhain, 2011; Gemmel and Schmidt, 2011, Chigor and Okoh, 2013). Additionally, these studies found high levels of faecal contamination indicator bacteria (coliforms and *E. coli*) and with human pathogens such as *Escherichia coli* O157:H7*, Salmonella* spp. and *Listeria monocytogenes*. Okoh *et al.* (2010) reported that inadequately treated wastewater is a source of human enteric viruses in the environment in South Africa. The use of such polluted water sources for irrigating produce may pose a risk of transferring foodborne pathogens onto crops, especially those which usually undergo minimal postharvest processing (Doyle and Erickson, 2008).

In 2011 approximately 2200 tonnes of vegetables were grown in South Africa (Department of Agriculture, Forestry and Fisheries (DAFF, 2011). This was fuelled by an increase in domestic and international market access for South African agricultural products with the bulk of produce exported to the European Union and Southern African Development Cooperation (SADC) countries (DAFF, 2010). Additional growth in the fruit and vegetable sector has been promoted by the World Health Organization and Food and Agricultural Organization (FAO) that recommends a minimum of 400 g of fruits and vegetables per day (WHO, 2011). This recommendation comes after release of evidence that regular intake reduces risk for obesity and diabetes and therefore effort has been made to encourage consumption worldwide, especially in developing countries (WHO, 2011).

Worldwide foodborne disease outbreaks have shown that fresh produce such as vegetables are highly susceptible to contamination with foodborne pathogens such as pathogenic *E. coli*

strains, *Salmonella* spp., *L. monocytogenes*, noroviruses (NoV I and II), hepatitis A virus (HAV) and protozoa (Herman *et al.*, 2008). Studies in South Africa showed that pathogens in irrigation water can be transferred onto produce in the field (Ijabadeniyi, 2010; Duhain, 2011). This presents a risk for outbreak of foodborne illnesses in the lucrative fresh produce market in South Africa. An example of the negative impact of foodborne disease outbreaks on a country is the widely published *E. coli* O104:H4 outbreak in the European Union in May 2011 associated with contaminated bean sprout consumption. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http://www.bbc.co.uk/news/world-europe-13683270); Anderson, 2011). Therefore ensuring microbiological safety of both local and exported produce is essential in keeping the fruit and vegetable industry vibrant through minimising contamination sources by using irrigation water of recommended microbiological quality.

4.2 RESEARCH FINDINGS

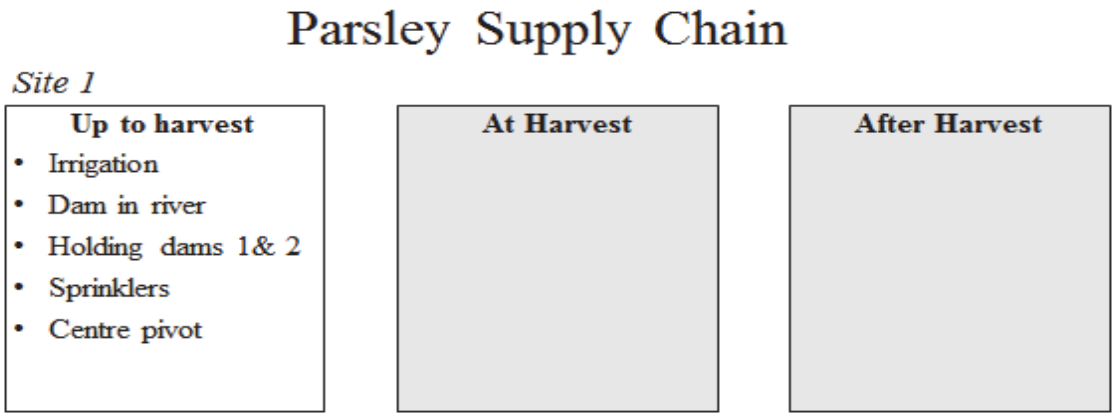
4.2.1 GAUTENG PROVINCE – Microbiological quality of water (including virological analysis) used to irrigate parsley from a farm in the Elandsdrift area

Specific aim

This study aimed to address the lack of scientific knowledge regarding the microbiological quality of fresh herbs at harvest and after harvest and the potential impact of irrigation water quality on fresh produce safety.

Description of sampling site

This study was conducted during 2011 on a vegetable/herb production farm in Gauteng using the Crocodile River for irrigation purposes. In this section only the microbiological quality of the irrigation water is reported on.



Refer to Chapter 1, section 1.2 of this volume.

Sampling and processing

Irrigation water samples were collected from the borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery. One litre water samples were collected in triplicate from each of the sampling points and filtrated through 0.45 µm pore size cellulose nitrate membranes.

Microbiological analysis

After filtering the irrigation water samples, the membranes were aseptically cut into small pieces, added to 9 mℓ TSB medium and serially diluted. To enumerate total viable bacteria and fungi/yeasts, the diluted samples were plated in duplicate onto Standard 1 agar (STD 1) and malt extract agar (MEA) (Merck, Johannesburg, South Africa) respectively. Plates were incubated at 25 °C for two (STD 1) and five days (MEA) respectively. The total coliform numbers and presence of *Escherichia coli* were determined by using a commercially available Colilert test kit according to the manufacturer's instructions (IDEXX Laboratory Inc., One IDEXX Drive, Westbrook, USA, Part no. 98-08877-00). All analyses (bacterial and viral) were performed in triplicate. Each of the water samples were enriched for detection of human pathogenic bacteria by incubation at 37 °C for 24 hours, before multiplex PCR analysis.

Samples enriched for determining the presence of *E. coli* (including *E. Coli* O157:H7) by incubating the 9 mℓ TSB medium with the filter membranes at 37°C for 24 hours and streaking onto Eosin methylene blue differential medium (EMB) (Merck) for detection of typical *E. coli* colonies. *Salmonella* spp. were detected by enriching each of the water samples in Salmonella enrichment broth for 24 hours at 37°C, followed by streaking onto Xylose lysine deoxycolate (XLD) agar for detection of typical colonies. *Listeria* spp. was enriched in Listeria enrichment broth at 37ºC for 24 hours, where after it was plated onto Listeria agar (all enrichment media were obtained from Oxoid, Johannesburg). All presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates from selective media were purified by re-streaking onto selective media and identified using PCR analysis and Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF) as described below.

Matrix Assisted Ionisation-Time of Flight (MALDI-TOF) analysis

Purified bacterial cultures isolated from the selective chromogenic media were transferred in duplicate directly to the MALDI-TOF steel polished target plate, and overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker, Bremen, Germany). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI-TOF in conjunction with Bruker Biotyper Automation Software and library. The MALDI-TOF was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. A SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of above 2.0 was used to determine the genus and probable species of an organism.

Determination of *E. coli* O157:H7, *S.* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* presence

DNA was extracted from each of the water samples after selective enrichment using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using the DNA as template using pathogen specific primers (Cebula *et al*., 1995; Standing *et al*., 2013).

Viral recovery

Irrigation water: Prior to viral recovery the water sample was seeded with a known titre of mengovirus which was used as a process control. Viruses were recovered from the 10 ℓ irrigation water samples using a glass wool adsorption-elution procedure based on the method of Vilaginès *et al*. (1993) as described by Wolfaardt *et al*. (1995) and Vivier *et al*. (2004) and further modified and optimised by Venter (2004) as described previously (Project K5/1773//4). Viruses were further concentrated to a final volume of 10 mℓ using a modification of the polyethylene glycol /sodium chloride (PEG/NaCl) precipitation method originally described by Vilaginès *et al*. (1997) and Minor (1985).

Virus Detection Methods

Nucleic acid extraction: Viral nucleic acid was extracted using two different technologies. Genomic viral nucleic acid was extracted from 1 mℓ of the recovered virus concentrate using the Nuclisens EasyMAG automated magnetic extraction platform (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's instructions and from a second 1 mℓ of the recovered virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC Robotic instrument (Roche), following the manufacturer's instructions. Purified nucleic acid was aliquoted and stored at-70°C.

Viral amplification and detection

The NoVs, HAV and mengoviruses were detected using optimised qualitative rt RT-PCR assays based on TaqMan technology and published primers and probes, namely: HAV (Costafreda *et al*., 2006); NoV GI (da Silva *et al*., 2007; Svraka *et al*., 2007); NoV GII (Kagejama *et al*., 2003; Loisy *et al*., 2005) and mengovirus (Pintó *et al*., 2009). Briefly complementary DNA (cDNA) was prepared using RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences, Burlington, Ontario) and amplification was

performed using the EXPRESS qPCR Universal Mix (Invitrogen, Carlsbad, CA) in a final volume of 20 µℓ. The reaction was carried out in sealed glass capillaries in a Lightcycler® v2.0 instrument (Roche). An amplification control (AC), which was designed and constructed as part of Project K5/1773//4, was included in each NoV GII reaction. An extraction negative control (nuclease-free water; Promega Corp., Madison, WI), a rt RT-PCR negative control and a rt RT-PCR positive control, which comprised of RNA from the target virus, were included in each assay.

Results

Microbiological analysis

Aerobic colony counts (ACC) – The total aerobic counts for all the irrigation water samples (borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery) ranged between 1.3 log CFU/mℓ (minimum) and 3.1 log CFU/mℓ (maximum) for sampling trip 1 (Table 22). The counts were higher during sampling trip 2 ranging between 2.9 log CFU/mℓ and 4.1 log CFU/mℓ.

Coliform and E. coli – coliform counts using the Colilert test ranged from 1550 MPN/100 mℓ to ≥ 2 419 MPN/100 mℓ. The *E. coli* counts were low, ranging between 3 MPN/100 mℓ to 81 MPN/100 mℓ (Table 22).

Fungi and Yeasts – the fungi/yeast counts for all seven irrigation water source samples collected during both trips were < 2 log CFU/mℓ (Table 22)

E. coli O157:H7 – not detected in any of the water samples analysed.

L. monocytogenes – not detected in any of the water samples analysed.

S. Typhimurium – not detected in any of the water samples analysed.

Norovirus GI, Norovirus GII and hepatitis A virus – None of the viruses were detected in water samples tested, i.e. Crocodile River, holding dam 1 and 2.

Table 22 Summary of microbiological analysis of water sources used to irrigate parsley

Water sources	Trip 1				Trip 2			
	ACC Log CFU/mℓ	**Fungi Log CFU/mℓ**	**Coliform MPN/100 mℓ**	***E. coli* MPN /100 mℓ**	**Bacteria**	**Fungi**	**Coliform MPN /100 mℓ**	***E. coli* MPN /100 mℓ**
Borehole	1.32	2.00	1200.4	113.5	3.24	1.09	*	3.4
Crocodile River	1.95	1.59	*	1.7	3.11	1.35	*	3.7
Holding dam 1	2.36	1.89	2190.7	110.5	4.10	0.88	909.7	36.9
Holding dam 2	2.53	1.71	*	7.8	3.24	1.94	980.4	47.7
Sprinklers	2.71	1.65	*	63.6	2.90	1.36	1245.6	37.1
Center-pivot	2.79	1.82	*	104.7	3.22	0.38	*	58
Nursery	3.06	1.85	*	79.3	3.03	0.98	1018.6	47.7

Discussion and conclusion

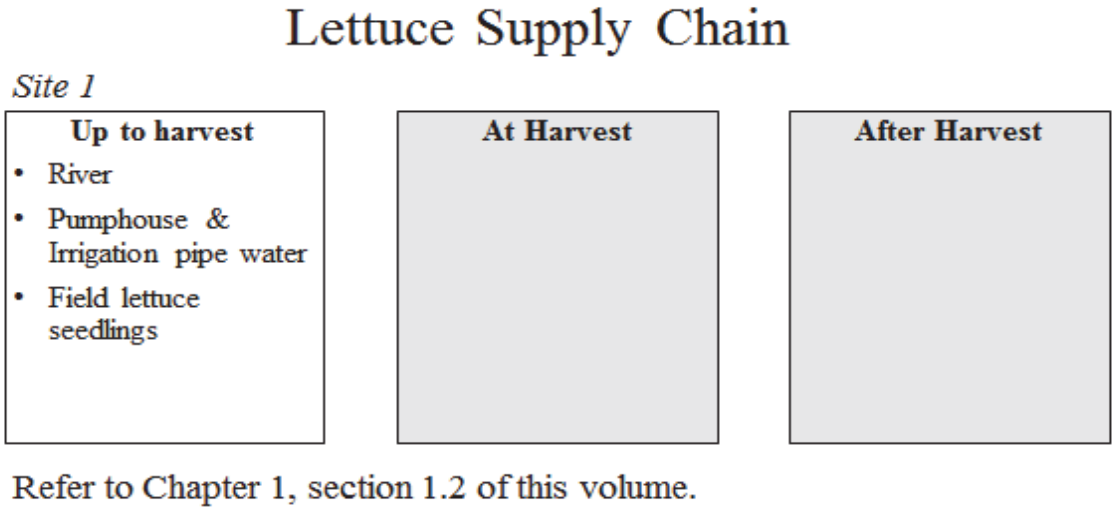
The *E. coli* levels in the irrigation water (borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery) were higher than the South African Department of Water

Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). None of the human pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium and viruses (Norovirus GI [NoV GI], Norovirus GII [NoV II] and hepatitis A virus [HAV]) tested for, were detected in any of the water samples.

4.2.2 GAUTENG PROVINCE – Microbiological quality of water used to irrigate lettuce on a farm in the Elandsdrift area

Sampling site

A fully operational commercial farm on the banks of the Crocodile River in the Crocodile (West) Marico Water Management Area, in Gauteng, was selected as the sampling area. River water on the farm is collected in a temporary dam which is then pumped to the farm through the pump house with ozone treatment. From the pump house, water is subsequently distributed to different areas of the farm for crop irrigation.



Refer to Chapter 1, section 1.2 of this volume.

Experimental procedures

Water sampling and processing

Three different water sources on the chosen farm were tested for quality monitoring purposes: (1) dam water, (2) pump house water, and (3) water from the irrigation sprinklers on the plot where lettuce samples were taken. Testing the water quality at these three points therefore gave an indication of the water quality at the critical distribution points.

Water quality monitoring

Triplicate 1ℓ water samples were collected aseptically from each of the water sources every week for a total of 12 weeks. Week 0 was considered as the week before seedlings were transplanted into the fields. Water samples were also collected on the day of harvest for comparison with samples taken at the start of the study. All water samples were membrane filtered through a 0.45µm cellulose nitrate filter (Sartorius, Johannesburg, South Africa). Concentrated cells were then dislodged from the filters through vortexing in 0.1% peptone

buffered water (Merck, Pretoria, South Africa) and a serial dilution performed. Cells were plated on Standard 1 Nutrient Agar (Merck, Johannesburg, South Africa) supplemented with 1% cyclohexamide (Sigma Aldrich, Johannesburg, South Africa) to quantify the number of viable bacterial cells present. Plates were incubated at 25°C for 48 hours after which colonies were recorded and transformed to log (x + 1) CFU $m\ell^{-1}$.

Enumeration of total coliforms and faecal coliform cells by Colilert-18®:

Triplicate 100 mℓ water samples were collected from each water source in sterile plastic bottles and transported to the laboratory for further analysis within 12 hours. Samples were collected from week 0 until the end of the study. An IDEXX Colilert-18® test using the Quanti-Tray®/2000 format (Dehteq, Johannesburg, South Africa) was performed on all samples according to manufacturer's instructions to detect levels of total coliforms and faecal coliforms. Positive (*E. coli* inoculated sterile water) and negative (sterile water) controls were also included. All Quanti-Tray®/2000 trays were then incubated at 37˚C for 18h. Positive wells were converted to MPN/100 mℓ values according to a tabulation of 95% confidence intervals provided by the manufacturer (IDEXX, Maine, USA).

Nucleic acid extraction and Polymerase Chain Reaction for pathogen detection

Filters from the water samples were enriched in Tryptone Soy Broth (Merck) for 24 hours at 37°C. DNA was extracted from each sample using a Zymo Quick-gDNA MiniPrep kit (Inqaba Biotech, Johannesburg, South Africa) as per the manufacturer's instructions. The DNA extracted was used as a template for PCR. A polymerase chain reaction for the detection of *Escherichia coli* and *Escherichia coli* O157:H7 was performed using the primers: Eco1 5'-GACCTCGGTTTAGTTCACAGA-3', Eco2 5'-CACACGCTGACGCTGACCA-3'(Schippa *et al.,* 2010) and UidAa 5'-GCG AAA ACT GTG GAA TTG GG-3', UidA 5'-TGA TGC TCC ATA ACT TCC TG-3'. A universal 16S reaction was also included in the multiplex PCR reaction to confirm the presence of DNA. The following primers were used: 8 forward 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 reverse 5'-ACGGCTACCTTGTTACGAC-3'. The PCR amplification was carried out in a GeneAmp 2400 PCR system (Applied Biosystems, Foster City, USA) using Takara EmeraldAmp Max HS PCR Master Mix (Separations, Johannesburg, South Africa). The PCR mixture contained 25µl EmeraldAmp MAX HS PCR Master Mix; 0.2µM each of Eco 1, Eco 2, UidAa, UidAb, 27 forward and 1492 reverse respectively; <500ng DNA Template; and sterile distilled water. PCR was performed for 30 cycles under the following conditions: 10s at 98˚C, 30 sec at 60˚C and 1 min/kb at 72˚C. The products of the amplification were then analysed by electrophoresis in a 2% (w/v) agarose gel containing 0.01% Ethidium bromide (Merck).

Statistical analysis

Data for lettuce phyllosphere, root and rhizosphere samples and for water samples were analysed using an analysis of variance (ANOVA) with SAS-9.2 software (SAS Institute Inc., Cary, United States of America). Means obtained were compared by the Fishers protected least significant difference (LSD) test at a 5% (P = 0.05) level of significance.

Results

Water analysis

The heterotrophic bacterial counts of the different water sources are presented in Table 23. Significant interactions occurred between the different water sources and time ($F = 35.18$; $P<0.0001$) and therefore this was considered for data analysis. 71% of the water testing periods showed a significant difference between the microbial quality of the dam water compared to the water that eventually reached the crops. In all cases, except one (week 4), the microbial content of the irrigation water was lower than the original source water. 50% of the dam water samples showed a significant decrease in the amount of bacteria present after passing through the pump house and subsequent ozonation. However, 43% of the samples showed no significant difference after ozone treatment with only 7% showing an increase in bacterial content after treatment. The majority of the pump house water samples showed an increase in bacterial content before it reached the irrigation point. However, only 50% of these increases were significant. Whereas 83% of the decreases in bacterial content between the pump house and the irrigation point showed significant decreases.

Enumeration of total coliforms and faecal coliform cells by Colilert-18® and PCR

Table 24 shows the total and faecal coliform counts of the water samples collected from the dam, the pump house and at the irrigation point during the study period. All of the faecal coliform values obtained were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting (DWAF, 1996). According to the WHO the faecal counts obtained showed that the water could be used for unrestricted irrigation of all crops (eaten both raw and cooked) without known ill effects (WHO, 1989). The total coliform values of the source water were all above 1000 counts/100 mℓ except for the reading taken at the start of the study. Four of the 14 test days showed an increase in the number of total coliforms between the dam and the irrigation point; 3 cases showed a decrease and the remaining 7 cases could not be determined as the limit of the testing method had been reached. Only 4 cases showed an overall increase in the number of faecal coliforms present between the dam and the irrigation point. No conclusive pattern could be identified for the increases/decreases in total and faecal coliform numbers. However, 4 distinct patterns could be seen: 1) gradual decrease in counts between the dam and irrigation point; 2) increase between the dam and pump house followed by a decrease to a value lower than the original starting value; 3) gradual increase in counts between the dam and irrigation point; and 4) decrease between the dam and pump house followed by an increase between the pump house and the irrigation point.

Table 23 Number of heterotrophic bacterial plate counts (Log_{10} (x + 1) CFU $m\ell^{-1}$) in dam water, pump-house water and irrigation-pipe water

Time (weeks)	Dam	Pump house	Irrigation pipe
0	3.40273 ijk	2.6735 st	2.8031 rs
1	3.35205 jkl	3.23006 lmno	3.08277 op
2	3.34678 jkl	2.80268 rs	3.13568 op
3	3.2986 klmn	2.63746 tu	2.6822 st
4	3.53999 efghi	3.06941 pq	4.22736 a
5	3.72211 bc	3.19601 mnop	3.57427 defgh
6	3.34199 jklm	3.33303 klm	3.35961 jkl
7	3.48493 fghij	2.94211 qr	3.43659 ghijk
8	3.37834 jk	3.74726 b	3.33508 klm
9	3.65847 bcde	3.33236 klm	3.16098 nop
10	3.58078 cdef	3.60843 bcdef	3.42839 hijk
11	3.73435 b	3.6509 bcde	3.70551 bcd
12	3.5479 efghi	3.66518 bcde	3.29092 klmn
At harvest	3.72185 bc	3.70349 bcd	2.51817 u

All means obtained from three replicates. All means followed by the same letter are not significantly different ($P < 0.05$). An analysis of variance indicated a highly significant difference between the water sources ($F = 104.30$; $P<0.0001$) as well as over time ($F = 64.29$; $P<0.0001$). As the interactions between the two variables were also significantly different ($F = 35.18$; $P<0.0001$), this relationship was used to analyse data.

Table 24 Total and faecal coliform (MPN/100 mℓ) detection in samples from the dam water, pump-house water and irrigation-pipe water through Colilert-18® analysis

Time (weeks)	Water Sources		
	Dam	Pump house	Irrigation pipe
0	549.73 (47.70)	275.33 (3.77)	>2419.6 (3.05)
1	2202.95 (154.20)	1123.17 (123.17)	>2419.6 (7.87)
2	>2419.6 (53.07)	1305.53 (22.20)	>2419.6 (14.83)
3	>2419.6 (34.23)	2046.27 (1.33)	>2419.6 (7.07)
4	>2419.6 (15.57)	1449.93 (2.03)	>2419.6 (38.57)
5	>2419.6 (19.63)	2275.17 (4.13)	2202.95 (18.83)
6	2130.77 (71.07)	>2419.6 (78.77)	>2419.6 (60.87)
7	1961.80 (31.03)	208.13 (4.67)	1613.03 (26.57)
8	>2419.6 (19.70)	>2419.6 (23.63)	1769.70 (20.73)
9	>2419.6 (39.10)	2202.95 (27.10)	>2419.6 (2.73)
10	>2419.6 (54.47)	>2419.6 (56.87)	>2419.6 (3.43)
11	2419.60 (148.33)	>2419.6 (194.93)	>2419.6 (277.4)
12	>2419.6 (437.13)	>2419.6 (771.17)	>2419.6 (112.23)
At Harvest	>2419.6 (109.53)	>2419.6 (160)	>2419.6 (1)

Discussion and conclusion

Current guidelines used by the international agricultural industry recommend ≤1000 faecal coliforms per 100 mℓ of irrigation source water (WHO, 1989). The South African irrigation water quality guidelines however recommend that water of this quality only be used on crops that are not eaten raw; the preferred Faecal Coliform count for crops eaten raw should be ≤1 (DWAF, 1996). The World Health Organization recommends that the faecal coliform limit be relaxed to 1000 faecal coliforms/100 mℓ for vegetables eaten raw. Despite the differences in guidelines, those set by the WHO are found to be more realistic as they were based on the actual quality of river water used for unrestricted irrigation of all crops in many countries around the world without known ill effects (WHO, 1989).

4.2.3 GAUTENG PROVINCE – Virological analysis and indicator organism levels of water used to irrigate strawberries

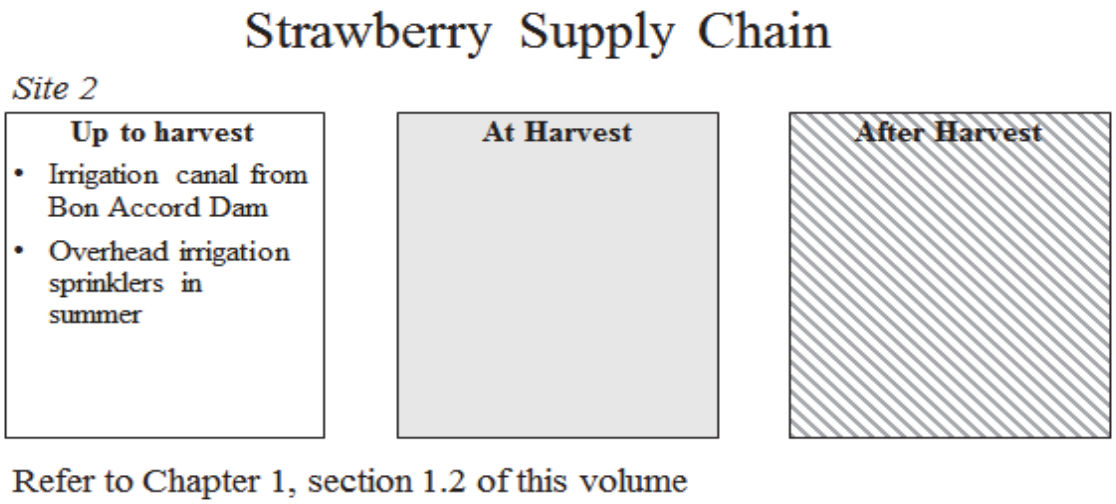
Specific aim

The aim of this study was: i) to assess the quality of surface water used for irrigation of strawberries; ii) to assess the sensitivity of two extraction procedures; iii) to determine the presence of selected enteric viruses (NoVs and HAV) on strawberries and in irrigation water(s) used; and iv) to characterise and determine the phylogenetic relationship of these viruses detected.

Experimental procedures

Site and sample selection

The samples were selected based on the recommended criteria that took the type of fruit or vegetable grown and corresponding irrigation water into account. A small scale strawberry farmer to the north of Pretoria, Gauteng, was approached for the collection of strawberries, and irrigation water from the canal used for the irrigation of the strawberries. It was noted that the farmer used overhead irrigation at the start of the study but changed to drip irrigation soon thereafter when he became aware of preliminary test results.



Irrigation water sampling

From September 2010 to August 2011 irrigation water samples were collected on a monthly basis from an irrigation canal flowing from the Bon Accord Dam, Pretoria. Water samples were collected in sterile containers (10 ℓ with an additional 1 ℓ).

Viral stock

Mengovirus (1 x 10^6 copies/mℓ) served as a process control to monitor the viral recovery and nucleic acid extraction from the strawberries and irrigation water. From March 2011 to August 2011, 1 mℓ and 10 µℓ aliquots of mengovirus were added to the irrigation water and inoculated onto the strawberry surface prior to viral recovery, respectively.

Primers and probes

Published sets of highly specific and sensitive primers and probes were used in this study. Furthermore, published sets of primers used for the genotyping of NoV GI and NoV GII are summarised in Table 25.

Table 25 Primer sequences used for genotyping of NoV GI and NoV GII

Virus	Primer Set	Primer		Reference
		Forward	Reverse	
Norovirus GI	1	QNIF4	G1SKR	Da Silva *et al*. (2007) and Kojima *et al*. (2002)
	2	G1SKF	G1SKR	Kojima *et al*. (2002)
Norovirus GII	3	QNIF2	G2SKR	Loisy et al. (2005) and Kojima *et al*. (2002)
	4	G2SKF	G2SKR	Kojima *et al*. (2002)

Nucleic acid extraction

Viral nucleic acid was extracted from 1 mℓ recovered viral suspensions of the water and strawberry samples using both of the following extraction platforms:

i) The fully automated MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) (Roche Diagnostics) in a MagNA Pure LC instrument (Roche Diagnostics), and

ii) The Nuclisens® easyMAG™ semi-automated magnetic extraction platform (Boom method) (bioMérieux SA) according to the manufacturer's recommendations. For each batch of extractions, a 1 mℓ aliquot of nuclease-free water (Promega Corp.) served as a negative control. The extracted nucleic acid was eluted in 100 µℓ and 10 µℓ aliquots and stored at -20°C.

Nucleic acid from 11 irrigation water samples collected, excluding 2011/01/21 due to insufficient material, were re-extracted using the MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) (Roche Diagnostics), aliquot in in 100µℓ and 10µℓ and stored at -20°C.

Real-time RT-PCR assays

Individual *rt* RT-PCR assays were performed for the screening analysis of NoV GI, NoV GII and HAV in the water and strawberry samples. A 10 µℓ aliquot of the extracted nucleic acid was used for cDNA synthesis using the RevertAid Premium™ First Strand cDNA synthesis kit (Fermentas Life Sciences) following the manufacturer's recommendations. A 60 min incubation period was used as recommended by the manufacturer. The EXPRESS qPCR Super Mix Universal kit (Invitrogen) with 4 µM and 8 µM TaqMan primer and probe concentrations, respectively, was used. Amplification was performed on a LightCycler v2.0 real-time PCR platform (Roche Diagnostics). Eleven irrigation water samples were reassessed using optimised multiplex *rt* RT-PCR assays. A negative *rt* RT-PCR control using nuclease-free water (Promega Corp.), an extraction negative control and a *rt* RT-PCR positive control, which consisted of the target viruses, were included in each batch of *rt* RT-PCR reactions.

For the characterisation of NoVs a highly conserved region at the 5'end of the NoV capsid gene was amplified and sequenced. A two-step conventional PCR amplification was performed with published sets of primers. The KAPA Taq Hot Start PCR kit (KAPA Biosystems, Cape Town, SA) with 5 mM dNTPs (Fermentas Life Sciences, Burlington, Ontario, Canada), 37.5 mM magnesium chloride (KAPA Biosystems), and 5 µℓ of cDNA was used for the first round of amplification. For NoV GI, primer set 1 (Table 4) and for NoV GII primer set 3 (Table 4) with 25 µM and 50 µM forward and reverse primer concentration, respectively, was used. The reaction mix was prepared to a final volume of 20 µℓ. Amplification was performed on a Little Genius thermal cycler (BIOER Technology, Hangzhou, China) using the following cycling conditions; pre-denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 50°C (NoV GI) or 55°C (NoV GII) for 45 sec, 72°C for 1 min, followed by a final extension step at 72°C for 5 min. A semi-nested PCR protocol was used for the second round of amplification. AmpliTaq® Gold DNA polymerase (0.025 U/µℓ) (Applied Biosystems) with 5 mM dNTPs (Fermentas Life Sciences), and 1 µℓ of the first round PCR product was used. For NoV GI, primer set 2 (Table 4) and for NoV GII primer set 4 (Table 4) with 5 µM and 25 µM forward and reverse primer concentrations, respectively, was used. Amplification was identical to that of the first round. The PCR products were analysed by 2% agarose gel electrophoresis (SeaKem® LE Agarose, Lonza, Rockland, NY) and visualised by EtBr staining and UV illumination using a 100 bp marker (O'GeneRuler, Fermentas Life Sciences) to determine the band size(s).

Viral Recovery

Irrigation water

A glass wool absorption-elution method, based on the method described by Vilaginès *et al*. (1997) and modified by Venter (2004) was used. The irrigation water was passed through the positively charged column using negative pressure at a rate of 10 ℓ/h. The viruses were eluted from the column to a final volume of 100 mℓ using a glycine-beef extract buffer (pH 9.5) 3.754 g/ℓ glycine (Merck) and 5 g/ℓ beef extract powder (Difco™ Becton, Dickinson and Company, MD). The solution was brought to a neutral pH (pH 7) using 1 M HCl (Merck) or 1 M NaOH (Merck). Secondary concentration of the viral suspension was achieved by a PEG_{6000} (Merck)/ NaCl (Merck) precipitation method based on the methods described by Vilaginès *et al*. (1997) and Minor (1985). The final pellet was resuspended in PBS (pH 7) (Sigma-Aldrich Co.) and stored (-20°C) prior to further analysis.

Analysis for indicator organism

Thermotolerant (faecal) coliforms

Thermotolerant (faecal) coliforms were enumerated from a 100 mℓ aliquot of sampled irrigation water (1 ℓ) using a membrane filtration method (0.45 µm Sartorius Stedim filters). Turbid water samples that could possibly restrain or inhibit the flow of the water through the filter were diluted ten-fold using Modified Scholtens' Broth (MSB). Faecal coliform selective agar plates (m-FC agar plate (55 mm diameter) (Selecta Media, Quantum Biotechnologies [Pty] Ltd, Ferndale, SA) were used onto which the filters were placed and incubated overnight at 44.5°C. Individual blue colonies on the surface of the filter(s) were used to calculate the number of colony forming units (CFU)/100 mℓ present for each water sampled. In cases

where individual colonies could not be counted due to the higher number present/over growth, serial ten-fold dilutions of the water were made with PBS (Sigma-Aldrich Co.) and the enumeration process repeated.

Escherichia coli

The process for the enumeration of *E. coli* from the irrigation water samples was identical to that for the thermotolerant (faecal) coliforms with the following differences; i) a 0.45 µm filter from Merck Millipore (Merck) was used; ii) m-ColiBlue24 *E. coli* specific broth (Merck) was used; and iii), the plates were incubated overnight at 35°C. The CFU/100 mℓ was calculated by counting the individual blue colonies present. Serial ten-fold dilutions of water were made in cases where individual colonies could not be enumerated.

Characterisation of NoVs

Cloning of PCR products

Amplified PCR fragments of approximately 300-400 bp were recovered from the gel and concentrated to 10 µℓ using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA). The purified PCR fragments were cloned into a plasmid vector using the CloneJET™ PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's recommendations. Four microliter of the ligation reaction was used to transform the *E. coli* competent cells (10 µℓ) (Lucigen Corp., Middleton, WI). The transformed *E. coli* cells were incubated at 37°C for 1 hour in 900 µℓ recovery media (Lucigen Corp.) under vigorous shaking. A 100 µℓ aliquot of the suspension was plated on LB enriched agar containing 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated overnight at 37°C. Ten individual colonies were randomly isolated from the plates and screened for the specific insert by PCR amplification. GoTaq® Flexi DNA Polymerase (5u/µℓ) (Promega Corp.) with 2.5 mM dNTP's (Fermentas Life Sciences) and 4 µM pJET 1.2/blunt specific primers according to the manufacturer's recommendations was used. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) using the following cycling conditions; pre-denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR fragments were analysed by electrophoresis in a 2% agarose gel (SeaKem® LE Agarose) and visualised under UV illumination after staining with EtBr. A 100 bp marker (O'GeneRuler) was used to determine the band size(s).

An additional ten clones were sequenced in those samples where NoV could be typed. The amplification process was repeated for those samples in which an appropriate fragment size could not be obtained with changes made to the nucleic acid concentration (serial tenfold dilution series), the Taq polymerase used for the first and second round amplification steps (KAPA Taq vs. AmpliTaq Gold), the volume of PCR product used for second round amplification (1 vs. 2 µℓ) and the cycling conditions of the PCR assays were adjusted.

Sequencing and sequence analysis of PCR products

Amplified PCR products were purified using the DNA Clean & Concentrator™-25 Kit (Zymo Research Corp.) and eluted in 10 µℓ. The fragments were sequenced based on the Sanger sequencing method using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) and pJET 1.2/blunt specific primers (3.2 pmol) according to the manufacturer's recommendations. A 2 µℓ aliquot of the purified PCR fragments were used in each PCR reaction. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) under the following cycling conditions; pre-denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 50°C for 10 sec and 60°C for 4 min. The ABI 3130 automated analyser (Applied Biosystems) was used to analyse the amplified PCR products. Sequencer™ v4.10.1 and MEGA version 5.1 (Tamura *et al*., 2011) was used for the analysis of the sequences Norovirus reference were strains obtained from (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/) (Table 26). MAFFT version 6 (http://0-mafft.cbrc.jp.innopac.up.ac.za/alignment/server/index.ht) was used to align the sequences. After manual adjustments of the alignment, phylogenetic analysis was performed with MEGA version 5.1 using the neighbour-joining method. Genotypes were assigned based on the clustering of the individual samples in the phylogenetic tree with a bootstrap support of >70%.

Table 26 Norovirus reference strains used in phylogenetic analysis of field strains.

Genotype	GenBank Accession no.	Strain	Year	Country of Origin
GII.1	HCU07611	Hawaii	1971	United States
GII.2	X81879.1	Melksham	1994	United Kingdom
GII.3	GU980585.1	CBNU1	2006	Korea
GII.3	HM635200.1	Seoul	2009	Korea
GII.4	X76716.1	Bristol	1993	United Kingdom
GII.5	AJ277607.1	Hillingdon	1990	United Kingdom
GII.6	AJ277620.1	Seacroft	1990	United Kingdom
GII.7	AJ277608.1	Leeds	1990	United Kingdom
GII.8	AF195848.1	98-18	1998	Amsterdam
GII.9	AY038599.2	VA97207	1997	United States
GII.10	AF427118.1	Erfurt	2000	Germany
GII.11	AB074893.1	Sw918	1997	Japan
GII.12	AJ277618.1	Wortley	1990	United Kingdom
GII.13	AY113106.1	Fayetteville	1998	United States
GII.14	AY130761.1	M7	1999	United States
GII.15	AY130762.1	J23	1999	United States
GII.16	AY502010.1	Tiffin	1999	United States
GII.17	AY502009.1	CS-E1	2002	United States
GII.18	AY823304.1	OH-QW101	2003	United States
GII.19	AY823306.1	OH-QW170	2003	United States
GII/Unknown	HM560937.1	205	2010	Taiwan

Results

Analysis of indicator organisms

The thermotolerant coliform and E. coli counts – ranged from 3100 (3.49 log) to 48 000 (4.68 log) and 30 (1.48 log) to 2 200 (3.34 log) CFU/100 mℓ, respectively (Table 27).

Table 27 Summary of thermotolerant coliform and *E. coli* counts for irrigation water sources

Sample	Collection Date	Thermotolerant coliforms (CFU/100 mℓ)*	*Escherichia coli* (CFU/100 mℓ)*
Irrigation water	2010/09/22	4.00	2.40
Irrigation water	2010/10/25	4.04	2.30
Irrigation water	2010/11/22	4.06	2.40
Irrigation water	2010/12/15	4.68	3.20
Irrigation water	2011/01/21	4.00	3.00
Irrigation water	2011/02/22	3.49	2.67
Irrigation water	2011/03/22	3.98	3.34
Irrigation water	2011/04/21	3.70	2.95
Irrigation water	2011/05/21	4.20	3.04
Irrigation water	2011/06/21	3.78	2.78
Irrigation water	2011/07/26	3.70	3.18
Irrigation water	2011/08/24	3.85	1.48

* CFU/100 mℓ = colony forming units (CFU)/100 mℓ

NoV GI, NoV GII and HAV – A summary of a 12 month survey of the Bon Accord irrigation canal for the presence of indicator organisms of faecal contamination is reflected in Table 28. From September 2010 to August 2011 a total 12 irrigation water samples were collected and analysed for the presence of NoV GI, NoV GII and HAV (Table 7). Norovirus GI, NoV GII and HAV were detected in 8.3% (1/12), 41.7% (5/12) and 0% (0/12), respectively, of the irrigation water samples.

The use of optimised multiplex *rt* RT-PCR assays allowed for the detection of the above and additional viral species (Table 28). Norovirus GI, NoV GII and HAV could be detected in 9.1% (1/11), 36.4% (4/11) and 0% (0/11) in irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR assays NoV GI, NoV GII and HAV were detected in 27.3% (3/11), 27.3% (3/11) and 0% (0/11) of the irrigation water samples, respectively. An additional three NoV GI positive irrigation water samples were detected by the multiplex *rt* RT-PCR assay. Human AstV, HRV and SaV were detected in 36.4% (4/11), 54.5% (6/11) and 9.1% (1/11) of the irrigation water samples collected. Mengovirus was consistently detected by both assays.

Table 28 Summary of results from the comparison of singleplex and multiplex *rt* RT-PCR assays for detection of selected enteric viruses

Sample	Sample date	Singleplex rt RT-PCR* assay				Multiplex rt RT-PCR* assays							Total viruses detected
		HAV	NoV GI	NoV GII	PC	HAV	NoV GI	NoV GII	PC	HAstV	HRV	SaV	
Irrigation water	2010.09.22	-	+	-	-	-	-	-	-	-	-	-	1
	2010.10.25	-	-	+	-	-	+	+	-	-	-	-	3
	2010.11.22	-	-	+	-	-	-	-	-	+	-	-	2
	2010.12.15	-	-	-	-	-	+	-	-	+	+	-	3
	2011.02.22	-	-	-	-	-	-	-	-	+	-	-	1
	2011.03.22	-	-	-	+	-	-	-	+	-	-	+	2
	2011.04.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.05.21	-	-	-	+	-	-	-	+	+	+	-	2
	2011.06.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.07.26	-	-	+	+	-	+	+	+	-	-	-	3
	2011.08.24	-	-	+	+	-	-	+	+	-	-	-	2

Phylogenetic analysis

The 5’ end of the NoV GII capsid gene (273 bp) was used in the phylogenetic analysis. The NoV GII strains could be genotyped from three of the 10 positive samples, however, no strains from the five NoV GI positive samples could be genotyped (Fig. 40).

A neighbour-joining tree using the amino acid sequence alignments of the reference NoV GII strains (Table 26) and unknown NoV strains from this study (Table 29) was drawn (Fig. 40). Phylogenetic analysis could closely match the NoV strain in the irrigation water as swine NoV GII.18 and that of the strawberry as human NoV GII.7. BLAST results of the irrigation water strains found an unidentified NoV GII strain (Table 30) recovered from an oyster in Taiwan as the closest match (99% nucleotide sequence identity) (Fig. 40) (Table 30). Analysis of this sequence strain and that of the individual irrigation water sequences closely grouped together with swine NoV GII.18. A pair wise alignment of the amino acid and nucleotide sequences of the Jul/Aug Bon Accord Irrigation Water (BIW) clones, the swine NoV GII.18 and the NoV strain from the oyster (Table 30) revealed an overall 81.8% amino acid and 76.3% nucleotide similarity of the clones to the swine NoV GII.18 strain (Table 9). An overall 99.5% amino acid and a 93.47% nucleotide sequence identity existed between the Jul/Aug BIW clones and that of the oyster NoV GII strain. In the majority of the Jul/Aug BIW clones, insignificant changes were noticed between the NoV GII strain collected in July and that in August 2011 (Fig.40; Table 30). Separate groupings of two clones (Gauteng Province (GP) July BIW clone 4 and GP July BIW clone 4) were noticed but these differences were not supported by bootstrap analysis. Similarly, Bon Accord Strawberries (BST) clones 3 and 7 (Fig. 40) grouped together but separate from the other clones, yet the differences noticed were not supported by bootstrap analysis.

Table 29 Summary of genotyping results of NoV GI and NoV GII strains detected in the irrigation water

Sample	Date	Norovirus genogroup	Genotyping
Irrigation water	2010.09.22	GI	Untypable
	2010.10.25	GII	Untypable
	2010.10.25	GI	Untypable
	2010.11.22	GII	Untypable
	2010.12.15	GI	Untypable
	2011.01.21	GII	Untypable
	2011.07.26	GII	GII.18
	2011.07.26	GI	Untypable
	2011.08.24	GII	GII.18

Table 30 Pairwise analysis of amino acid and nucleotide sequences of four swine NoVGII.18 strains, with two additional clones of each of the samples respectively, were used to determine the relatedness of the strains to each other and to the two swine NoVGII.18 reference strains (AY823304 and HM560937). A 306 bp region of the NoVGII capsid protein was aligned and used for analysis.

		AY823304	HM560937	Jul 11 BIW				Aug 11 BIW				
		Swine	Oyster	Clone 1	Clone 2	Clone 3	Clone 4	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
AY823304 Swine			82.18	82.18	82.18	83.17	81.12	82.18	82.18	82.18	79.21	82.18
HM560937 Oyster		77.23		100	100	99	99	100	100	100	97.03	100
Jul 2011 BIW	**Clone 1**	76.24	93.34		100	99	99	100	100	100	97.03	100
	Clone 2	76.57	93.73	99.34		99	99	100	100	100	97.03	100
	Clone 3	76.57	93.4	98.68	99		98.02	99	99	99	96.04	99
	Clone 4	75.91	93.1	99.97	99	98.35		99	99	99	96.04	99
Aug 2011 BIW	**Clone 1**	76.57	93.37	99.67	99.67	99	99.39		100	100	97.03	100
	Clone 2	76.57	93.4	98.69	98.35	98.02	98.35	98.35		100	97.03	100
	Clone 3	76.57	93.73	99.67	99.67	99	99.34	100	98.35		97.03	100
	Clone 4	75.58	93.1	98.35	98.68	98.35	98.02	98.68	97.69	98.68		97.03
	Clone 5	76.24	93.73	99.67	99.34	99	99.34	99.34	99	99.34	98.68	

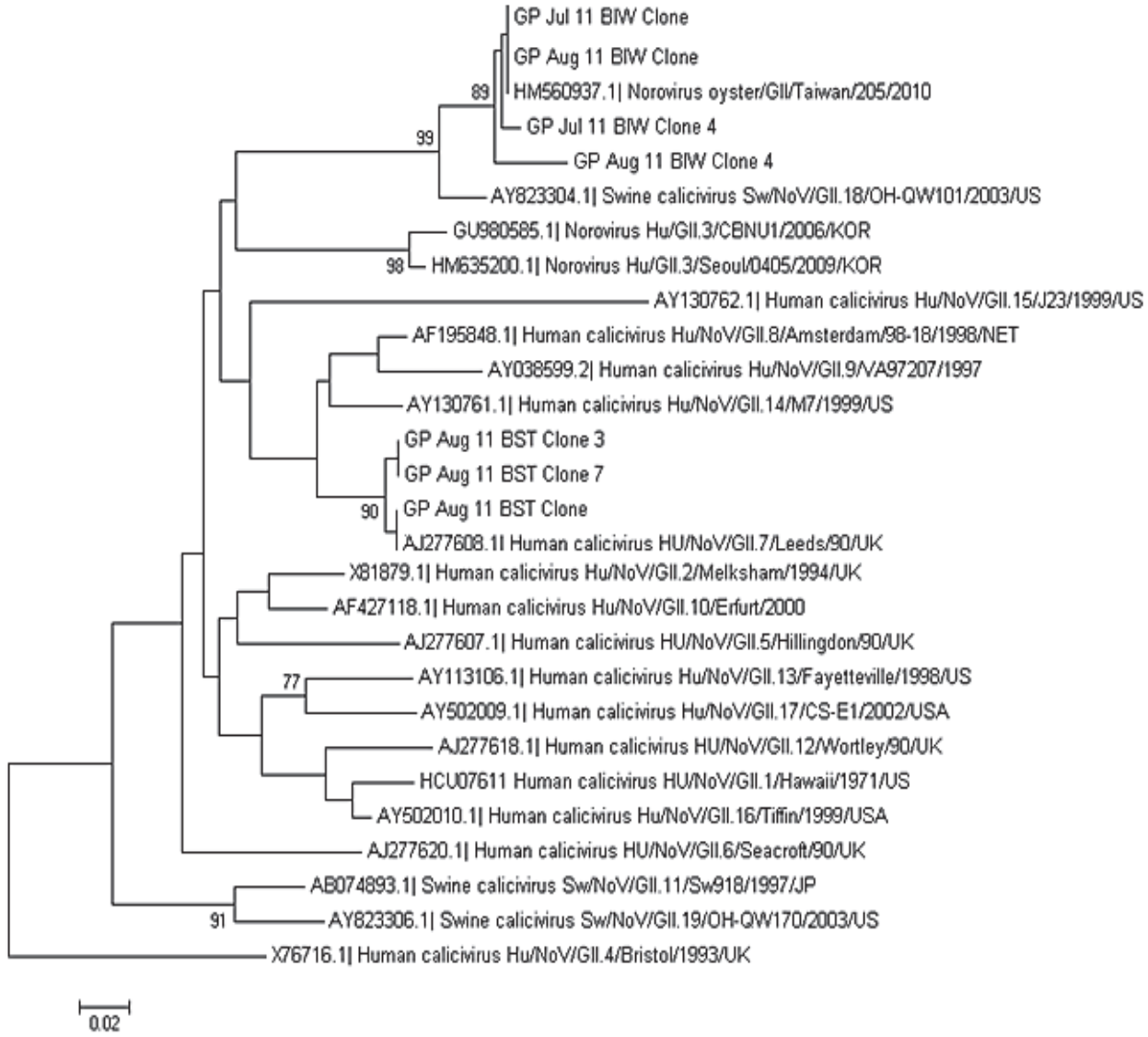


Figure 40 Neighbour-joining phylogenetic tree based on the amino acid sequence of the NoV capsid gene region. The NoV strains from irrigation water and strawberry samples along with reference NoV GII strains were used to draw the tree. Norovirus GII.4 was used to root the tree. Bootstrap percentages are indicated with the bar representing the nucleotide changes. BIW (Bon Accord Irrigation Water) and BST (Bon Accord Strawberries)

Discussion and conclusion

Escherichia coli and thermotolerant coliforms are used as indicator organisms for the presence of enteric viruses in food and water samples because of the cost effectiveness and effortlessness in their detection. These organisms were used to assess the quality of irrigation water and to correlate their presence with that of selected enteric viruses. The findings indicate that the quality of the water used for irrigation does not meet the standards (thermotolerant coliforms: <1000 colony forming units per 100 mℓ and *E. coli* counts: ≤1 counts/100 mℓ) set by the Department of Water Affairs and Forestry of SA in 1996. The use of bacterial indicator organisms is considered by some as imperfect for the indication of the presence of enteric viruses (Sinclair *et al*., 2009).

A wide range of NoVs have been documented to circulate in SA with NoV GII.4 being the most prevalent strain both in the surface water and in paediatric patients in SA (Mans *et al*., 2010, 2013). Real-time RT-PCR assays in the form of multi- and singleplex assays were used to determine the presence of NoV GI, GII and HAV in paired irrigation water and strawberry samples. Noro viruses, in particularly NoV GII, were most frequently detected in the irrigation water (Table 29). The multiplex RT-PCR assays applied in this study could detect three additional NoV GI positive samples. However, this could partially be due to the re-extraction of nucleic acid. Additional enteric viruses (HAstV, HRV and SaV) were detected in the irrigation water used for the strawberries. Human RV was frequently detected in the irrigation water samples (54.54%) (Table 28).

Environmental sources could potentially be seen as reservoirs for animal pathogens, more specifically RNA viruses, which could potentially amount to cross-species transmission (Heeney, 2006). The genus *Norovirus* comprises of viruses capable of infecting humans, pigs, cattle and mice species (Patel *et al*., 2009). A risk of zoonotic transmission between human and animal NoV strains could possibly exist. In such an event a novel virus with a new host tropism and increased or decreased pathogenicity may arise (Almanza *et al*., 2008). Swine NoVs are genetically and antigenically related to the most prevalent human NoVs strain (GII) (Wang *et al*., 2005). Mattison *et al*. (2007) demonstrated the presence of NoV GII.4 human-like strains in pigs whereas Wang *et al*. (2005) identified a potential human-swine recombinant NoV strain that could infect genotobiotic pigs. The possibility for pigs as potential reservoirs for the emergence of new human NoVs strains therefore exists (Wang *et al*., 2005).

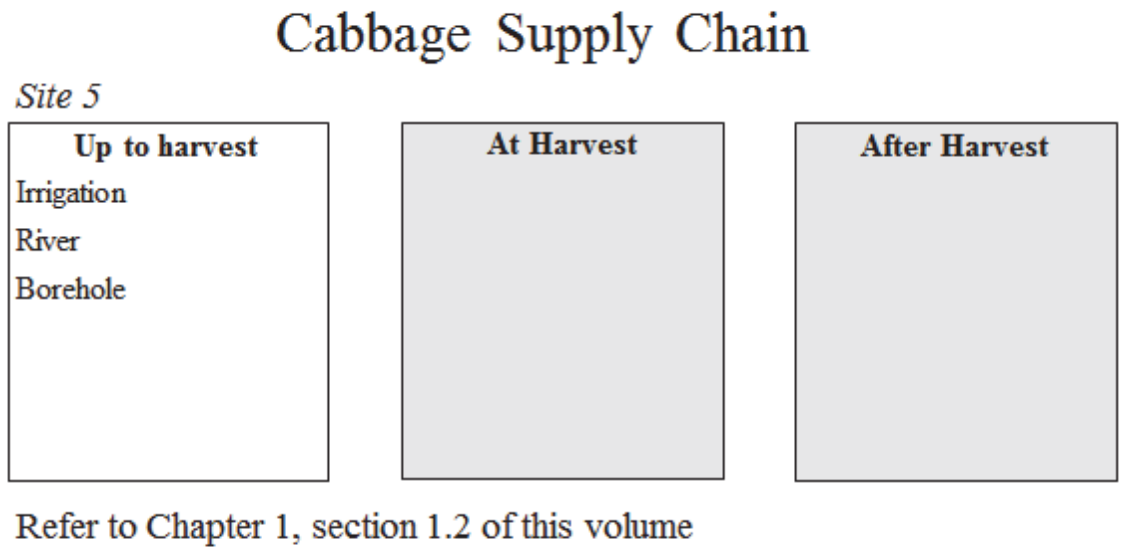
During the period Sept 2010 to Aug 2011, 12 water samples were collected of which 42% (5/12) tested positive for NoV GII. Swine NoV GII.18 was genotyped from two irrigation water samples and human NoV GII.7 from a single strawberry sample. Mans *et al*. (2013) had similar success rates in the genotyping of NoVs from surface water. To our knowledge, swine NoV has not been detected in SA (Mans *et al*., 2010, 2013) and thus makes this the first reported incidence of swine NoV GII.18 in SA. BLAST analysis of this strain indicated a 94% sequence identity with a NoV strain detected in 2010 in Taiwan from an oyster (Fig. 40). Two clones; GP Jul BIW 11 clone 4 and Aug BIW 11 clone 4 grouped separately from the other strains but these differences were not supported by bootstrap values (Fig. 40). Despite these differences, the closest representatives remained swine NoV GII.18 and human NoV GII.7 detected in the water and on the strawberries, respectively. This was the first report on the presence of swine NoV GII.18 in SA.

4.2.4 GAUTENG PROVINCE and KWAZULU-NATAL PROVINCE – The extent of virological contamination and indicator organism levels in water used to irrigate cabbages from two farms (small scale and commercial)

Specific aim

The objective of this study was to establish if the presence of NoVs and HAV on produce is due to using contaminated irrigation water.

Sampling site



Experimental procedures

Irrigation water sampling

Water (10 ℓ) and associated cabbages were collected from selected farms in KwaZulu-Natal (river water) and near Pretoria (borehole and river).

Microbial indicator analysis

Faecal (Thermotolerant) coliforms, *E. coli*, viral analysis including nucleic acid extraction, viral amplification and detection were as described for water used for irrigation of strawberries in the previous section (section 4.2.3).

Results

Microbial indicator levels in irrigation water

Faecal (thermotolerant) coliforms and *E. coli* were detected in both the river water samples while faecal coliforms were detected at a low level (4 colony forming units (CFU)/ 100 mℓ) in the borehole water (Table 31).

Table 31 Number of colonies counted for faecal coliform and *E. coli*

Farm		Faecal coliform*	*E. coli* *
KwaZulu-Natal (Farm)	Irrigation water (River)	29	24
Pretoria (Farm)	Borehole water	4	0
	River water	70	40

* colony forming units (CFU)/ 100 mℓ

Virological analysis

The river water sample from the KZN farm tested positive for HAV and negative for NoV GI and NoV GII. Water samples from the farm near Pretoria tested positive for the process control virus, i.e. mengovirus, and negative for the target viruses, i.e. NoV GI, NoV GII, and HAV.

Hepatitis A virus characterisation

BLAST analysis of a ~ 390 bp region of the VP1/2B junction of the HAV strain detected in the irrigation water (KZN) showed a high percentage genetic relatedness, i.e. 82% nucleotide sequence identity, to a Tunisian strain (Tun40-03) belonging to sub-genotype IA (Gharbi-Khelifi *et al*., 2006). However, the neighbour-joining phylogenetic tree based on the VP1/2B region sequence grouped the strain within genotype V (Fig. 41).

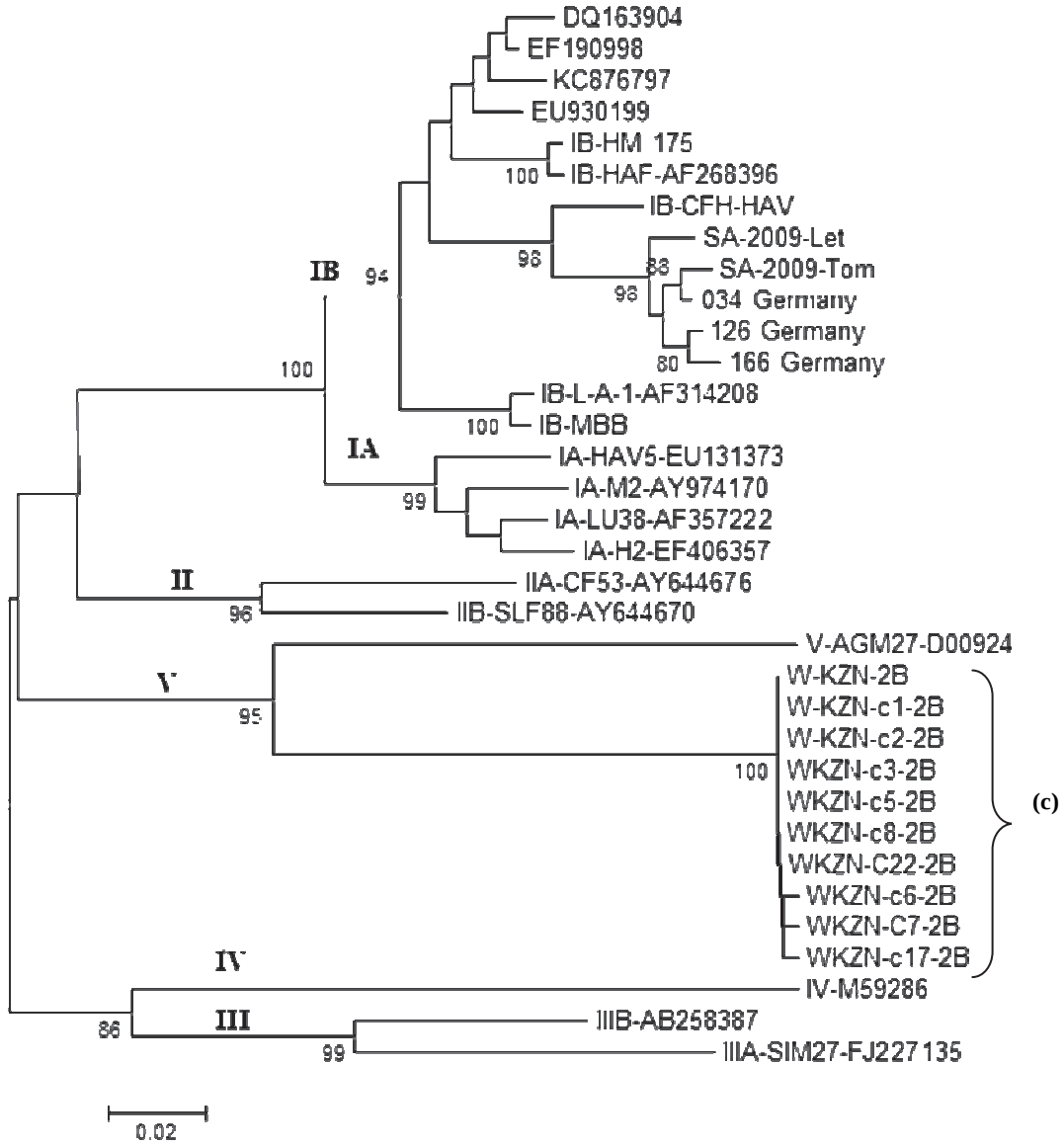


Figure 41 Neighbour-joining phylogenetic tree based on the ~ 390 base-pair VP1/2B region of the HAV strain detected in the irrigation water sample from the farm in KwaZulu-Natal and HAV reference strains. Bootstrap percentages are indicated.

Discussion and conclusion

The *E. coli* counts were generally low < 40 CFU/100 mℓ, which makes it suitable for irrigation of crops not to be eaten raw (DWAF). The presence of HAV in the irrigation water sample implicated potential faecal contamination of human origin. BLAST analysis showed the strain to belong to genotype IA with genetic relatedness of 82% (nucleotide sequence identity) with a strain that was detected in Tunisia (Tun40-03) (Gharbi-Khelifi *et al.*, 2006). However, the neighbour-joining phylogenetic tree based on the ~ 390 bp VP1/2B region grouped the strain within genotype V with a bootstrap value of 95% (Fig. 41). The latter finding suggests the strain to be associated with non-human primates, i.e. simian species (Costa-Mattioli *et al.*, 2003). Genotype V strain (AGM-27) was first described after isolation from an African green monkey from Kenya. Another study with a report of genotype V strain

was conducted in India, in which simian HAV was isolated from a faecal sample from a rhesus monkey in 1995 (Aranakalle and Ramakrishnan, 2009). This was an interesting result as Taylor *et al.* (1997) showed subtype IB to be the predominant strain in SA.

4.2.5 KWAZULU NATAL – Microbiological quality of water used to irrigate raw produce (broccoli, bell pepper, cabbage, Chinese cabbage, red cabbage, cauliflower, Crisphead lettuce, jam tomatoes parsley and spinach) from three farms near Cato-Ridge, Camperdown and Richmond respectively

Specific aim

This study investigated the microbial quality of irrigation water and fresh produce from three local farmers in KwaZulu-Natal (KZN).

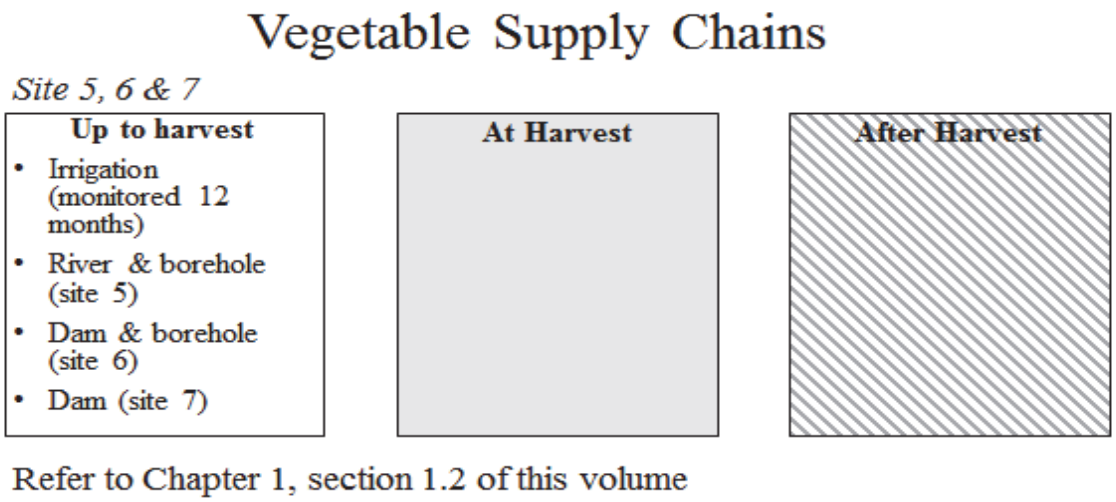
Experimental procedures

Sampling sites

Three different farms, designated A, B and C were used in this study. Farm A (130 hectares) is located in Camperdown. Crops planted are broccoli, spinach, jam tomatoes, Crisphead lettuce, cauliflower and cabbage. Crops are not washed and packed on site. The farm has two sources of irrigation water, namely river (A1) and borehole water (A2), and used weekly to irrigate the plants.

Farm B (60 hectares) is located in Cato Ridge. Crops planted are broccoli, spinach, lettuce, cauliflower, Chinese cabbage, parsley, bell pepper and red cabbage. These crops are irrigated weekly. Crops are washed and packed on site. The farm's source of irrigation water is a mixture of borehole and dam water (B).

Farm C (360 hectares) is located in Richmond. Crops planted are oranges, cabbage (planted in winter) and jam tomatoes (planted in summer). Crops are washed and packed on site. The farm's source of irrigation is dam water (C).



Sample collection and processing

Microbiological analysis

One litre water samples (x 3) from the respective irrigation sources were collected monthly from the farms for a period of 1 year from July 2009 to June 2010. Serial dilutions were prepared from each water sample using sterile distilled water. Hundred and fifty millilitres of the appropriate dilutions were filtered through a membrane filter (0.45 µm), serial dilutions prepared and plated onto selective media and the membranes were placed onto selective media. Selective media used for growth of coliforms/*Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Shigella* spp. were Coliform-chromo agar, Agar Listeria Ottaviani and Agosti (ALOA) Agar respectively.

Results

Microbiological analysis of the irrigation water

The microbial load of the irrigation water sources from farm A and B increased during the summer months (October 2009 to March 2010; Fig. 42a and b. Fig. 43a). Overall the microbial numbers in the dam water from farm C were low (Fig. 43b).

Farm A (river A1) – the coliform counts ranged from 2500 (3.40 log) CFU/100 mℓ to 7000 (3.84 log) CFU/100 mℓ from July 2009 to June 2010. *E. coli* was present during July and August 2009 only at values below 1000 CFU/100 mℓ (Fig. 42a).

Farm A (borehole A2) – the coliform counts ranged from zero to 5000 (3.70 log) CFU/100 mℓ over the entire twelve month sampling period. *E. coli* counts ranged from zero for all the water sampling periods, except once during October 2009 when the count was 6000 (3.78 log) CFU/100 mℓ (Fig. 42b).

Farm B (mixture borehole and dam water B) – the coliform counts ranged from 3 (0.47 log) CFU/ 100 mℓ to 9 000 (3.95 log) CFU/100 mℓ over the entire twelve month sampling period. *E. coli* counts were zero (Fig. 43a).

Farm C (dam water C) – Coliform bacteria were counted only once at < 740 (2.87 log) CFU/100 mℓ and *E. coli* counts were zero during the twelve month sampling period (Fig. 43b).

L. monocytogenes – Typical growth on selective media was not observed for all the water source samples, except once during October 2009 for the borehole water sample (A2).

Salmonella spp. – Typical growth on selective media were observed once for each of the samples, i.e. river water (A1), borehole water (A2) as well as mixed dam and borehole water (B) during the twelve month sampling period.

Shigella spp. **–** Typical growth was observed for all the water samples, except for sample C (dam water).

Campylobacter – Presumptive *Campylobacter* spp. was only detected in water sample C (dam water), and the population increased when the irrigation water was not in use, and the dam was allowed to be stagnate. This could be due to survival and growth of the *Campylobacter* spp. which is a microaerophile in the stagnant water with limited oxygen levels (Moore *et al*., 2006).

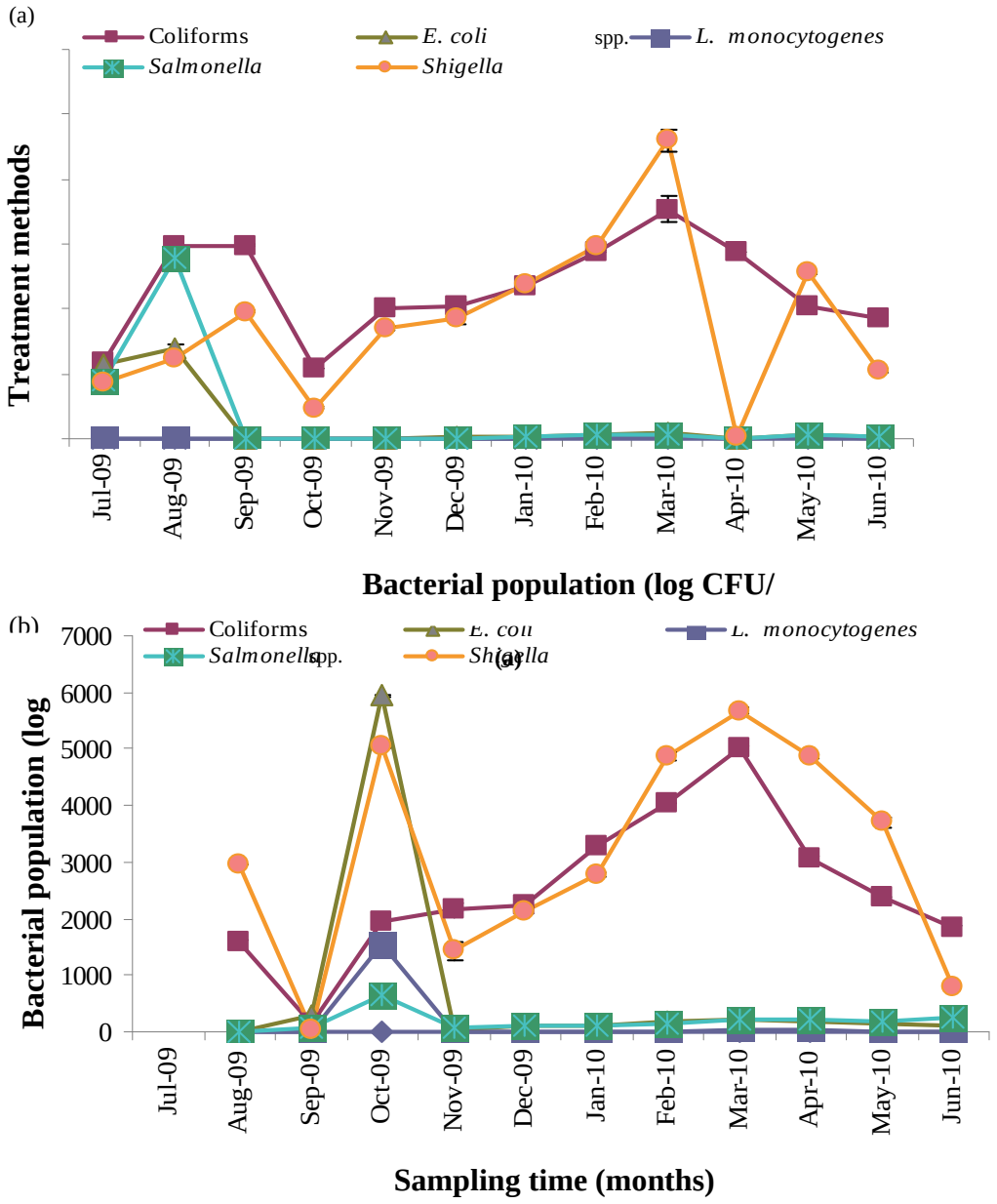


Figure 42 The microbial quality of irrigation water collected from farm A (a) and (b), over a one-year period.

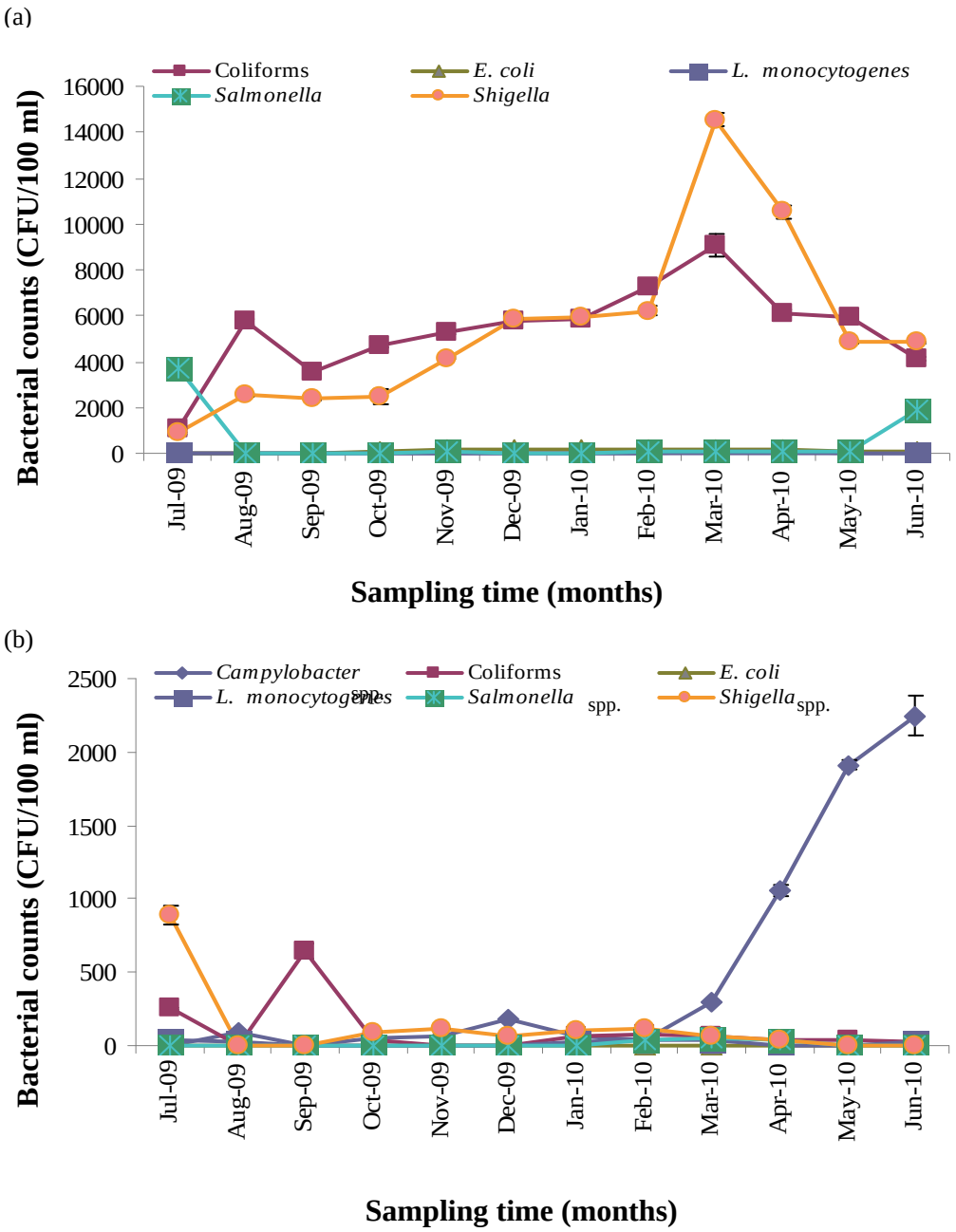


Figure 43 The microbial quality of irrigation water collected from farm B (a) and C (b), over a one-year period.

Discussion and conclusion

The guideline limit for the incidence of *E. coli* in irrigation water is ≤1000 CFU/100 mℓ for crops not to be eaten raw and allowed to dry (DWAF, 1996). According to these standards the irrigation water sources (river, borehole, dam) on all three farms were acceptable for irrigation of fresh produce to be eaten raw. The majority of the water samples tested from farms A1, A2 and B showed high levels of these presumptive pathogens during summer. Presumptive *Shigella* spp. were detected in all the water samples from all three farms A, B and C. Presumptive *Campylobacter* spp. was only detected in water sample C (dam water), and the population increased when the irrigation water was not in use, and the dam was allowed to stagnate. This probably allowed for the growth and survival of *Campylobacter* spp., being a microaerophile (Moore *et al.*, 2006), since stagnant waters limit the entrance of oxygen.

Furthermore, temperature and solute concentration govern the water solubility of oxygen. It has been noted that the water quality depends partially on land use and how these water resources are managed and protected, as some pathogenic microorganisms may survive longer in water or soil when conditions are optimal than what has been considered to be the norm.

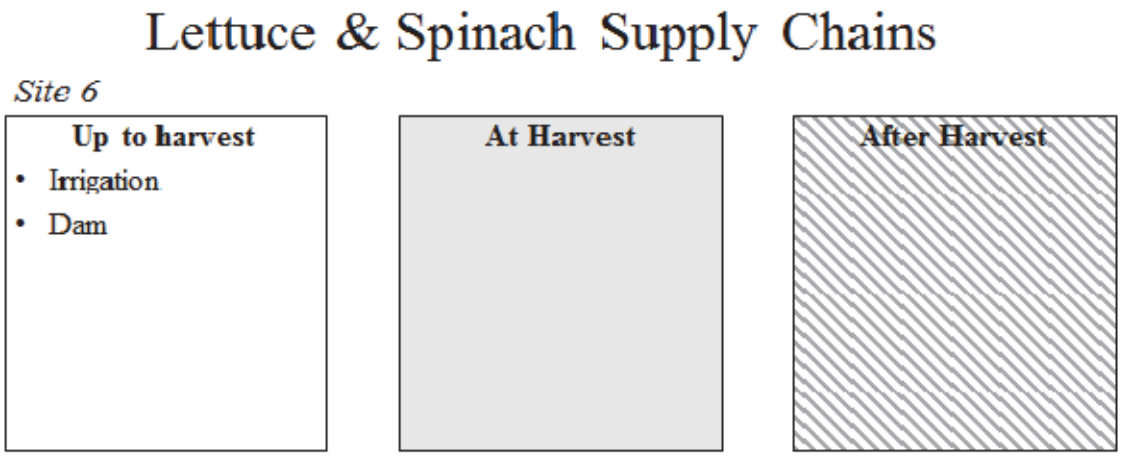
4.2.6 KWAZULU-NATAL PROVINCE – Microbiological quality of water (including agrichemical spray water) used to irrigate spinach and lettuce on a farm near Cato Ridge

Specific aim

The motivation of this study was to gain a broad understanding of the microbial ecology of fresh produce and how factors such as irrigation water and pesticides used during cultivation could result in contamination by food-borne pathogenic bacteria resulting in a decrease in food quality and shelf life.

Sampling site

Farm B (60 hectares) is located in Cato Ridge. Crops planted are broccoli, spinach, lettuce, cauliflower, Chinese cabbage, parsley, bell pepper and red cabbage, and these crops are irrigated weekly. Crops are washed and packed on site. The farm's source of irrigation is a mixture of borehole and dam water (B).



Refer to Chapter 1, section 1.2 of this volume

Experimental procedures

Sample collection and processing

Samples of irrigation water were collected in 5 ℓ sterile plastic bottles once a month from a farm in the Cato Ridge area, KwaZulu-Natal for a period of 3 months (August-October,

2013). The size of the farm is 60 hectares and their source of irrigation is dam water. Water was collected from the tap used for irrigation using ethanol-sterilised 5 ℓ plastic bottles.

Microbiological analysis of irrigation water samples

Sample water collected from the farm was enumerated by carrying out 10-fold serial dilutions and spread plating onto selective media as described in section 4.2.5.

Measuring the physical parameters of water

BOD (biological oxygen demand) and COD (chemical oxygen demand) was determined using HACH HQ40d Portable Meter with HACH (LC101 probe) and a spectroquant NOVO 60 photometer respectively, according to standard methods (Clesceri *et al*., 1998). Total dissolved solids (TSD), turbidity and pH were determined using a HACH HQ40d portable meter with a HACH probe CDC 401, 2100 P HACH turbimeter and Beckman 50 pH meter.

Influence of pesticides in agrichemical spray water on the microbial load found in irrigation water

Pesticide mixed with irrigation water was collected from the farm. This mixture was then used to enumerate microorganisms found in the solution by carrying out 10-fold dilutions. The samples were plated by taking 0.1 mℓ of the sample at different time intervals (0 hours, 6 hours, 12 hours, 24 hours and 48 hours) and plated on media as described above.

Results

Aerobic colony counts (ACC): the ACC plate counts ranged between 2.8 log CFU/ mℓ (August 2013) to 3.6 log CFU/mℓ (October 2013) (Fig. 44). The lower counts observed during August for the microorganisms, might have been due to the low temperatures during early season of spring as indicated by the water temperature measured at 20^0C. The gradual increase in *E. coli* species detected in irrigation water could possibly be attributed to the increase of BOD, COD, TDS and turbidity from August to October (Table 32).

E. coli – the *E. coli* counts gradually increased from 2.25 log CFU/mℓ to 2.5 log CFU/mℓ (Fig. 44).

Presumptive *L. monocytogenes* and *Salmonella* spp. were present in water sampled from August 2013 to October 2013. Presumptive *Shigella* spp. were only isolated during September and October (Fig. 44).

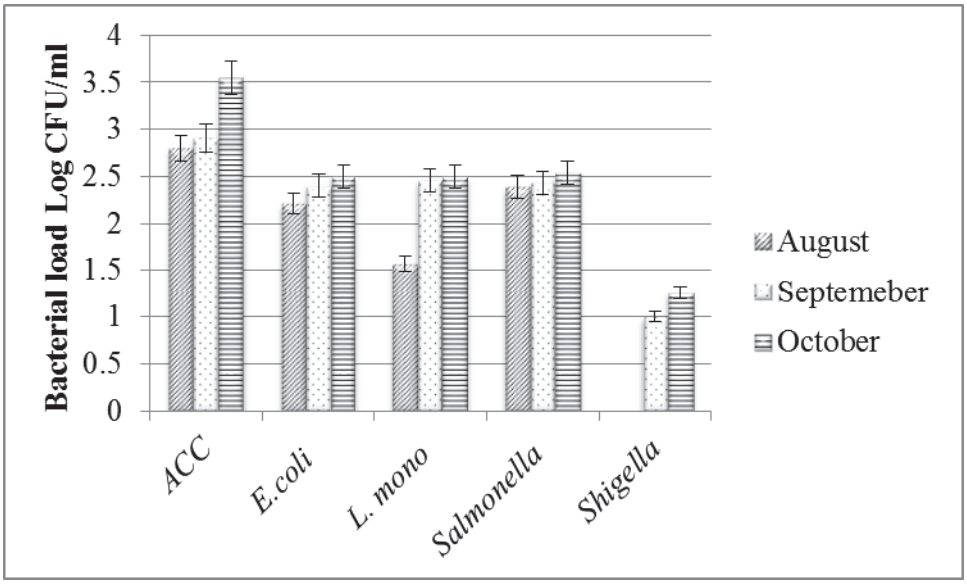


Figure 44 Presumptive bacterial species detected on irrigation water for 3 months

Table 32 Physical parameters of irrigation water

Physical parameters	August	September	October
Temperature °C	20	23	26
pH	6.9	7.1	7.23
Salinity ‰	0.23	0.25	0.28
Turbidity UNT	228	238	286
TDS mg/ℓ	16.8	17. 5	18. 4
BOD mg/ℓ	6.5	6.6	7.02
COD mg/ℓ	250	253	262

Influence of pesticides in agrichemical spray water on the microbial load found in irrigation water

Agrichemical sprays used on the farm showed the ability to inhibit growth of some of the microorganisms found in irrigation water. As indicated in Figure 45 below, *E. coli* and the ACC were the only species detected in the pesticide solution. *E. coli* increased from 2.5 log CFU/mℓ at time 0 to 3.4 log CFU/mℓ after 6 hours of incubation. The *E. coli* of water compared to the pesticide solution is low, indicating that pesticides do have the potential to promote growth of some microorganisms. ACC counts of water were very low, when compared to the pesticide solution. This can be attributed to the fact that these contain a wide range of microorganisms of which some have the ability to utilize the active ingredient found in the pesticide as a nutrient source. After 12 hours the *E. coli* levels decreased. However, the ACC counts started decreasing at 24 hours.

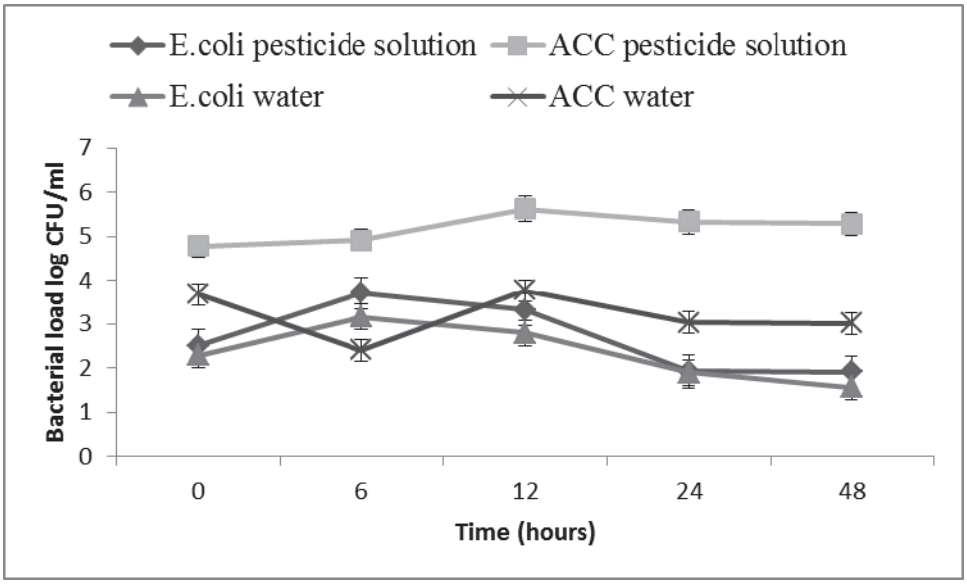


Figure 45 Presumptive microbial species detected on alpha-trin pesticide mixed with irrigation water.

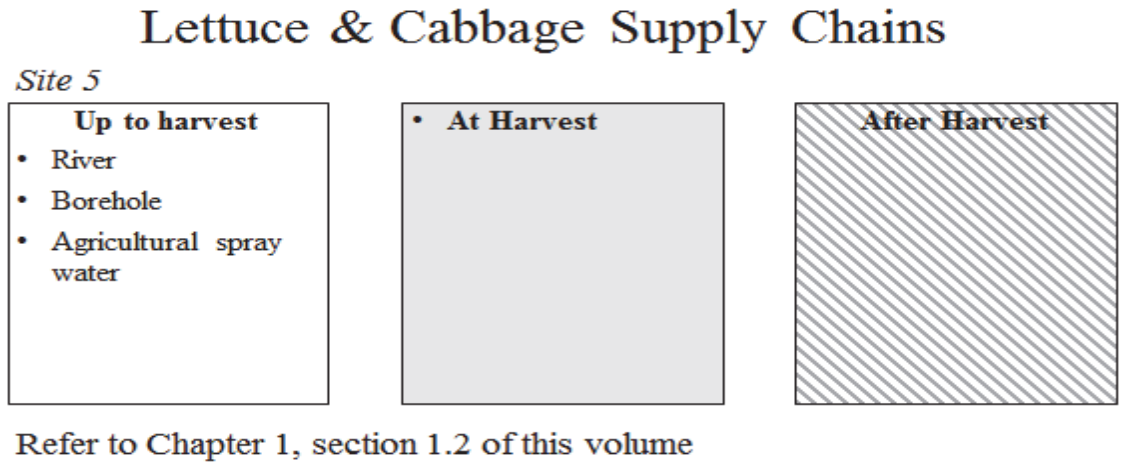
Discussion and conclusion

The results presented above show the correlation between irrigation water quality and pesticide solution in relation to the contamination of fresh produce. As the amount of bacteria detected on water increased, the amount found in irrigation water also increased. Therefore, it can be assumed from these results, that irrigation can indeed be the source of contamination of fresh produce. A study done by The Department of Water Affairs and The Water Research Commission (2012) in conjunction with the University of Stellenbosch showed that most South African rivers used as a source of irrigation water are highly contaminated with faecal coliforms (Mouton, 2013). This study showed that the concentrations found in rivers were more than 1 million microbes per 100 mℓ of water measured (Mouton, 2013). Department of Water Affairs guidelines (1996) state that TDS of water should not be greater than 40 mg/ℓ, but from the physical analysis carried out throughout the sampling period it can be noted that the TDS value far exceeded the guideline values. Temperature plays a pivotal role in the growth of bacteria, as bacteria have different optimum temperatures for growth. The low counts observed for most of the bacteria in August might have been due to the low temperature of water and the atmospheric temperature, as this was still the late stage of winter. The four bacterial species detected in irrigation water and fresh produce are of great concern as they have been linked with a number of foodborne illnesses in the past. The increase in the total ACC and *E. coli* counts in the irrigation water mixed with pesticides is similar to results obtained by Peter *et al*. (2005). They showed the ability of pesticides to promote growth of some microorganisms when mixed with irrigation water.

4.2.7 KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of fresh produce (lettuce and cabbage) on two farms near Camperdown

Specific aim: To determine the microbiological quality of irrigation water, fresh produce and agricultural spray water and to show relationships among all possible pairs of physico-chemical parameters [BOD (biological oxygen demand), COD (chemical oxygen demand), total dissolved solids (TSD), turbidity and pH] and the bacterial counts found on the crops.

Sampling site



Experimental procedures

Sample collection

Irrigation water and agricultural spray water samples were collected from two farms in KwaZulu-Natal for a period of three months (July-September 2014). Irrigation water and pesticide solutions were collected using sterile 2 litre plastic bottles pre-washed with 70% ethanol and rinsed with the sample before collection. After collection, all the samples were stored on ice during transportation to the laboratory where they were stored at 4°C and processed for analysis within 24 hours (Gemmell and Schmidt, 2012).

Microbiological analysis of irrigation water samples

Sample water collected from the farm was enumerated by carrying out 10-fold serial dilutions and spread plating onto selective media (as described in section 4.2.6).

Measuring the physical parameters of water

BOD (biological oxygen demand), COD (chemical oxygen demand), total dissolved solids (TSD), turbidity and pH were determined (as described in section 4.2.6).

Impact of pesticides on the microbial load found in irrigation water

Pesticides mixed with irrigation water were collected from both farms. One hundred microliters of the pesticide solution were placed in sterile flasks and incubated at 37 °C, 24 hours and shaking at 150 rpm. Samples were then taken at 0, 12 and 24 hour intervals and bacteria enumerated as previously mentioned. Microbial counts were expressed as CFU/ml. Table 33 illustrates the pesticide mixtures and their compositions based on the sampling period. At each sampling period the combination of pesticides was different.

Table 33 Different pesticides used and their active ingredients.

Farm	Pesticides used (600 L)	Type	Active ingredient
A1	Metfos 585 SL	Insecticide	Methamidophos
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Aphox	Insecticide	Pirimicarb
B1	Biohumate	Fertilizer	Humic acids
	Dimethoate	Insecticide	Dimethoate
	Urea HB	Fertilizer	Nitrogen
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
A2	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Aphox	Insecticide	Pirimicarb
	Nu-Film P	Non-toxic sticker – spreader	Poly-1-p-menthene
	Metfos 585 SL	Insecticide	Methamidophos
	Liquid boron	Fertilizer	Boron
B2	Allice 20 SP	Insecticide	Acetamiprid (neonicotinoid)
	M45 dithane	Fungicide	Mancozeb
	Biohumate	Fertilizer	Humic acids
	Urea HB	Fertilizer	Nitrogen
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate

Farm	Pesticides used (600 L)	Type	Active ingredient
A3	Prev – AM	Fungicide / insecticide /miticide	Borax; Orange oil
	Aphox	Insecticide	Pirimicarb
	Belt	Insecticide	Flubendiamide (phthalic acid diamide)
	Bolldex	Biological bollworm control	Helicoverpa armigera nucleopoly-hedrovirus (HearNPV)
	Nu-Film	Non-toxic sticker – spreader	Poly-1-p-menthene
	Agromectin	Insecticide	Abamectin
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
B3	Steward 150	Insecticide	Indox acarb – oxadiazine
	Allice 20 SP	Insecticide	Acetamiprid (neonicotinoid)
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Odeon 720 SC	Fungicide	Chlorothalonil
	Urea HB	Fertilizer	Nitrogen
	Afrikelp LG-1	Plant growth stimulant	Ecklonia maxima (liquid seaweed concentrate)
	Cyperin 200 EC	Insecticide	Cypermethrin (pyrethroid)
A4	Prev – AM	Fungicide / insecticide /miticide	Borax; Orange oil
	Aphox	Insecticide	Pirimicarb
	Belt	Insecticide	Flubendiamide (phthalic acid diamide)
	Bolldex	Biological bollworm control	Helicoverpa armigera nucleopoly-hedrovirus (HearNPV)
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Decis Forte	Insecticide	Deltam ethrin (pyrethroid)
B4	Urea HB	Fertilizer	Nitrogen

Farm	Pesticides used (600 L)	Type	Active ingredient
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Agromectin	Insecticide	Abamectin
	Voema Calmag and TE	Fertilizer	Microelements
	Diclorvos 1000 EC	Insecticide	Dichlorvos
	M45 dithane	Fungicide	Mancozeb

A and B = Farm A and Farm B respectively; 1-4 = Sampling periods

Confirmation of presumptive pathogen identities

DNA isolation

Genomic DNA isolation was done using the boiling method according to Ahmed and Shimamoto (2014).

PCR amplification and confirmation of isolates

PCR amplification of DNA from *L. monocytogenes* was performed according to Jeyaletchumi *et al*. (2010). For the amplification of the *InvA* gene in *Salmonella* spp., the protocol by Gassama-Sow *et al*. (2006) was used. Details of primers used are summarised in Table 34.

Table 34 The description of the primers, sequences and target genes.

Organism	Target gene	Primers	Primer sequence (5' ⟹ 3')	Product size (bp)
E. coli	*mdh*	*mdh*-F	CGTTCTGTTCAAATGGCCTCAGG	392
		mdh-R	ACTGAAAGGCAAACAGCCAAG	
L. monocytogenes	*hlyA*	*hlyA*-F	CAAGTCCTAAGACGCCAATC	1412
		hlyA-R	ATAAAGTGTAGTGCCCCAGA	
Salmonella spp.	*InvA*	*InvA*-F	TGCCTACAAGCATGAAATGG	450
		InvA-R	AAACTGGACCACGGTTGACAA	

Statistical analysis

Data of the log averages and associated standard deviations (SD) from each set of three replicates were calculated for each sample time using Excel 2010 (Microsoft). The data was statistically analysed using mean and standard deviations. P-values associated with T-test and One-way ANOVA was taken as an indicator of significant differences among the data using

GraphPad Prism software (version 5.00). In all statistical analyses, confidence level was held at 95% and P<0.05 (at 5% level of significance) was considered as significant. Pearson product-moment correlation coefficient using SPSS (SPSS, Inc., Illinois 21.0) was used to show relationships among all possible pairs of physico-chemical parameters and the bacterial counts found on the crops.

Results

Water:

Total Heterotrophic bacteria (THB) – the THB plate counts in water from both farms were similar and ranged between 2.91 log CFU/ml -3.49 log CFU/ml. (Fig. 46).

E. coli – the *E. coli* counts ranged between 0.52 log CFU/ml and 1.4 log CFU/ml (Fig. 47).

Presumptive *L. monocytogenes* and *Salmonella* were present in the irrigation water samples (Fig. 47).

The identities of presumptive *E. coli*, *Salmonella* and *L. monocytogenes* were confirmed using PCR analysis and pathogen specific primers as described in Table 34.

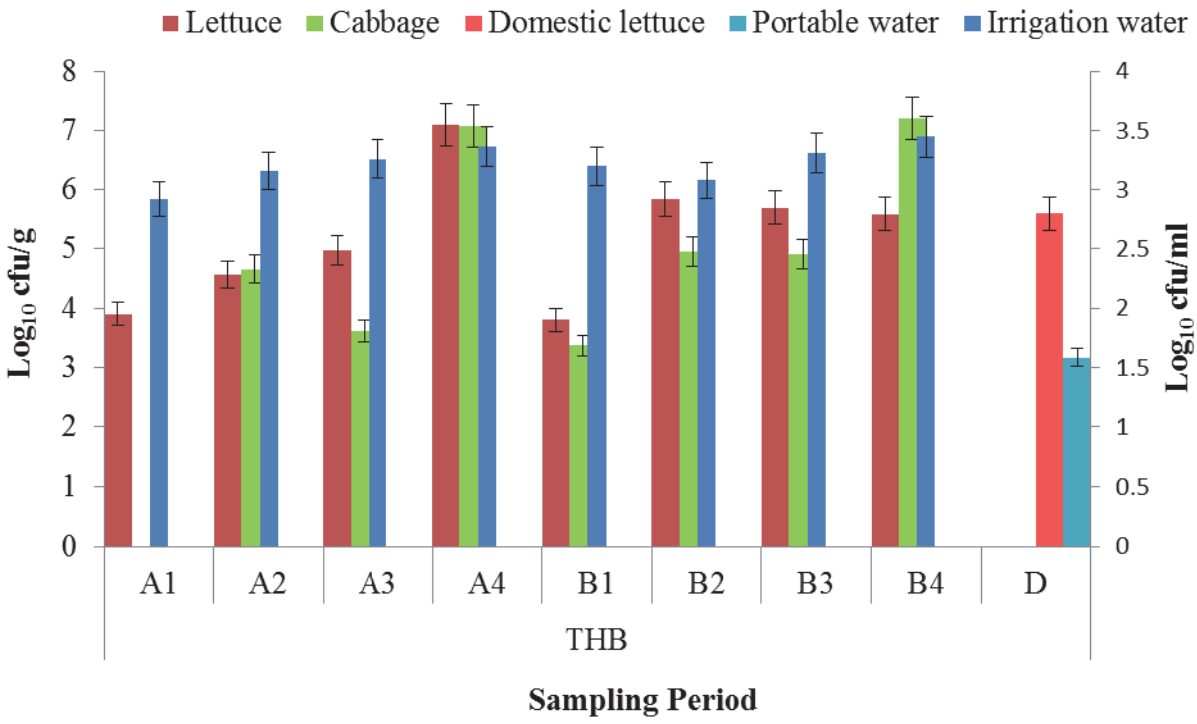


Figure 46 Microbial load of total heterotrophic bacteria in irrigation water and fresh produce.

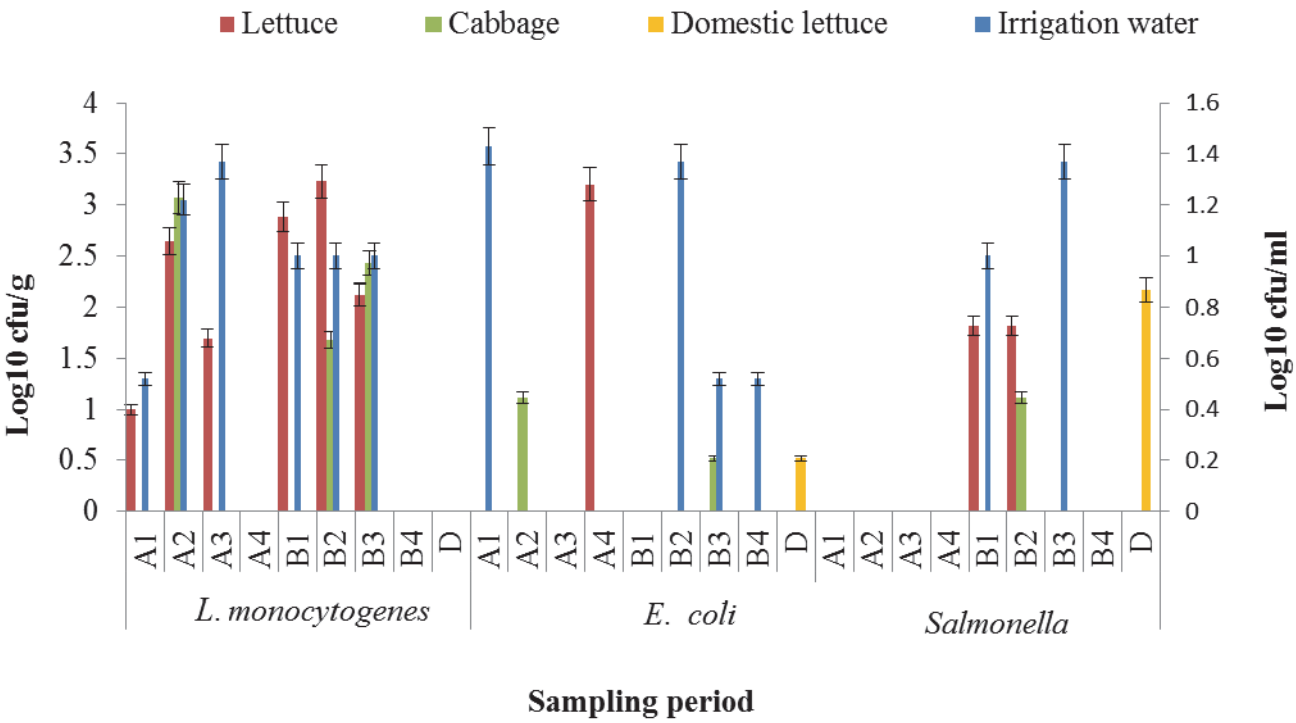


Figure 47 Microbial load of selected bacteria in irrigation water and fresh produce.

Physico-chemical properties of water samples

The data for the physic-chemical parameters of water obtained for both farms and the potable water are summarised in Table 35. The water temperature from all three sites was similar and ranged from 15-20 °C during the research period. The pH of the irrigation water from farm A ranged between 7.02-7.98 while it ranged between 7.0.4-7.47 on farm B. TDS of the water samples differed significantly ($p < 0.01$) during the four sampling periods for both farms. The turbidity of the water samples from both farms differed significantly ($p < 0.05$). Farm B had a higher mean turbidity of 2.94 NTU compared to farm A which had a mean turbidity of 2.74 NTU. The COD and BOD values of the water samples were not significantly different ($p > 0.05$). Farm A had a higher mean COD of 155.7 mg/l compared to farm B which had a lower mean COD of 80.54 mg/l. The physico-chemical parameter data for the portable water was within the standards for drinking water.

Table 35 Physico-chemical parameters of irrigation (A & B) and potable water (D) samples

Sample	Temp (°C)	pH	Turb (NTU)	TDS (mg/l)	COD (mg/l)	BOD_5 (mg/l)
A1	17	7.12	2.48 ± 0.04	150.93 ± 0.40	302 ± 4.58	0.79 ± 0.01
A2	16	7.02	2.60 ± 0.08	158.70 ± 0.61	86 ± 3.00	0.96 ± 0.01
A3	17	7.58	2.83 ± 0.03	155.67 ± 0.15	107.33 ± 5.69	0.62 ± 0.01
A4	20	7.98	3.00 ± 0.21	161.43 ± 0.06	127.33 ± 19.09	3.01 ± 0.01
B1	16	7.04	1.85 ± 0.04	193.33 ± 0.15	50.5 ± 2.12	0.67 ± 0.02
B2	15	7.43	2.99 ± 0.01	198.97 ± 0.15	219.33 ± 2.31	0.99 ± 0.01
B3	16	7.27	3.75 ± 0.05	192.50 ± 0.20	12.00 ± 3.00	4.99 ± 0.02
B4	20	7.47	3.15 ± 0.05	193.3 ± 0.10	40.33 ± 1.15	1.17 ± 0.01
D	19	7.35	0.5 ± 0.11	72.07 ± 0.15	8 ± 1.00	6.75 ± 0.01

Turb: Turbidity, Temp: Temperature, TDS: Total dissolved solids, COD: Chemical oxygen demand, BOD: Biochemical oxygen demand

The data in Table 36 showed that there was a positive and significant correlation ($p \leq 0.05$) between pH and turbidity (r= 0.952), between pH and temperature (r= 0.899), and between temperature and turbidity (r= 0.782). Positive correlation was also noted between COD and *E. coli* counts in the water samples (r= 0.985). There was a positive and highly significant ($p \leq 0.01$) correlation between the COD and THB counts (r= 0.995), and between the THB and *E. coli* counts (r= 0.998) in the water samples. TDS influenced the growth of *E. coli* (r= 0.650), *L. monocytogenes* (r= 0.381) and THB (r= 0.616). This was illustrated by the positive correlation between these factors. There was a positive correlation between various parameters, including BOD and temp, pH and turbidity (r= 0.926, 0.764 and 0.725, respectively).

Table 36 Correlation matrix of physical parameters of irrigation water for farm A.

	Temp	pH	Turb	TDS	COD	BOD	THB	LM	EC
Temp	1								
pH	.899	1							
Turb	.782	.952*	1						
TDS	-.760	-.703	-.786	1					
COD	-.030	-.301	-.579	.555	1				
BOD	.926	.764	.725	-.921	-.200	1			
THB	-.127	-.396	-.658	.616	.995**	-.281	1		
LM	-.670	-.284	-.073	.381	-.551	-.681	-.493	1	
EC	-.192	-.459	-.709	.650	.985*	-.331	.998**	-.454	1

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

SAL (*Salmonella* spp.) is constant. THB: total heterotrophic bacteria, LM: *L. monocytogenes*, EC: *E. coli*

From the data in Table 37 it can be seen that there is a highly significant ($p \leq 0.01$) correlation between COD and TDS on farm B (r= 0.998). Significant correlation ($p \leq 0.05$) was observed for *E. coli* with TDS and COD, as indicated by r= 0.971 and r= 0.956, respectively. A positive correlation was observed between *Salmonella* spp. and pH (r= 0.983), and turbidity (r= 0.591). There was a negative correlation between *L. monocytogenes* and all the physico-chemical parameters with the exception of temperature (r= 0.980), with which there was a significant positive correlation ($p \leq 0.05$).

Table 37 Correlation matrix of physical parameters of irrigation water for farm B.

	Temp	pH	Turb	TDS	COD	BOD	THB	EC	SAL	LM
Temp	1									
pH	-.717	1								
Turb	-.075	.466	1							
TDS	-.079	-.428	.170	1						
COD	-.093	-.390	.224	.998**	1					
BOD	-.337	.057	-.857	-.435	-.474	1				
THB	-.293	.840	.807	-.382	-.329	-.423	1			
EC	-.066	-.543	-.071	.971*	.956*	-.231	-.581	1		
SAL	-.594	.983*	.591	-.442	-.398	-.095	.925	-.589	1	
LM	.980*	-.596	-.084	-.273	-.285	-.257	-.187	-.260	-.471	1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).
SAL: *Salmonella* spp., THB: total heterotrophic bacteria, LM: *L. monocytogenes*, EC: *E. coli*

Pesticide analysis

The data in Figure 48 showed an increase in the microbial load from 0-24 hours for A1, A3, A4 and B1, while A2, B2, B3 and B4 showed a decrease. The pesticide mixtures used at each sampling interval was different. In B4 no initial counts were observed at 0 hours, however there were very high counts at 12 hours followed by a sharp decrease in the microbial count at 24 hours of incubation. In A2 there was total inhibition of the microbes at 24 hours, with B2 having no growth at 12 hours. The bacterial counts ranged from 1.99-5.42, 2-7.25, and 0.52-8.07 at 0, 12 and 24 hours respectively. Data recorded for the 24 hour incubation were not significant ($p > 0.05$).

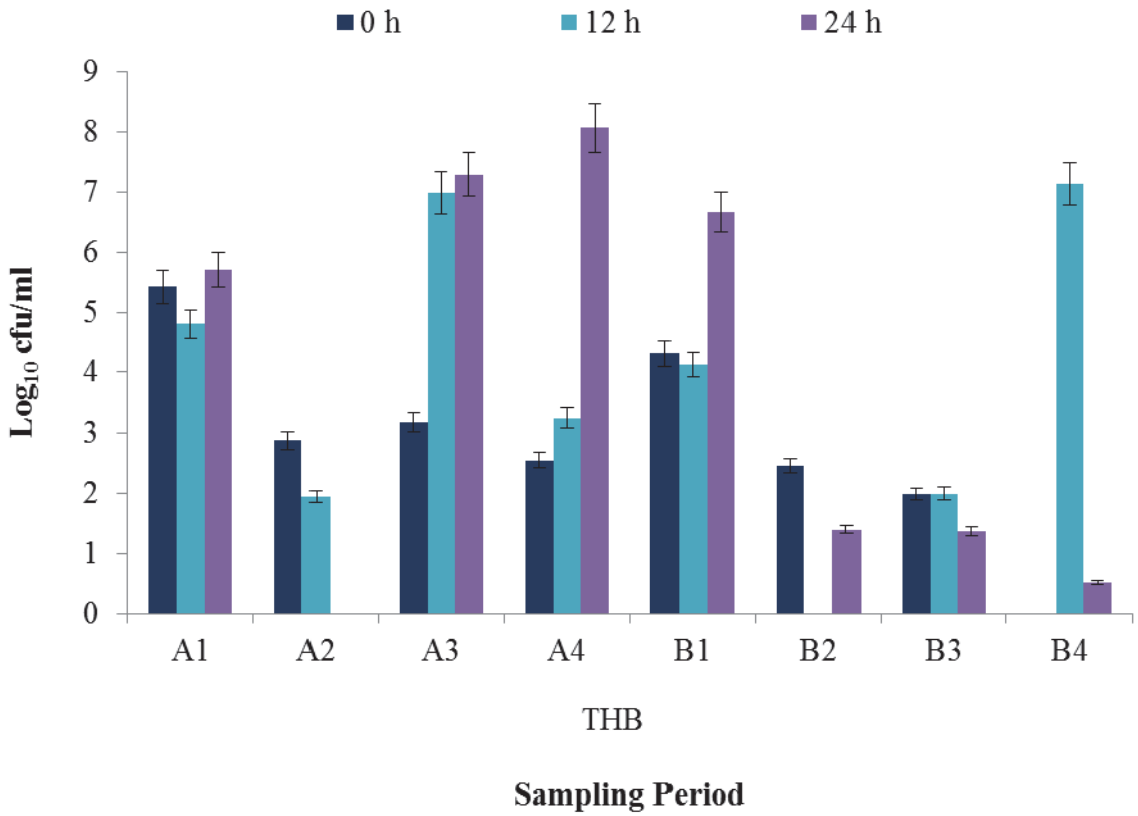


Figure 48 Microbial load of THB in irrigation water mixed with pesticides at different time periods.

The data in Table 38 shows that there was an initial inhibition of the selected microbes at 0 hours, however at 12 hours *E. coli* and *Salmonella* were detected in A3 and B4 sampling intervals. Growth of all three microbes was observed at 24 hours for A1 and A3 sampling intervals. A common trend was observed with pesticides used in A1 and A3 sampling intervals.

Table 38 Microbial load of selected bacteria in irrigation water mixed with pesticides at different time periods in log CFU/ml.

Organism	Sampling Period	Pesticide solution sampling time (h)		
		0	**12**	**24**
L. monocytogenes	A1	0	0	0.52
	A2	0	0	0
	A3	0	0	0.52
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	0
E. coli	A1	0	0	2.54
	A2	0	0	0
	A3	0	1.12	3.26
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	0
***Salmonella* spp.**	A1	0	0	2.34
	A2	0	0	0
	A3	0	2	2.70
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	1.12	0

A and B = farm A and farm B; 1-4 = sampling times.

The pH of all the irrigation water samples fell within Food and Agriculture Organization of the United Nations (FAO) guidelines of 6.5-8.4 (FAO, 1996). Water temperature was in the range of 15-20 °C, including the potable water sample. This was expected as the sampling was done during the months of July to September when temperatures are not very high. The pH affects functioning of the cell. Since the pH was close to neutral values, this created an ideal environment for the growth of most of the organisms, including enteric pathogens. The mean turbidity levels in the irrigation water exceeded the international turbidity of 0-1 NTU standard for water (DWAF, 1996). The turbidity of the potable drinking water was within the range of 0-1 NTU. The high turbidity levels of the irrigation water could be the result of soil erosion and runoff. There was a positive correlation between the BOD, temperature and pH, and the turbidity levels on farm A. The values for TDS recorded on both farms were within the recommended range of 1000 mg/l for TDS in drinking water and 40 mg/ml for irrigation water as specified by WHO (WHO, 1996). There was a positive correlation between the TDS and COD levels in the irrigation water from both farms. *E. coli* counts appeared to be influenced by TDS and COD on farm B. Microbial growth could have been supported by the presence of organic matter in the irrigation water.

The BOD levels were lower than the COD levels which is expected since BOD only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels on both farms. COD and TDS values were the only two parameters of water that seemed to have influenced the survival of the THB in the irrigation water on farm A. TDS seemed to have also influenced the survival of *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. There was a significant positive correlation between the COD levels and the THB and *E. coli* counts in the water samples. South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by WHO of 10 mg/l. This is an indication that the water contains organic pollutants which could help promote the growth of certain microbes in the water. There was a positive correlation between the THB and *E. coli* in the water on farm A while there was a negative correlation between the two in water from farm B. THB counts were also positively correlated wth the *Salmonella* counts. This could be as a result of mutualistic relationships between *Salmonella* and some of the other bacteria in the sample. These other bacteria could be breaking down nutrients for *Salmonella* or it could be using the by-products of these other organisms that are present in the environment.

South Africa does not have standards for the amount of aerobic counts allowed in irrigation water; however the guidelines for domestic water specify a heterotrophic plate count of ≤ 100 CFU/ml (DWAF, 1996). The difference in mean counts was found to be significant ($p \leq 0.05$) for irrigation water and vegetables from farm A, and highly significant ($p < 0.01$) for irrigation water and vegetables from farm B. There was no significance ($p \geq 0.05$) in the mean values of potable water and the lettuce sample. The heterotrophic bacterial counts are in general used as an indication of the microbial quality of water. THB counts are not a representation of the total number of bacteria present in a sample.

Contamination of water sources by other bacterial pathogens, namely, *L. monocytogenes* and *Salmonella* showed that the irrigation water samples are of poor microbial quality. The presence of the indicator *E. coli* in the water samples evaluated, indicates possible contamination of the water sources by faecal matter. The international standard for faecal coliforms allowed, is 1000 CFU/100 ml in irrigation water, and this was exceeded in the majority of the samples. The microbial load on lettuce was generally higher compared to cabbage samples. Slight differences in the micro-architectural structure of the vegetable may also contribute to differences in pathogen attachment and survival. Supporting this statement, higher numbers of *Salmonella* spp. attached to Romaine lettuce compared to cabbage surfaces (Monaghan and Hutchison, 2012). Lettuce irrigated with non-potable water has been shown to have significantly higher rates of total coliforms and presumptive *Salmonella* contamination than lettuce irrigated with drinking water (Strawn *et al*., 2013). Chai *et al*. (2011) showed that *Salmonella* spp. present on the surface of plants can survive for extended periods and might overcome the immune response of plants and actively enter through the stomata. This was demonstrated for lettuce. No presumptive *Shigella* spp. was detected in all of the samples analyzed. *Shigella* is not ubiquitous in the environment and contamination of fresh produce by this species has been mostly linked to improper sanitation and human handling (Nygren *et al*., 2013).

The use of pesticides to control weeds, insects, and other pests has resulted in a range of benefits, including increased food production. South Africa has one of the largest markets for pesticides in Sub-Saharan Africa (Quinn *et al*., 2012). The data for the pesticide analysis showed that the active ingredients of the pesticides could act as inhibitors or promoters of microbial growth. These findings correlate with previous studies which showed that pesticide solutions provide a suitable environment for the survival and growth of pathogenic microbes, such as *L. monocytogenes*, *E. coli*, *Salmonella* and *Shigella* spp., whereas others are inhibitory to these microorganisms (Compas *et al*., 2013). The common trend was that the microbes' growth was favoured mainly by the pesticides with organophosphates and carbamates as the active ingredients. Organophosphate pesticides contain diazinon and chlorpyrifos. Other active ingredients were abamectin (16-membered macrocyclic lactone derivative), phthalic acid diamide and poly-1-p-methene (insect growth regulator). The effect of combining more than one pesticide in the water might be different than the effects of each individual pesticide alone. The frequency of the application of pesticides and the degree of adsorption has been shown to affect microbial degradation of pesticides and utilising it as a source of nutrients.

4.2.8 LIMPOPO PROVINCE – Microbiological quality (including virological analysis) of water used to irrigate onions and tomatoes on a commercial farm near Polokwane

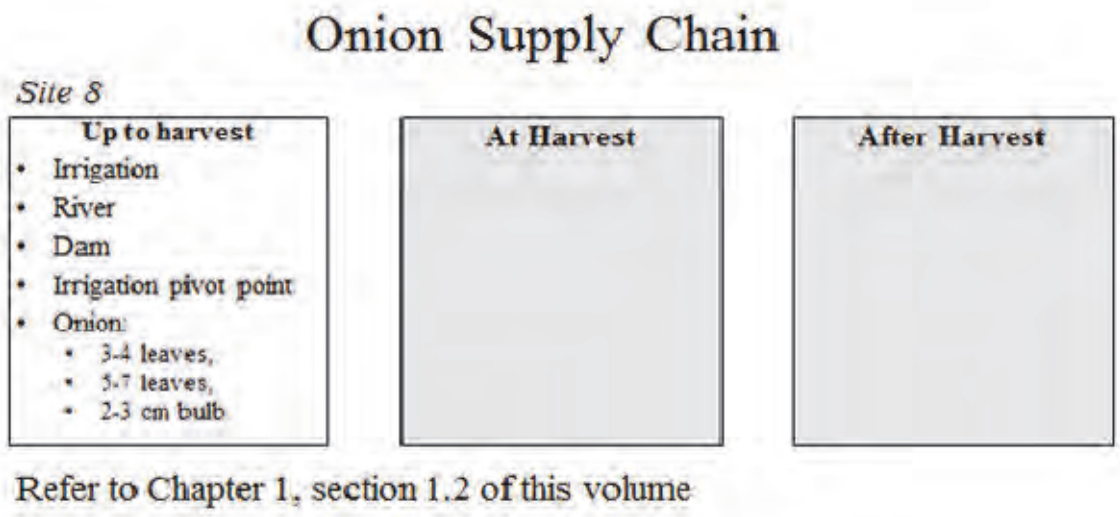
Specific aim

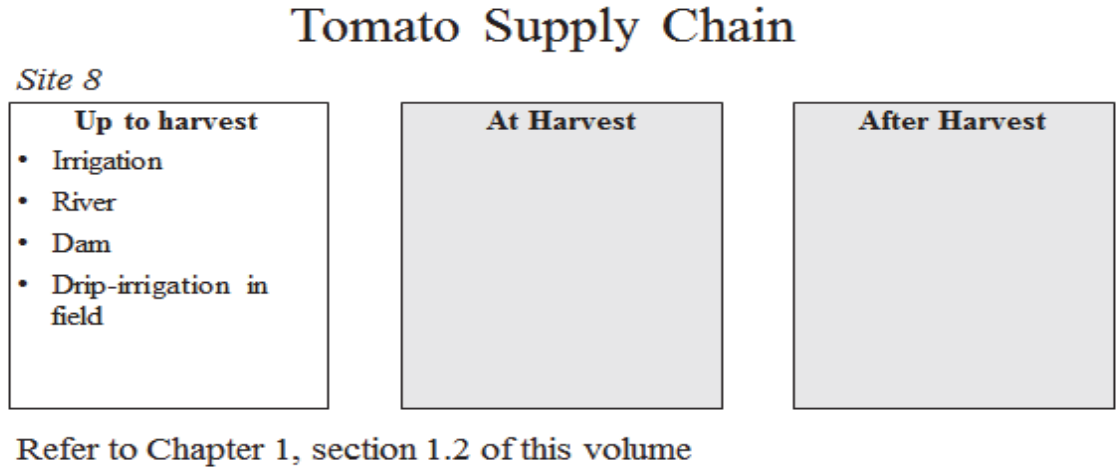
The aim of this project was to investigate the quality of irrigation water and onions during the various onion growth stages, at harvest and after harvest at the fresh produce market.

Experimental procedures

Experimental site

A commercial onion/tomato production farm situated near Polokwane in the Limpopo Province. Irrigation water was pumped from the Sand River to holding dams and used for overhead irrigation of the onions.





Water sampling and processing

Three water sampling sites (river, dam and irrigation pivot point) linked to the irrigated field where onions were cultivated, were microbiologically monitored over a full onion growing cycle. Water samples, (3 x 1 ℓ) per sampling site, were collected in air dried plastic bottles sterilized with 70% ethanol from each of the sites. All analyses were performed in triplicate. Each of the one litre water samples was filtered through sterile cellulose nitrate filters (0.45 µm pore size) (Sartorius, Goettingen, Germany) for further microbiological analysis.

Microbiological analysis

After filtering the irrigation water, the membranes were aseptically cut into small pieces, added to 9 mℓ TSB medium and serially diluted. To enumerate total viable bacteria and fungi respectively, the diluted samples were plated in duplicate onto Standard 1 agar (STD 1) and malt extract agar (MEA) (Merck, Johannesburg, South Africa). Plates were incubated at 25 °C for two (STD 1) and five days (MEA) respectively. *E. coli*/ coliform count plates (3M Petrifilm, Merck) were used for enumeration after incubation at 37°C for 24 hours. The data was transformed to log CFU/100 mℓ of the water samples.

Samples enriched for determining the presence of *E. coli* (including *E. coli* O157:H7), *Salmonella* spp. and *Listeria* spp. as described in section 4.2.1 for water used to irrigate parsley. *Listeria* spp. was enriched in Listeria enrichment broth at 37ºC for 24 hours, where after it was plated onto Listeria agar (all enrichment media were obtained from Oxoid, Johannesburg). All presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates from selective media were purified by re-streaking onto selective media and identified using PCR analysis and Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF).

Molecular methods

DNA was extracted from single colonies of presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates using the Quick-gDNA miniprep kit (Zymo Research, California, USA). The universal primers F-27 and R-1492 were used to amplify the 16s rDNA genes of the bacterial isolates (Lane *et al*., 1985). The PCR products were sequenced and a search of nucleotide databases conducted to confirm the microorganism identities.

Results

Microbiological analysis of water

Aerobic colony counts (ACC) – The ACC counts varied between 2.5 log CFU/mℓ to 6.5 log CFU/mℓ (Sand River); from 3.5 log to 6 log CFU/mℓ (dam) and from 3.1 log CFU/mℓ to 5.5 log CFU/mℓ (onion irrigation pivot point) and 3.1 to 3.8 log CFU/mℓ (micro irrigation pipe in the tomato field). ACC reflects the total microbial content including spoilage organisms (Sela and Fallik, 2009).

Coliforms and E. coli – Coliform and *E. coli* counts were higher during summer than during winter (Fig. 49).

River water – *E. coli* counts on the farm exceeded 1000 CFU/100 mℓ during the summer months. However, the *E. coli* counts during the winter months were close to or at acceptable levels according to the guidelines of ≤ 1-1000 CFU/ 100 mℓ for irrigation water (DWAF, 1996).

Dam water – *E. coli* counts exceeded the irrigation water guidelines only once, during week 7, (February 2012). This water source is used to irrigate onions and tomatoes.

Irrigation pivot point water sampled when the onions were irrigated during March (week 13), May (week 20) and June (week 25) *E. coli* counts complied with guidelines of ≤ 1-1000 *E. coli* CFU/100 mℓ.

Drip irrigation pipe water – the tomatoes were produced and irrigated in summer and the *E. coli* levels for one of the sampling intervals exceeded 1000 CFU/100 mℓ.

Fungi – Fungal counts were low ranging from a minimum of 0.1 log CFU/mℓ (pivot point water) to a maximum of 2.6 log CFU/mℓ (dam water).

Yeast – Yeast counts ranged from a minimum of 0.75 log CFU/mℓ (dam) to a maximum of 4.5 log CFU/mℓ (river)

E. coli O157: H7 – None isolated.

Salmonella spp. – Isolated from 22% of the water samples tested included *Salmonella enterica* subsp. enterica serovar Typhimurium.

Listeria spp. – None isolated.

Noro viruses (NoV GI, NoVG1) and Hepatitis A virus (HAV) –

River – all three viruses were detected in the river water for 5/6 (January, March, May, June, August) sampling intervals.

Dam – all three viruses were detected in the river water for 3/6 sampling intervals

Irrigation pivot point – Nov GI was detected in 1/3 (May 2012) sampling intervals
Nov GII and HAV was detected for 3/3 (March, May and June 2012) sampling intervals

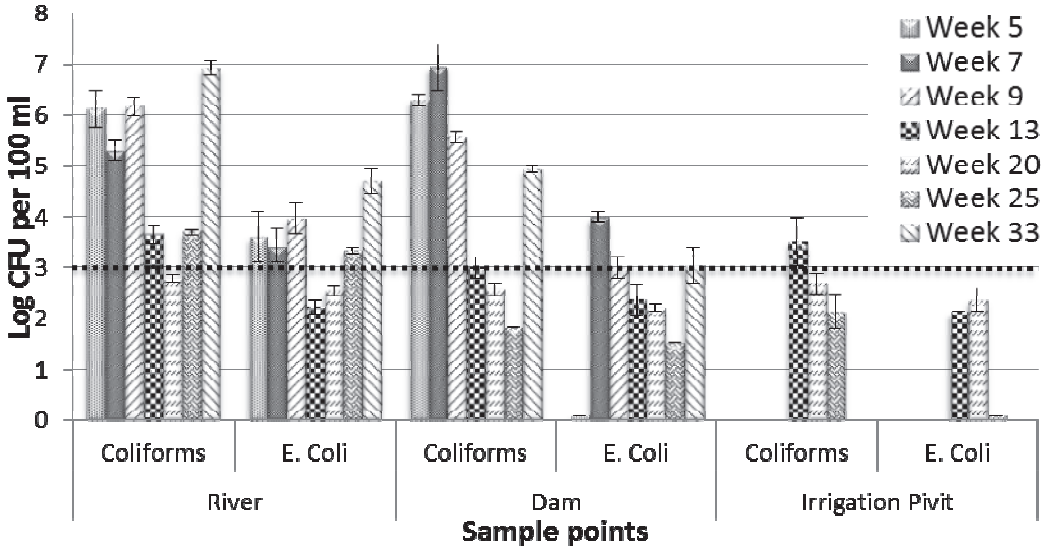


Figure 49 Coliform and *Escherichia coli* counts for river, dam and irrigation pivot point water used to irrigate onions. The broken line indicates the Department of Water and Health (1996) guideline limit in South Africa for agricultural water used for irrigation of fresh produce vegetables and fruit not to be eaten raw, i.e. 1,000 *E. coli* CFU/100 mℓ.

Determination of the physical parameters in the irrigation water

The pH, conductivity, ammonium, nitrate, nitrite, orthophosphate and COD of all the water samples were determined and summarised in Table 39. The electrical conductivity (EC) of the water samples ranged from 70.9 m Siemens/m to 115.2 mS/m which can be regarded as good and tolerable respectively, according to the DWAF irrigation water guidelines (DWAF, 1996). Nitrogen occurs predominantly as nitrate in irrigation water. The ammonium form is usually a result of contamination with wastewater. The highest level of ammonium (25.5 mg/ℓ) was indeed recorded for the water sampled from the dam 3 outflow just below the Polokwane waterworks (sample 5). The waterworks often overflow after rain and subsequently lead to contamination of the river water downstream which is used for irrigation purposes on the farm. The pH of all the water samples tested were within the recommended pH range for irrigation water in South Africa, i.e. from pH 6.5 to pH 9.5 (DWAF, 1996). However, this range also promotes growth of enteric pathogens.

Table 39 Summary of physical properties of the irrigation water sources.

	Sample number		**1**	**5**	**7**	**9**	**16B**	**17B**	**23**
Physical and aggregate properties									
Date		**Unit/s**							
2012/01/31	pH @ 25 °C	pH units	7.5	7.4	7.7	7.8	8.5	8.1	7.2
2012/08/13			7.5	7.4	7.7	8.7	8.4	8.6	7.5
2012/01/31	Conductivity @ 25 °C	mS/m	78.6	108.8	83.3	113.6	115.2	70.9	89.6
2012/08/13			124.2	138.7	132.6	115.0	126.0	119.5	114.6
Inorganic non-metallic constituents									
2012/01/31	Ammonium as NH4-N	mg/l	4.8	25.5	10.6	<0.03	<0.03	7	9.3
2012/08/13			18.5	36.8	26.8	0.08	0.37	21.1	18.5
2012/01/31	Nitrate as NO3-N	mg/l	2.7	<1.4	1.8	8.1	10	2.1	5.6
2012/08/13			3.8	<1.4	1.5	2.2	10.8	<1.4	13.3
2012/01/31	Nitrite as NO2-N	mg/l	0.45	0.01	0.57	0.07	0.09	0.64	0.98
2012/08/13			0.27	<0.01	0.09	0.04	0.15	0.19	0.59
Phosphorus									
2012/01/31	Orthophosphate as PO4-P	mg/l	2.23	4.92	2.91	0.56	0.06	2.52	4.3
2012/08/13			5.79	5.01	4.63	0.62	0.12	4.42	6.18
Aggregate organic constituents									
2012/01/31	COD as O2	mg/l	42.4	70.7	27.4	<18.1	30.6	41	54.7
2012/08/13			49.5	141	104	<18.1	38	75	97.5

Discussion and conclusion

Although the *E. coli* counts measured for the primary irrigation water source (farm river) exceeded 1000 CFU/100 mℓ during the summer months, the *E. coli* counts in the river, dam and irrigation pivot point water during the winter months (during the onion production cycle) were close to or at acceptable levels according to the guidelines of ≤ 1-1000/100 mℓ for irrigation water. The presence of noroviruses and hepatitis A virus in river, dam and irrigation

pivot point water used for irrigation of onions and tomatoes could however pose a health risk to consumers. However, Noroviruses were not detected on tomato surfaces.

4.2.9 LIMPOPO PROVINCE – The microbiological quality of water used to irrigate peaches on a commercial peach farm near Mookgophong

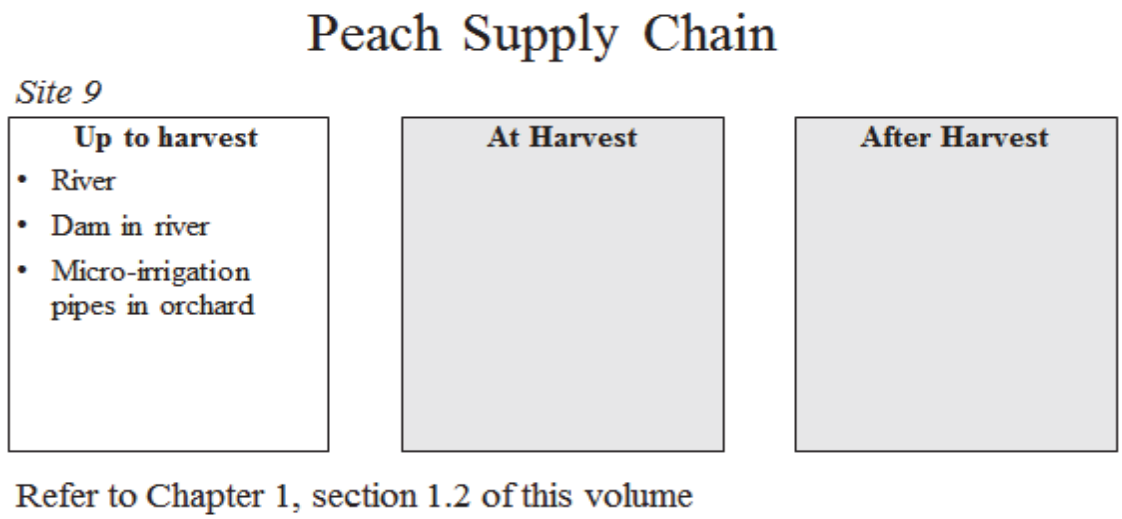
Specific aim

The aim of this study was to investigate the microbiological quality of peaches and water used for irrigation purposes with the ultimate aim of establishing whether a link exists between the irrigation water and fresh fruit.

Experimental procedures

Experimental site

- The study was conducted on a Global GAP (Good Agricultural Practices) certified commercial farm. Peaches (Cultivar=Sunette) were sampled from a peach farm located in the Mookgophong (Naboomspruit) area in the Northern Province, South Africa.
- The peach farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Sterk River and dams in the river are used for irrigation.
- Peaches were irrigated by use of micro irrigation and pivots.
- For the sake of our study, a single orchard (HH) on the farm was selected for microbiological assessment of peaches.



Sampling

Description of water sampling sites

Four water sampling sites linked to the orchard (Orchard HH) where the peaches were harvested, were chosen for determining the microbiological quality and safety of the irrigation water. These included water from the Sterk River, dam in the river, irrigation pipe water after filters in the field and from micro irrigation pipes.

Water sampling

Water samples (100 mℓ, 1 ℓ and 10 ℓ respectively) were collected in ethanol (70%) sterilized, air dried plastic bottles from each of the sites. Samples were transported in a sterilized (70% ethanol) cooler box using ice bricks and stored at 4 °C until analysis started. All analyses were performed in triplicate.

Microbiological analysis

Total aerobic colony counts, coliform/*E. coli*, fungi and yeast counts were determined as described previously (section 4.2.8). Slanetz and Bartley medium (Oxoid) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC solution, Oxoid) and Staph Express count system (3M Petrifilm) were used for the enumeration of Intestinal enterococci (IE) (red, maroon, pink or brown colonies) and *S. aureus* (red-violet colonies, and black colonies which may or may not be *S. aureus*), respectively. Colonies were enumerated after incubation of plates at 37 °C for 24-48 hours. Presumptive intestinal enterococci) colonies were transferred onto Bile Esculin Azide Agar (Oxoid) and incubated at 44°C for 2 hours (ISO 7899-2:2000). Enrichment for the detection of *E. coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* were done as described in section 4.2.1. All presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates from selective media were purified by re-streaking onto selective media and identified using PCR analysis and Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF) as described in section 4.2.1.

Results

Aerobic colony counts (ACC) – Total average bacterial counts were higher after filters installed in irrigation pipes in the orchard and in micro irrigation pipes in the orchard when compared to river and dam water counts.

Yeasts – Total average yeast counts were higher after filters installed in irrigation pipes in the orchard and in micro irrigation pipes in the orchard when compared to river and dam water. The highest yeast count was 4.49 log CFU/mℓ.

Fungi – The average fungi counts ranged between zero and 1.65 log CFU/mℓ

Coliforms and E. coli – The *E. coli* counts ranged between 3.2 log CFU/100 mℓ and 4 log CFU/100 mℓ The *E. coli* levels in the irrigation water (Sterk River, dam, micro irrigation pipes in the orchard and filters in the irrigation pipes) exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ (Fig. 50)

Enterococcus – Counts were very low ranging from 0.5 log CFU/mℓ to 1 log CFU/mℓ.

E. coli O157 (including H7) – none isolated

Salmonella – none isolated

Listeria spp. – none isolated

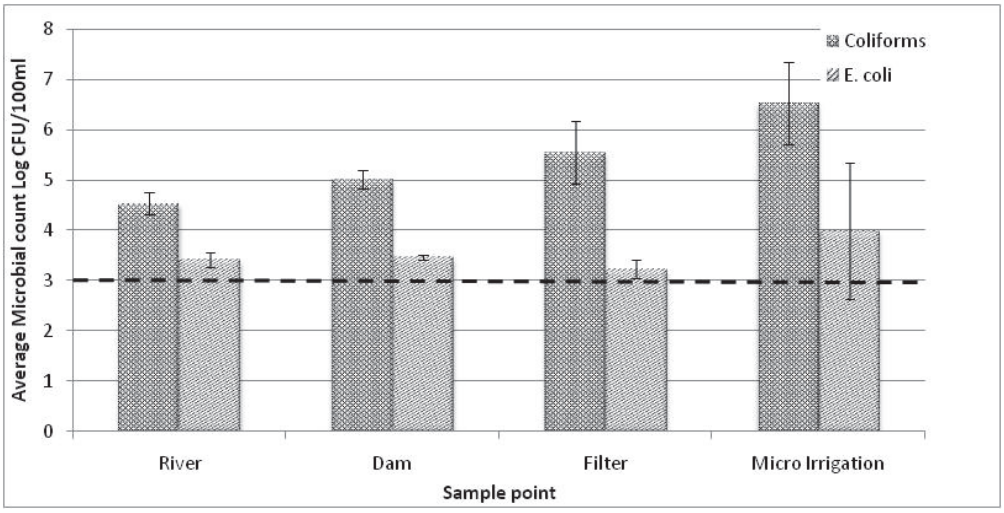


Figure 50 Coliform and *E. coli* counts of irrigation water sources used to irrigate peaches. The broken line indicates the South African Department of Water and Health (1996) guideline limit of *E. coli*, i.e. 1 000 CFU/100 mℓ.

Microorganism identification

The dominant bacteria identified were *Pantoea agglomerans, Bacillus cereus, Enterobacter cowanii* and *Enterococcus hirae*. Dominant fungi isolated from MEA medium were identified as *Fusarium* spp., *Alternaria* spp. and *Epicoccum nigrum.*

Discussion and conclusions

The total number of aerobic bacteria, yeast and coliform numbers of the water sampled after filters in irrigation pipes in the orchard and from micro irrigation pipes were higher when

compared to the water from the river and dam. This could indicate an accumulation of microorganisms as opposed to improving the water quality by filtering the water.

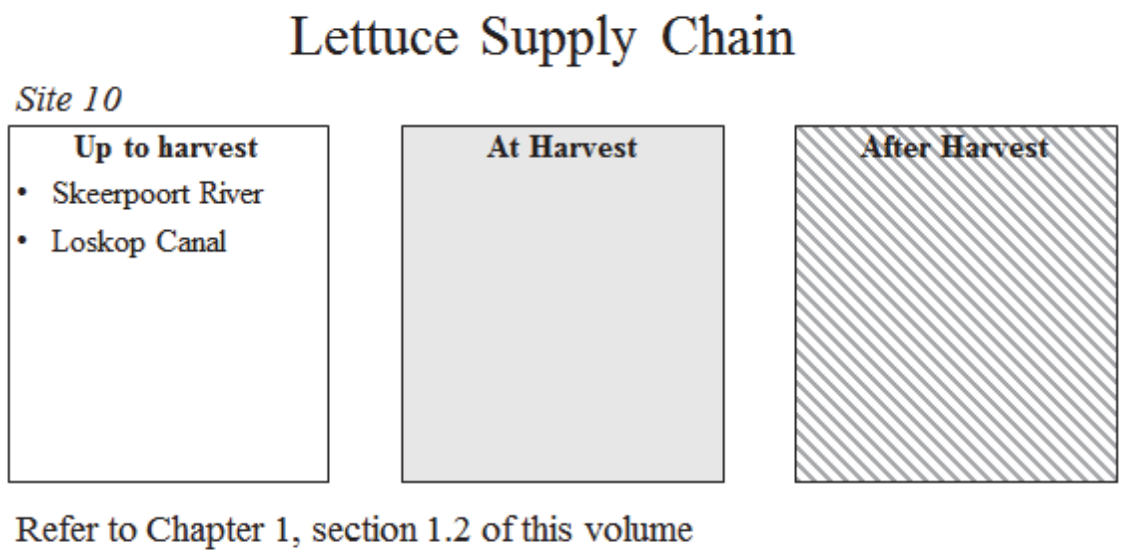
4.2.10 NORTH WEST PROVINCE and LIMPOPO – Microbiological quality of water from the Loskop Dam Canal and the Skeerpoort River used for lettuce irrigation

Specific aim

This study aimed at comparing the prevalence of bacterial foodborne pathogens in two irrigation water sources within South Africa, located in predominantly rural (the Loskop canal) and urban (the Skeerpoort River) areas respectively. *E. coli* isolates from each irrigation water source were characterized based on phenotypic (antibiotic resistance, toxin production) and genotypic relatedness. An additional aim was to determine whether irrigation water from the Skeerpoort River located in the North West Province of South Africa was a source of foodborne bacterial pathogens on irrigated lettuce over two seasons (10 months).

Site Selection

Irrigation water was collected from the Skeerpoort River (North West Province) and the Loskop canal (Limpopo Province). The Skeerpoort River is downstream the Hartbeespoort Dam, a major tourist location surrounded by an urban settlement. The Loskop canal carries irrigation water throughout the Loskop irrigation scheme which is located in a predominantly rural area. A vegetable growing farm using water from a dam containing water diverted from the Skeerpoort River for irrigation purposes was chosen as the sampling site for lettuce.



Experimental procedures

Sample Collection

One water sample was aseptically collected (SANS, 2006) on a monthly basis from the Loskop canal and the Skeerpoort River for 10 months from January to October, 2011.

Physico-chemical parameters

Temperature (at sampling site) and pH (on arrival at the laboratory) (Check temp 1, Hanna instruments Inc., Rhode Island, USA) for each water sample were measured.

Microbiological analysis

Bacterial indicators (Aerobic colony counts, ACC; Anaerobic spore formers, AnSF; Aerobic spore formers, ASF; Intestinal enterococci, IE; Total coliforms, TC; Faecal coliforms, FC) and pathogens (*E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp.) were enumerated and identified (Table 40).

Table 40 Methodology for detection of bacterial organisms in irrigation water

Organism	Media	Temperature/Time of incubation	Company	*Reference
Aerobic colony count (ACC)	Standard Plate Count Agar	30°C for 48 to 72 h	Oxoid Ltd, Basingstoke Hampshire, UK	SABS ISO 4833 (1991)
Aerobic spore formers (ASF)	Trypticase Soy Agar	35°C for 48 h	Biolab Diagnostics (Pty) Ltd, Wadeville Gauteng, South Africa	MFLP-44
Anaerobic spore formers (AnSF)	Trypticase Soy Agar	35°C for 48 h	Biolab	Health Canada, 1998
Total coliforms	Lauryl Tryptose (LST) Broth	35°C for 24 to 48h	Oxoid	MFHB19 (Health Canada, 2002)
Faecal coliforms	Brilliant Green Bile 2% Broth (BGLB)	35°C for 24 to 48h	Oxoid	
	E.C Broth	35°C for 24 to 48h	Oxoid	
E. coli	Eosin Methylene Blue Agar (Levine) (EMB)	35°C for 24h	Oxoid	MFHB19 (Health Canada, 2002)
	E. coli/chromogenic Medium	35°C for 18 to 24h	Oxoid	
	Agar Chrom ID ™ 0157 H7 (O157 ID-F)	35°C for 18 to 24h	BioMerieux, Marcyl-l' Etoile, France	
Intestinal enterococci	Slanetz and Bartley Medium	35°C for 44h	Oxoid	SANS ISO 7899-2 (2004)
	Bile Esculin Agar	44°C for 2h	Oxoid	
S. aureus	Baird-Parker Agar	35°C for 48h	Oxoid	SABS ISO 6888-1 (1999)
§*Salmonella* spp	Brilliance™ Salmonella Agar Base	35°C for 24 to 48h	Oxoid	SABS ISO 6579 (2003)
	Rapid Salmonella	35°C for 24 to 48h	Biorad, Marnes-la-Coquette, France	
†*L. monocytogenes*	Listeria Selective Agar (Oxford formulation)	35°C for 48 h	Oxoid	SABS ISO 11290-1 (1996)
	Listeria Selective Agar (Palcam Selective Supplement)	35°C for 48 h	Oxoid	

§-Final confirmation of presumptive *Salmonella* spp. was by serotyping at the Agricultural Research Council (ARC)-Onderstepoort, Pretoria, South Africa.

†-Final confirmation of presumptive *L. monocytogenes* was done using Omnilog® Data Collection Software Identification System Version 2.1 (Biolog Inc. Hayward, California)

*-SABS-South African Bureau of Standards; ISO-International Standards Organization; SANS-South African National Standard; MFHB19 ─Health Products and Food Branch Methods for the microbiological analysis of foods: determination of coliforms, faecal coliforms and *Escherichia coli* in foods; MFLP44 ─ Laboratory Methods for the Microbiological Analysis of Foods. Determination of aerobic and anaerobic endospore formers

Characterisation and identification of most prevalent aerobic bacteria

To determine the most prevalent aerobic bacterial species within both irrigation water sources, for each sample analyzed, 3 different colonies (visual discrimination) were randomly picked from the highest dilution standard plate count agar Petri dish. Ten samples were analyzed monthly for 10 months. Gram stain and catalase tests were done. Sixty (60) bacterial isolates were identified from the Loskop canal (30) and the Skeerpoort River (30). Isolates were identified to species level with the Omnilog® data collection software identification system version 2.1 (Biolog Inc. Hayward California) following prior isolation on plate count agar (Oxoid) and subsequently on Biolog Universal Growth medium (BUG) (Biolog).

Antibiotic resistance in *E. coli*

A total of 31 *E. coli* isolates from the Loskop canal (19) and the Skeerpoort River (12) were analyzed. From the total group of isolates, 13 were isolated on Eosin Methylene Blue agar (Levine) (EMB) (Oxoid) and identified with selective *E. coli*/chromogenic medium (Oxoid) and Agar Chrom ID ™ 0157 H7 (O157 ID-F) (BioMerieux, Marcyl-l' Etoile, France) prior to isolation from the Most Probable Number Method (Table 13). Additionally 18 isolates (selected from aerobic plate counts) were identified using Omnilog® Data Collection Software Identification System Version 2.1 (Biolog Inc. Hayward California) following prior isolation on plate count agar (Oxoid) and subsequently Biolog Universal Growth medium (BUG) (Biolog). Eleven antibiotics (Oxoid) at single concentrations were selected according to Da Silva *et al*. (2011) and antimicrobial susceptibility was done using Multiple Antibiotic Resistance (MAR) analysis (Vantakaris *et al*., 2006). Antibiotics used included Amikacin (30µg), Gentamicin (10µg), Chloramphenicol (30µg), Nalidixic Acid (30µg), Norfloxacin (10µg), Neomycin (30µg), Nitrofurantoin (300µg), Amoxicillin (25µg), Ampicillin (10µg), Cephalothin (30µg) and Oxytetracycline (30µg). Antibiotic susceptibility testing was determined by disc diffusion method using Mueller-Hinton agar (Oxoid).

Virulence genes in *E. coli*

The same *E. coli* isolates used for antibiotic resistance were tested for the following virulence genes: Shigatoxin 1 (*stx 1*), Shigatoxin 2 (*stx 2*) and intimin (*eae*) (Bio-Rad, Hercules, California). The thermocycler (C1000 Touch ThermalCycler CFX96^{TM} Real Time System) (Bio-Rad) and software (CFX Manager IDE) (Bio-Rad) were set up for analysis using iQ-Check™ STEC VirX catalogue # 357-8139 (Bio-Rad).

$(GTG)_5$-Rep-PCR fingerprinting of *E. coli*

The same isolates used for antibiotic resistance and virulence gene analysis were subjected to $(GTG)_5$-Rep-PCR. DNA preparation was performed using ZR Fungal/Bacterial DNA Kit^{TM} (Zymo Research, Irvine, California) from single colonies. The 20 µL reaction consisted of 17.6 µL, 1.1 times HotStarTaq Plus Master Mix Kit (Qiagen, Limburgh, Netherlands), 0.2 µM (GTG) 5 primer, 1.6 µL DNA template and 1% dimethylsulphoxide. Amplification was carried out as follows: initial denaturation at 95° C for 10 min; 35 cycles of 95 °C for 30 s, 40 °C for 60 s and 65° C for 3 min; and a final elongation step at 65 °C for 8 min. The PCR

amplified DNA was separated on 1.6% agarose gel containing SYBR® safe gel (Invitrogen, Carlsbad, California) by electrophoresis and visualized on the UVTEC Cambridge image (Bio-Rad) under UV light. The molecular marker 1500bp (Invitrogen) was used to estimate the length of amplicons.

Statistical analysis

Analyses of variance was performed to test for significant differences in antibiotic resistance patterns for *E. coli* isolated from the Loskop canal, Skeerpoort River and lettuce at 95% confidence interval. For numerical classification, antibiotic measurements (mm) were used to determine inter-isolate relationships by weighted pair-group average euclidean distances (Sneath and Sokal, 1973). Clusters were defined at euclidean distance of 2.0. All analyses were done with Statistica© software for Windows version 10 (Statsoft Inc., Tulsa, 2011).

The resulting fingerprints from $(GTG)_5$-Rep-PCR were analyzed using the GelCompare III version 5.10 (Applied Maths, Saint-Marten-Latem Belgium) software package. The similarity among digitized bands was calculated using the Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles.

Results

Physicochemical quality indicators

The pH ranged from 5.4 to 9.9 and 7.9 to 9.2 in the Loskop canal and the Skeerpoort River respectively. The pH in the Loskop canal exceeded South African national guidelines (DWAF, 1996) for irrigation water twice during the 10 month study. Water temperature in the Loskop canal ranged from 8.5 to 20.5°C with the lowest and highest noted in September and February respectively. Temperature in the Skeerpoort River ranged from 10.7 to 26.0°C with

the lowest and highest noted in May and January respectively. Higher mean monthly rainfall was noted in areas around the Skeerpoort River (74.7 mm) than the Loskop canal (0.1 mm) (Personal communication: Lucky Dlamini, South African Weather Service, July 2012).

Results

Bacterial indicators

Mean monthly aerobic colony counts (ACC) in the Loskop canal and the Skeerpoort River were 3.2 and 3.4 log CFU/mℓ respectively (Table 41). Mean monthly anaerobic spore formers (AnSF) and aerobic spore formers (ASF) ranged from 1.8 to 2.5 log CFU/mℓ in both water sources. However, high counts (5.1 log CFU/mℓ) of ASFs were noted in the Loskop canal in April and October (Figure 51).

Additionally high counts of AnSF (5.3 log CFU/mℓ) were noted in June and October in the Skeerpoort River (Figure 51).
Low mean faecal coliforms (FC) (1.3 log CFU/mℓ) were noted in the Loskop canal and the Skeerpoort River (Table 41). FC surpassed South African national guidelines for irrigation

water once (March) in the Skeerpoort River (Figure 52).
Counts of intestinal enterococci (IE) ranged from 0.1 to 0.4 log CFU/mℓ in the Loskop canal and the Skeerpoort River (Table 41). Prevalence of *E. coli* was 40% at both irrigation water sites (Figures 52 and 53). Other than May *E. coli* was isolated in January, April and August in the Loskop canal and in March, September and October in the Skeerpoort River. *E. coli* was isolated from the Loskop canal and the Skeerpoort River in months when the highest faecal coliform counts were recorded (Figures 52 and 53). *E. coli* O157:H7 was isolated in September in both water sources as well as in October in the Skeerpoort River. *S. aureus* was noted in a few months at both irrigation water sites. In the Loskop canal, *S. aureus* was noted in March, April, August and September (Figure 51). In the Skeerpoort River *S. aureus* was noted in February, March, April and May (Figure 52). Additionally, the pathogen was noted in 3 (the Skeerpoort River) and 4 (Loskop canal) months during the ten month study (Figures 51 and 52). However when *S. aureus* was noted, counts ranged from 1.6 to 3.0 log CFU/mℓ in the Loskop canal and 0.6 to 2.0 log CFU/mℓ in the Skeerpoort River (Figures 51 and 52). *Salmonella enterica* subsp *salamae* (typed as *Salmonella* II 13, 22, 23) was isolated from the Loskop canal (Table 42).

Diversity of aerobic bacterial species

Seven bacterial genera/species were isolated from the Loskop canal. They included *Bacillus* spp, *Enterobacter* spp, *E. coli*, *Klebsiella* spp, *Kluyvera ascorbata*, *Enterococcus gallinarum* and *Serratia marcescens* ss. *marcescens* (Table 42). Eight genera/species were isolated from the Skeerpoort River. They included *E. coli*, *Bacillus* spp., *Enterobacter* spp., *Klebsiella* spp., *Raoultella* spp., *Burkholderia* spp., *Buttiauxella* spp. and *Salmonella* spp. (Table 42). *Bacillus* spp., *E. coli and Enterobacter* spp. were the most prevalent bacteria in the Loskop canal and the Skeerpoort River (Figures 53 and 54). *E. coli* was most prevalent in winter and *Bacillus* spp. in summer at both irrigation water sites. Low prevalence (3.4%) for *Kluyvera ascorbata*, *K. oxytoca*, *Enterococcus gallinarum*, *K. pneumoniae* subsp. *pneumonia* and *S. marcescens* subsp. *marcescens* was noted in the Loskop canal (Figure 53). Similarly low prevalence (3.3%) of *Salmonella* spp., *Klebsiella* spp., *Raoultella* spp., *Burkholderia* spp., *Buttiauxella* spp. was noted in the Skeerpoort River (Figure 54).

Table 41 Physico-chemical parameters, bacterial counts and incidence of bacterial contaminants in irrigation water from the Loskop canal and the Skeerpoort River for samples (n=20) collected monthly over 10 months.

				Indicator Parameters (log_{10}CFU/mℓ, g)						% samples positive for bacterial contaminants	
	No. of samples	**Temp (˚C)**	**pH**	**Aerobic Colony Count**	**Anaerobic Spore Formers**	**Aerobic Spore Formers**	***S. aureus***	**IE**	**FC**	***E. coli***	***Salmonella* spp.**
Loskop canal[1]	10	15.4±3.7	7.4±1.1	3.2 ±0.7	1.8±1.9	2.1±2.1	0.9±1.2	0.1 ±0.2	1.3±1.0	40	10
Skeerpoort River	10	18.2±6.2	8.4±0.4	3.4±0.6[a]	2.5±1.8	2.5 ± 1.2	0.5 ± 0.8[a]	0.1 ±0.4	1.3 ± 1.1	40	ND
Average	20	16.8±2.0	7.9±0.7	3.3±0.1	2.2±0.5	2.3±0.3	0.7±0.3	0.1±0.0	1.3±0.0	40	5

FC ─ Faecal coliforms (log_{10} MPN/100 mℓ, g)

S. aureus ─ *Staphylococcus aureus*

IE ─ Intestinal enterococci

[1]*Salmonella enterica* subsp *salamae* was isolated from the Loskop canal

± Standard deviation

ND ─ not determined

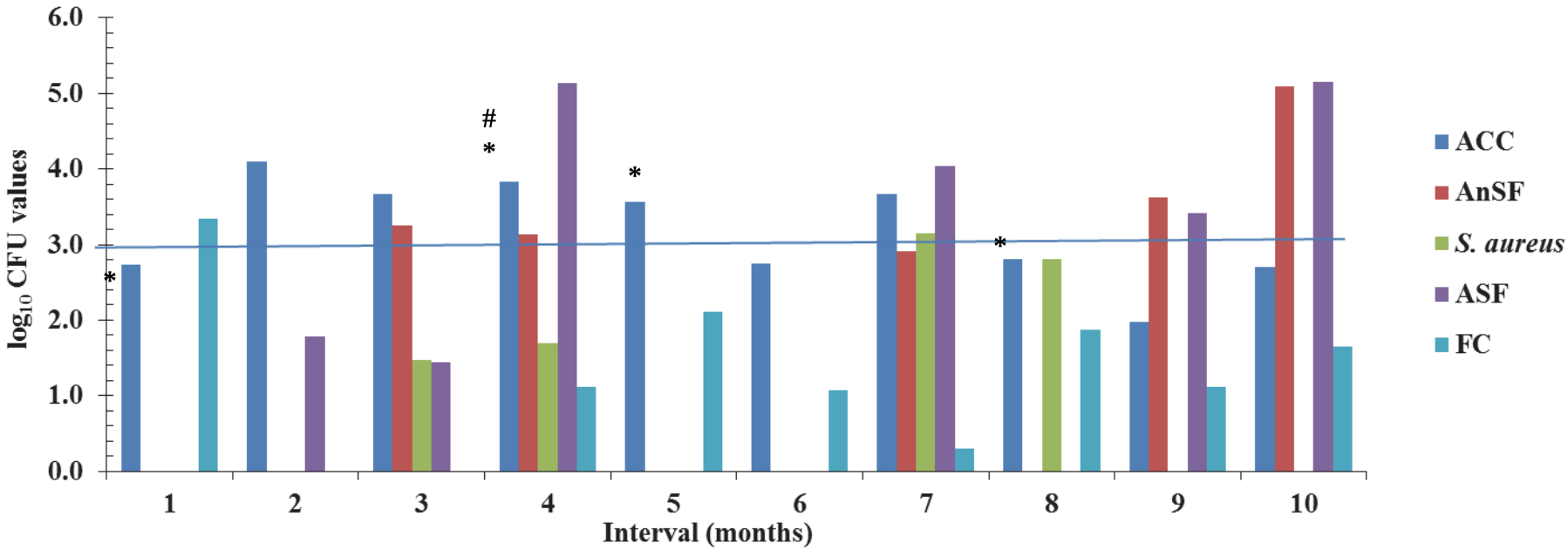


Figure 51 Bacterial counts and incidence of *E. coli* and *Salmonella* spp. in irrigation water from the Loskop canal for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*, (#) Interval positive for *Salmonella enteric* Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and faecal coliforms (FC: $\log_{10}$ MPN/100 mℓ). Horizontal line represents upper limit for faecal coliforms in South African irrigation water (Department of Water Affairs and Forestry (DWAF), 1996).

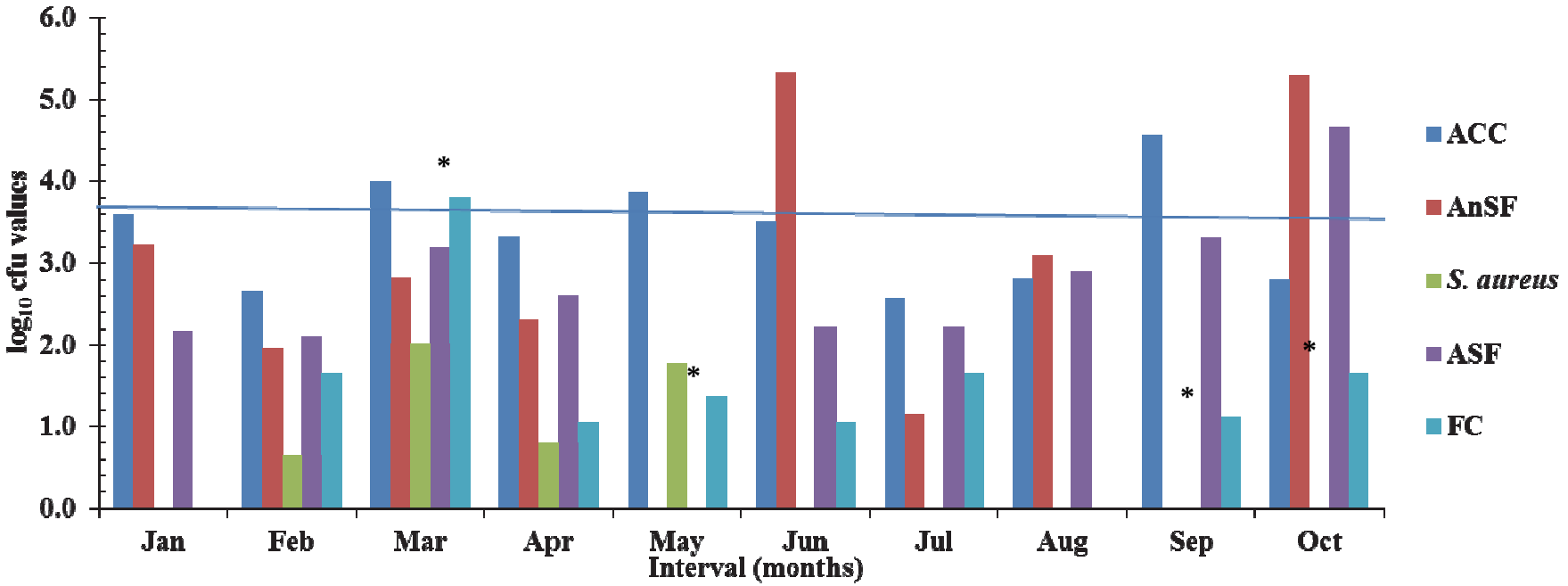


Figure 52 Change of bacterial counts and incidence of *E. coli* with time in irrigation water from the Skeerpoort River for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC: ($\log_{10}$ MPN/100 mℓ)). Horizontal line represents upper limit for faecal coliforms in irrigation water (DWAF, 1996).

Table 42 Prevalence of aerobic bacteria in irrigation water from the Loskop canal and the Skeerpoort River for isolates (n=60) collected monthly over 10 months

Month	1		2		3		4		5		6		7		8		9		10		
Sample Percentage isolates	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	Total number of isolates
E. coli	33	–	33	–	–	–	–	–	–	67	67	100	–	33	33	–	67	100	–	67	18
Bacillus spp	33	33	33	100	100	33	33	33	33	33	33	–	–	–	–	33	–	–	67	–	18
Enterobacter spp	33	33	33	–	–	–	33	67	33	–	–	–	67	33	33	–	33	–	–	33	13
Klebsiella spp	–	–	–	–	–	67	–	–	33	–	–	–	–	–	33	–	–	–	–	–	4
Enterococcus spp	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	–	–	1
Salmonella spp	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Serratia spp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	1
Raoultella spp	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	–	1
Kluyvera ascorbata	–	–	–	–	–	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Burkholderia caprophylil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	1
Buttiaxella agrestis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	1
Total number of isolates	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60
ACC (log_{10}CFU/mℓ)	2.7	3.6	4.1	2.7	3.7	4.0	3.8	3.3	3.6	3.9	2.8	3.5	3.7	2.6	2.8	2.8	2.0	4.6	2.7	2.8	

L C, the Loskop canal; SR, the Skeerpoort River; -, Not determined; ACC, aerobic colony count

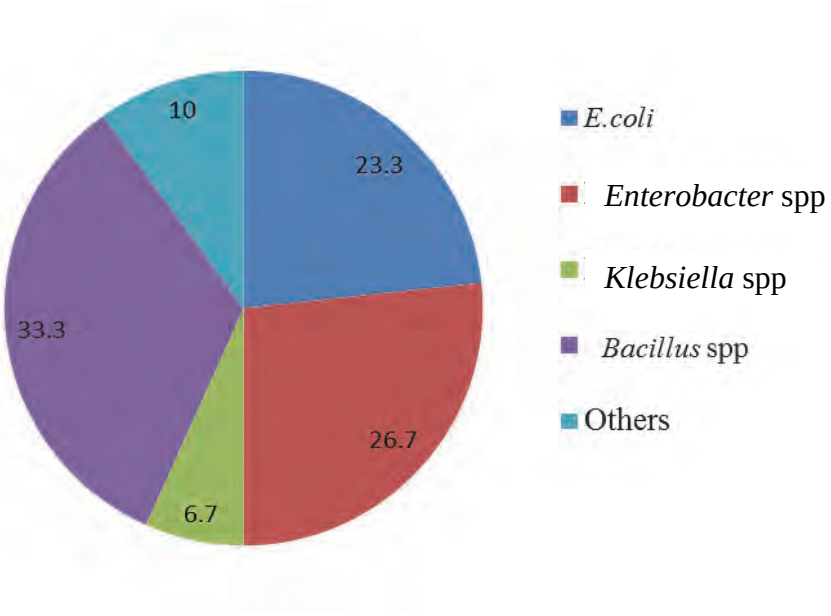


Figure 53 Prevalence of aerobic bacteria (n=30) in irrigation water from the Loskop canal for samples (n=10) collected monthly over 10 months. Others: *Kluyvera ascorbata* and *Serratia marcescens* ss. *marcescens*

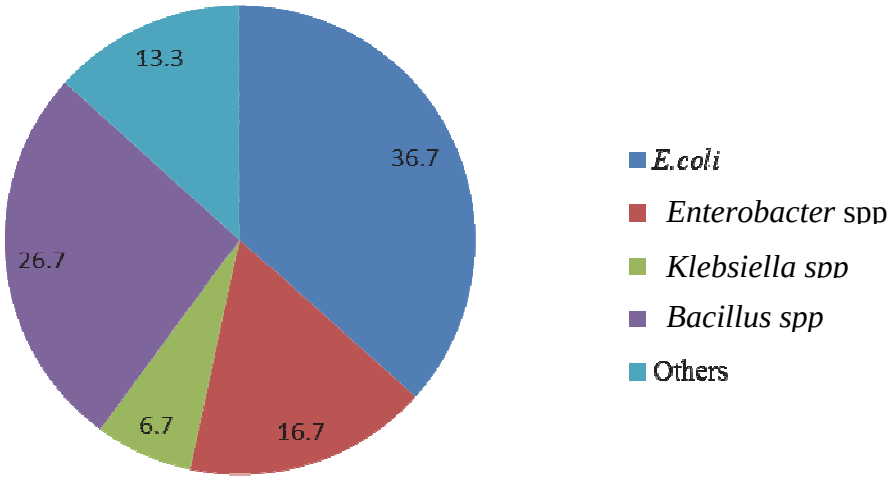


Figure 54 Prevalence of aerobic bacteria (n=30) in irrigation water from the Skeerpoort River for samples (n=10) collected monthly over 10 months. Others: *Raoultella terringena, Burkholderia caprophylil, Buttiauxella agrestis* and *Salmonella enterica*

Antibiotic resistance in *E. coli*

E. coli from the Loskop canal was resistant to nitrofurantoin, ampicillin, nalidixic acid, gentamicin, oxytetracycline, amikacin, cephalothin, neomycin and amoxicillin. Similarly *E. coli* from the Skeerpoort River showed resistance to nalidixic acid, norfloxacin, neomycin, amoxycillin, ampicillin, cephalothin and oxytetracycline. Resistance of *E. coli* to at least one antibiotic was 84.2% and 83.3% in the Loskop canal and the Skeerpoort River respectively. Highest resistance to single antibiotics in both water sites was to cephalothin and ampicillin (Table 43). Resistance to more than one antibiotic in the Loskop canal and the Skeerpoort River was 42.1% and 50% respectively. However 5.3% of isolates in the Loskop canal and 33.3% in the Skeerpoort River were resistant to more than two antibiotics.

Table 43 Prevalence of antibiotic resistant *E. coli* collected over 10 months in irrigation water from the Loskop canal and the Skeerpoort River

	% of Resistant isolates	
Antibiotic	**Loskop canal (n=19)**	**Skeerpoort River (n=12)**
Amikacin, 30µg	5.3	ND
Gentamicin, 10µg	5.3	ND
Chloramphenicol,30µg	ND	ND
Nalidixic acid, 30µg	5.3	16.7
Norfloxacin, 10µg	ND	8.3
Neomycin, 30µg	5.3	8.3
Nitroforantin, 300µg	10.5	ND
Amoxycillin, 25µg	5.3	16.7
Ampicillin, 10µg	21.1	50
Cephalothin, 30µg	73.7	50
Oxytetracyline, 30µg	5.3	25

ND – No resistance

There was a significant difference ($p \leq 0.05$) in resistance to antibiotics in *E. coli* from the Loskop canal and the Skeerpoort River. *E. coli* in the Loskop canal was susceptible to norfloxacin and chloramphenicol. *E. coli* from the Skeerpoort River was susceptible to amikacin, gentamicin, chloramphenicol and nitrofurantoin. Eight antibiotic resistant patterns in the Loskop canal compared to 6 patterns in the Skeerpoort River (Table 44). *E. coli* from the Skeerpoort River showed more resistance to multiple antibiotics (Table 44).

Table 44 Multiple resistance to antibiotics in *E. coli* isolated over 10 months from irrigation water in the Loskop canal and the Skeerpoort River

	% of isolates with indicated resistance pattern	
Pattern of antibiotic resistance	**Loskop canal (n=19)**	**Skeerpoort River (n=12)**
KF-AK	5.3	ND
KF-AMP	15.8	8.3
KF-F	5.3	ND
KF-OT	5.3	8.3
KF-CN	5.3	ND
F-OT	5.3	ND
AMP-N	5.3	ND
AMP-KF-AML-F	5.3	ND
KF-AMP-NA	ND	8.3
KF-AMP-AML	ND	8.3
KF-AMP-OT	ND	8.3
KF-OT-NA-AMP	ND	8.3

AK – Amikacin; KF –Cephalothin; F – Nitrofurantoin; OT – Oxytetracycline; AMP – Ampicillin; AML – Amoxycillin; NA – Nalidixic Acid; CN –Gentamicin; ND, No resistance

Virulence genes in *E. coli*

In the Loskop canal 15.7% of isolates were positive for at least one virulence gene. Additionally 10.5% of isolates were positive for *stx1*/*stx2* and *eae* and 5.2% for *stx1*/*stx2* (Table45). In the Skeerpoort River, 41.6% of isolates were positive for at least one virulence gene with 25% of them positive for *stx1*/*stx2* and *eae* (Table 45). Additionally 8.3% of isolates were positive for only *stx1/stx2* and *eae* respectively. All isolates having virulence genes in the Loskop canal showed antibiotic resistance while 80% of isolates possessing virulence genes in the Skeerpoort River showed antibiotic resistance.

Table 45 Prevalence of virulence genes in *E. coli* isolated over 10 months from the Loskop Canal and the Skeerpoort River

stx1 – Shigatoxin 1 gene *stx*2-Shigatoxin 2 gene *eae*-Intimin gene ND-not determined

Source	% occurrence of virulence genes		
	Stx1*/*Stx2* and *eae	***Stx 1*/*Stx 2***	***eae***
Loskop Canal	10.5	5.2	ND
Skeerpoort River	25.0	8.3	8.3

E. coli from the same irrigation water source showed close phenotypic relatedness based on resistance to antibiotics (Figure 55). However some isolates from different sources (7 and 18 in the Loskop canal) and (f and d in the Skeerpoort River) showed close relatedness respectively (Figure 55).

Genetic fingerprinting

E. coli in the Loskop canal (47.3%) and the Skeerpoort River (67%) showed complete (100%) genetic relatedness (Fig. 56). Additionally 53% of isolates from the Loskop canal and 75% in the Skeerpoort River showed relatedness higher than 80% (Figure 56).

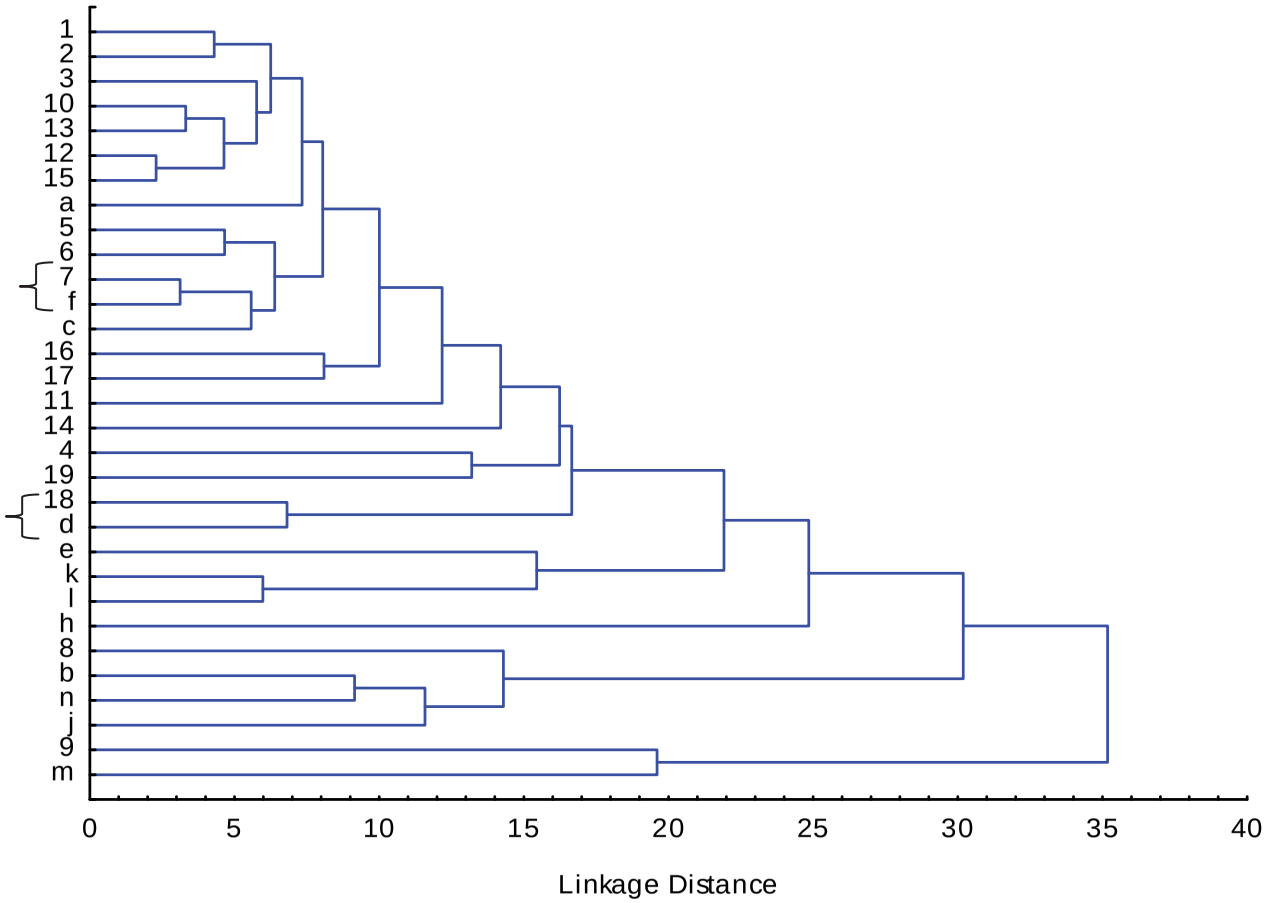


Figure 55 Dendogram showing cluster formation for *E. coli* collected over 10 months from the Loskop canal and the Skeerpoort River. Numbers represent isolates from the Loskop canal and alphabetical letters isolates from the Skeerpoort River. Brackets enclose closely related isolates from different sites.

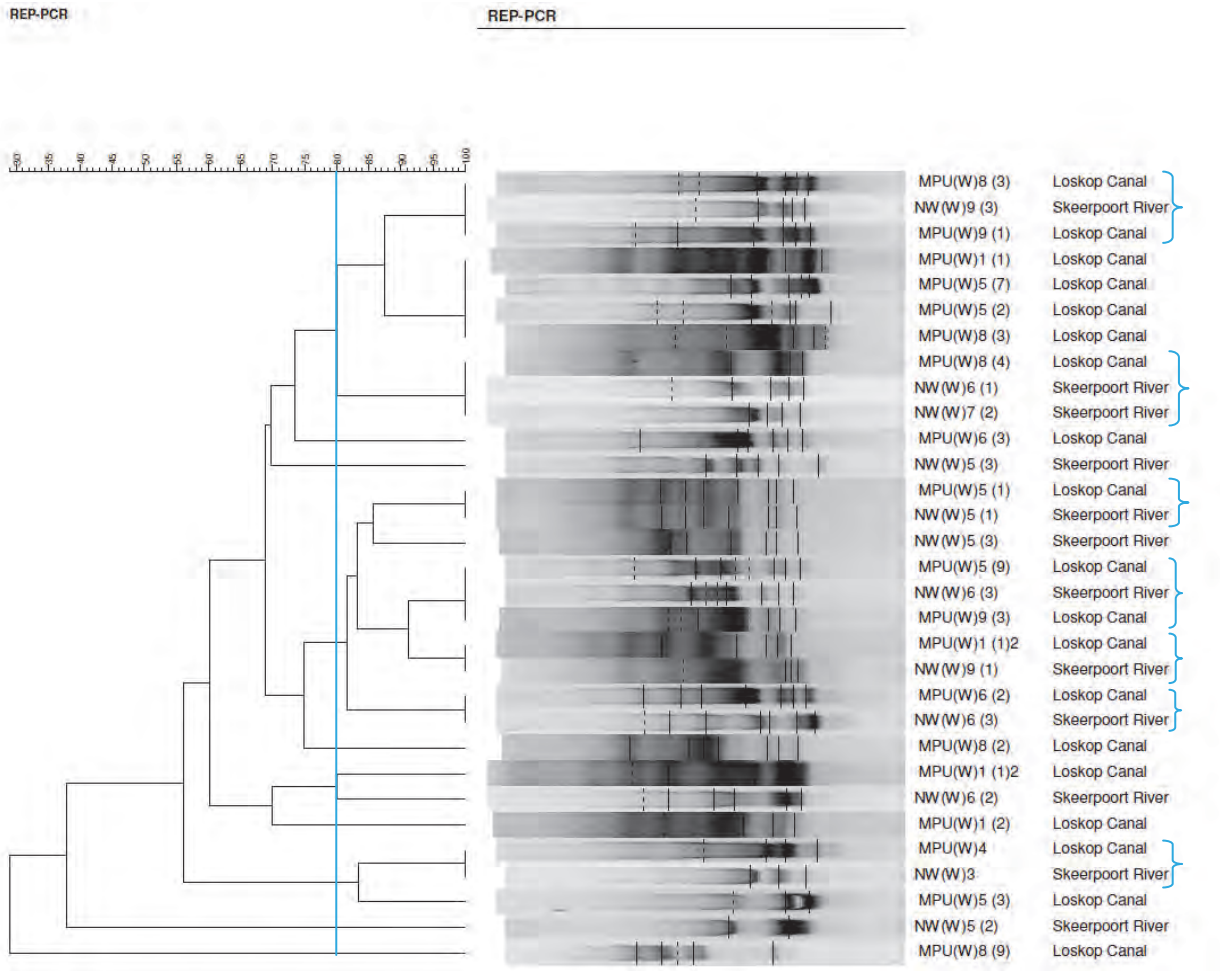


Figure 56 Cluster analysis of $(GTG)_5$-PCR fingerprints of *E. coli* isolates collected from the Loskop canal (19) and the Skeerpoort River (12). The vertical blue line depicts clusters of isolates that showed 80% similarity which was taken as the threshold for closely related isolates. Isolate codes are in line with the source of isolation.

Discussion and conclusion

Physico-chemical and bacterial indicators

The fluctuations noted in water temperature from the Loskop canal and the Skeerpoort River were due to seasonal changes resulting from summer and winter. Such fluctuations may have played a role in selecting for *E. coli* and *Bacillus* spp. Temperature has been noted as a major factor influencing proliferation of bacterial pathogens (Pachepsky *et al*., 2011). The pH fluctuations out of the recommended range noted in the Loskop canal may have resulted from the surrounding land use practices. There are defunct, flooded underground coal mines which contribute to pollution in water sources of the upper Olifants River catchment area located upstream the Loskop canal (Centre for Science Innovation and Research (CSIR), 2010). Therefore, effects of mining such as acid drainage and industrial effluents may have caused changes in pH of water in the Loskop canal.

Changes in ACC, ASF and AnSF in the Loskop canal and the Skeerpoort River which did not follow a particular trend may have been due to isolated events of pollution at different times. Similar sporadic pH values were previously noted in the Loskop Canal (Ijabadeniyi *et al*., 2011). Bacterial indicator counts may vary as a result of availability of nutrient growth requirements, settling to sediment, chemical reactions, decay or due to seasonal changes (Pachepsky *et al*., 2011). Low mean counts for bacterial indicators in the Loskop canal may have been due to the fencing around the canal, concrete flooring and side walls which might have reduced bacterial contamination from extraneous sources such as animals and runoff. Use of a reservoir dam for collecting irrigation water in the Skeerpoort River may have protected it from contamination sources commonly associated with free flowing rivers such as runoff, animal faecal material, domestic sewage and industrial effluent. Low and sporadic counts of ACC, ASF and ASF were previously noted in the Loskop Canal (Ijabadeniyi *et al*., 2011).

High FCs noted during summer when high rainfall was also noted suggests a positive linear relationship between faecal contamination and rainfall. This was possibly due to a larger volume of runoff generated, which increased contamination within the water source as well as the churning of sediment (Korajkic *et al*., 2010). High prevalence of *E. coli* noted at the two sites is indicative of possible faecal contamination and ability of the pathogen to stay viable in irrigation water during winter (Islam *et al*., 2004). This may increase possible risk of transfer and proliferation of pathogenic bacteria on irrigated produce. *E. coli* was isolated from irrigation water and on fresh produce irrigated with water from the Loskop canal (Ijabadeniyi *et al*., 2011) and the Skeerpoort River (Duhain, 2011). Predominance of *E. coli* during winter at both sites suggested adaptation to low environmental temperature compared to other isolated bacterial species. *E. coli* has been suggested to apply a dual regulation system enabling selection and production of scarce metabolic requirements, which assist and maintain growth in secondary environments such as water and the plant phyllosphere (Seurinck *et al*., 2005). Additionally *E. coli* have petrichous pili-flagella (Welch, 2006) that aid movement in liquid environments, an adaptation that may enhance survival by enabling transfer to nutrient rich areas. These adaptations may help *E. coli* stay viable in irrigation

water increasing likelihood of contaminating irrigated produce. High prevalence of *E. coli* has previously been noted at lower temperatures of 8˚C (Berry and Wells, 2010).

High prevalence of *Bacillus* spp. noted in the Loskop canal and the Skeerpoort River could be attributed to its ubiquitous nature. *Bacillus* spp. is widely distributed in sediment, runoff from soil and decaying matter (Ells and Hansen, 2006), contaminants which usually end up in irrigation water sources as a result of environmental, human and/or animal contamination. The high prevalence of *Bacillus* spp. during summer as opposed to winter may be due to warmer temperatures that favour spore germination into vegetative cells and hence proliferation. During winter, *Bacillus* spp. may revert to spores as a means of protection against unfavourable conditions.

Enterobacter spp. and *K. pneumonia* are common opportunistic pathogens. *K. pneumonia* proliferates in human intestines and is found in faeces (Centers for Disease Prevention and Control, (CDC, 2012), therefore its presence may indicate possible faecal contamination at both irrigation water sites. *K. pneumonia* has been isolated from irrigation water and lettuce (Olayemi, 1997) as well as from raw lettuce on sale in markets (Puspanadan *et al*., 2012). *K. Pneumonia* spp. is usually associated with healthcare-associated infections, wounds or surgical site infections and meningitis in individuals with compromised immunity such as infants, the ill and aged (CDC, 2012). *K. pneumonia* has been noted as a cause of infections among
new-born babies in South Africa (Ballot *et al*., 2012) and other developing countries (Zaidi *et al.*, 2009; Decré *et al*., 2011) with sources of infection linked more to the environment than maternal hygiene (Zaidi *et al*., 2009).

High prevalence of *Enterobacter* spp. (Zamxaka *et al*., 2004; Lin and Biyela, 2005; Lötter, 2010) and *Klebsiella* spp. (Samie *et al.*, 2011) have been noted in South African water sources.

Antibiotic resistance, virulence genes and genotypic fingerprinting of *E. coli*

High resistance noted in *E. coli* isolated from the two sites to cephalothin and ampicillin may have suggested extended exposure to these antibiotics possibly resulting from common use within surrounding areas. Extended exposure of *E. coli* to antibiotics has been noted to increase resistance whereby resistance genes may be transferred by horizontal genetic transfer occurring on mobile genetic elements such as plasmids (Da Silva and Medonça, 2012). Beta-lactam antibiotics, to which ampicillin and cephalothin belong, have low toxicity, a factor that has resulted in over-use of these drugs within the medical community (Olaniran *et al*., 2009). High resistance to ampicillin and cephalothin was also noted in *E. coli* isolated from the Umgeni and Palmiet Rivers located in South Africa (Olaniran *et al*., 2009).

Higher multiple resistances in *E. coli* isolated from the Skeerpoort River than the Loskop canal to antibiotics may be linked to the existence of a large pool of antibiotic resistance bacteria. Rivers flowing through urban areas may be exposed to higher levels of pollution compared to rural areas through domestic and industrial waste disposal (Walters *et al*., 2011)

increasing the risk of contamination with antibiotic resistant bacterial pathogens. Olaniran *et al*. (2009) suggested contamination of surface water sources with pollutants as a factor influencing increased prevalence of antibiotic resistant pathogens. Therefore irrigation water sources may serve as reservoirs of antibiotic resistant pathogens thereby posing a food safety and public health threat in case new pathogens emerge. Multiple resistances to antibiotics were noted in *E. coli* isolated from rivers close to urban/densely populated areas (Olaniran *et al*., 2009; Da Silva *et al*., 2011).

Phenotypic (antibiotic resistance) relatedness shown by some *E. coli* from the Loskop canal and the Skeerpoort River may have resulted from extended exposure to similar antibiotics. *E. coli* from the Loskop canal and the Skeerpoort River may have been exposed to similar antibiotics in spite of the geographical difference. This may have been due to spontaneous events of contamination resulting from point and non-point pollution possibly influenced by surrounding land use practices. Therefore, in spite of different geographical locations, the Loskop canal and Skeerpoort River may have been exposed to similar sources of contamination.

Antibiotic resistance can be used to determine relatedness of environmental *E. coli* as well as the source of contamination (Wiggins *et al*., 1999). Similarly genotypic relatedness among *E. coli* isolates from the two sites suggest similar sources of contamination. This observation is in line with results from antibiotic resistance analysis that suggested that similar sources of faecal contamination were responsible for contaminating the Loskop canal and the Skeerpoort River in spite of the geographical and possible land-use difference.

Fingerprinting using $(GTG)_5$-Rep-PCR has been used to track sources of bacterial faecal contamination within water sources (Mohapatra and Mazumder, 2008). The method was more resolute in discriminating *E. coli* strains from different animal sources than Rep-PCR using BOX and ERIC primers (Mohapatra and Mazumder, 2008). The combination of phenotypic and genotypic methods to determine sources of bacterial contamination has been recommended because it provides a more accurate mode of assessing environmental bacterial contamination (EPA), 2005).

The presence of genes associated with Enterohaemorrhagic *E. coli* (EHEC) infections within *E. coli* from the two sites, suggested ability to cause shigatoxin-related human infections. Additionally, resistance to antibiotics noted within strains with virulence genes may heighten risk and severity of illness due to the reduced effectiveness of antimicrobial therapy. Therefore such water is unsuitable for irrigating fresh produce undergoing minimal processing (Pachepsky *et al*., 2011). Studies have reported high resistance to antibiotics in shigatoxin producing *E. coli* (STEC) isolated from animal wastewater (Schroeder *et al*., 2002; Da Silva and Medonça, 2012). *E. coli* coding for *stx1*, *stx2* and *eae* genes was noted as resistant to ampicillin and streptomycin (Da Silva and Medonça, 2012).

This study has shown that surface irrigation water sources, regardless of geographical location and surrounding land-use practices, can be reservoirs of bacterial pathogens that may

compromise the safety of irrigated, minimally processed produce. Therefore continuous and consistent monitoring of the bacteriological quality of irrigation water sources is essential in order to assess levels of pathogenic bacterial contamination, determine potential sources of contamination and provide reliable information for use in remediation.

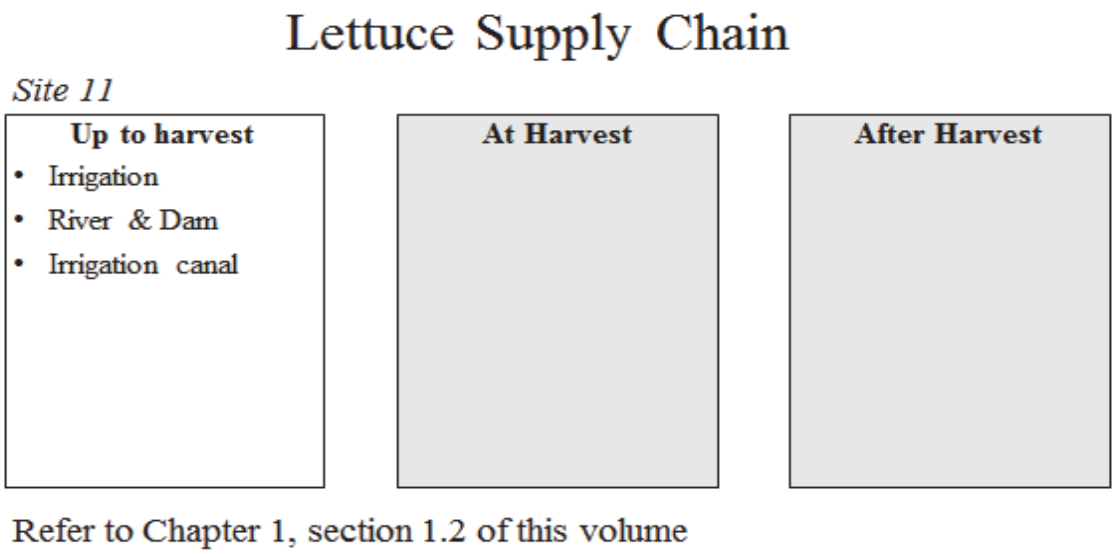
4.2.11 NORTH WEST – Microbiological analysis of water from an irrigation channel (fed by water from the Crocodile River and Hartbeespoort Dam) used for overhead irrigation of lettuce

Specific aim

The main aim of this study was to evaluate the microbiological quality of irrigation water and associated field lettuce from planting to end of shelf life. The microbiological quality of the irrigation water is reported in this section.

Experimental site

- The lettuce farm is located in the Brits area of the North West Province, South Africa.
- The lettuce farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Crocodile River and Hartbeespoort Dam feeds the irrigation canal channelled to the farm for irrigation.
- Lettuce plants were irrigated by use of overhead sprinklers.
- For the sake of our study, a single plot on the lettuce farm was selected for microbiological assessment.



Refer to Chapter 1, section 1.2 of this volume

Experimental procedures

Sampling and sample preparation

On the farm, irrigation water samples were taken once every two weeks during week 1 (a day after planting), weeks 3, 5, 7 and 10 when the mature lettuce heads were harvested.

Microbiological analysis

Total aerobic colony counts (ACC), Intestinal enterococci counts (IE), total coliform counts (TC), *E. coli* counts and *Staphylococcus aureus* counts were determined as described in the experimental procedures for lettuce in the previous section (4.2.10). Typical *E. coli* isolates were further assessed for the presence of Shiga Toxin producing *E. coli* (STEC: stx1, stx2, ehx, eae and O157:H7) and enterohaemorrhagic *E. coli* serotypes (O145, H7, O26, O111 and O103) by real time PCR using gene discs (GeneSystems, France). *Salmonella* spp. and *Listeria* spp. were detected as described in section 4.2.10.

Statistical analyses

Six replicate water samples were collected at each sampling point. Results presented were subjected to one way ANOVA and student t-test to evaluate significant differences ($P \leq 0.05$) between average log values. Statistica version 9 (Statsoft) was used to conduct statistical analyses.

Results

Aerobic colony counts (ACC) – the ACC in irrigation water ranged between 3.5 log CFU/mℓ and 5 log CFU/mℓ (Table 46). Bacterial spp. isolated from irrigation water is summarized in Table 47.
Faecal coliforms – the coliform count ranged between 2.8 log CFU/100 mℓ and 4.1 log CFU/ 100 mℓ
Intestinal enterococci (IE) – the IE counts ranged from zero to1.9 log CFU/mℓ.
Listeria spp. – not detected in irrigation water samples
Salmonella spp. – present in irrigation water samples. High incidence of *Listeria* spp. was observed in field lettuce and the corresponding soil samples. *E. coli* incidence was high in irrigation water (60%). The predominant IE species were *E. faecium* and *E. avium.* These were present in both the irrigation water, soil and on lettuce throughout the cultivation period. Other species isolated included *E. faecalis, Aeromonas viridians* and *Globicatella saguinis* (Table 47). Unlike *E. faecium, E. faecalis* was not detected in irrigation water.

Table 46 Summary of bacterial incidence in water and on lettuce from planting to end of shelf life.

Sampling points	**N**	**Log_{10} CFU/gor mL**			**% Incidence**			
		Aerobic Plate Count	**Total Coliforms**	**Intestinal Enterococci**	***S. aureus***	***E. coli***	***Listeria***	***Salmonella***
Irrigation water	5	4.2 (0.54)	3.6 (0.48)	0.9 (0.89)	1.5 (1.11)	60 (3)	ND	40 (2)
Soil	24	6.6 (0.34)	5.7 (0.44)	2.0 (0.93)	4.6 (0.39)	25 (6)	87.5 (21)	ND
Lettuce:								
Farm	30	6.6 (0.64)	5.9 (0.60)	1.6 (1.24)	4.2 (0.76)	10 (3)	63 (19)	ND
Processing factory	30	5.3 (1.44)	4.8 (1.15)	0.7 (0.91)	3.3 (1.21)	ND	10 (3)	ND
Shelf life storage	30	4.1 (0.56)	3.8 (0.71)	0 (0)	0.5 (0.76)	ND	ND	10 (3)

ND: Not Detected

Table 47 Bacterial *spp*. isolated from irrigation water over the lettuce cultivation period.

Organism type	Isolates	Pathogenic status
Pathogens	*E. coli*	
	Salmonella spp.	NP
Intestinal Enterococci	*Enterococcus faecium*	OP
	Enterococcus avium	NP
Coliforms	*Aeromonas caviae*	OP
	Pseudomonas monteilii	OP
	Raoultella ornithinolytica	OP
	Stenotrophomonas spp.	

Pathogen status: P (Pathogenic), NP (Non-pathogenic), OP (Opportunistic pathogen), U (Unknown)

Discussion and conclusion

In this study, faecal coliform levels in irrigation water often exceeded the upper limit (≤ log 3 CFU/100 mℓ) suggested for faecal coliform counts in irrigation water used for irrigation of vegetables eaten uncooked (DWAF, 1996). The use of overhead irrigation on the lettuce production farm contributes significantly to the risk of contamination with human pathogenic bacteria and viruses. Irrigation water from the Crocodile River and Hartbeespoort Dam was susceptible to faecal contamination from informal settlements and wild animals. Proper monitoring of the microbiological quality and partial treatment prior to use could reduce risk of produce contamination. High incidence of *Listeria* spp. was observed in field lettuce and the corresponding soil samples. *E. coli* incidence was however low in field lettuce (10%) and soil (25%) but high in irrigation water (60%).

4.2.12 NORTH WEST PROVINCE – Microbiological quality of water used to irrigate basil on a farm near Brits

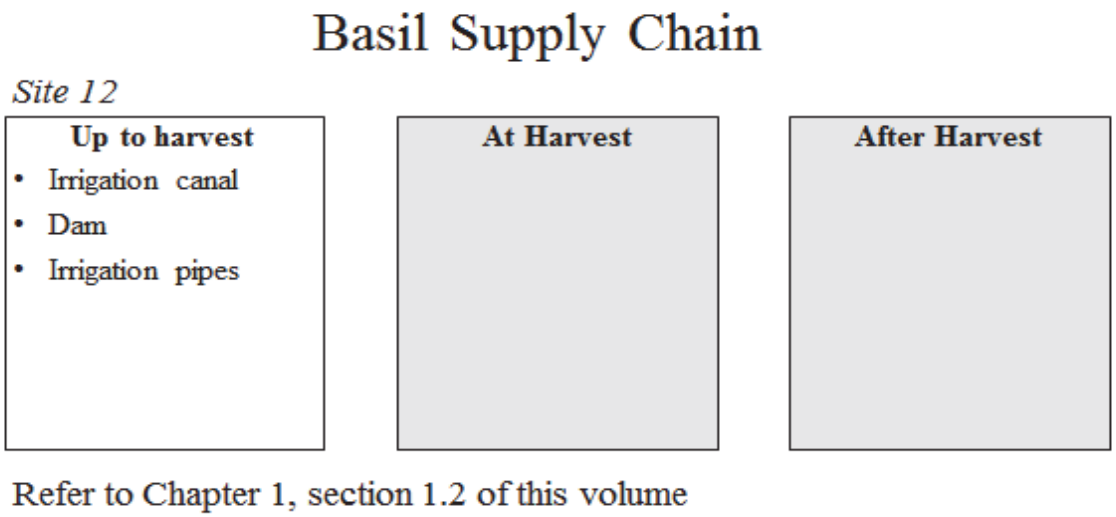
Specific aim

The aim of this study was to evaluate the microbiological quality of the water used for basil irrigation.

Experimental procedures

Sampling site

Irrigation water and basil were sampled on a commercial farm near Brits (North West Province). Irrigation water is drawn from an irrigation channel, stored in a dam and used for overhead irrigation of the basil.



Water sampling and processing

Three different water sites were sampled on each farm, i.e. the water source used, the on-farm storage dam and the outlet of the irrigation pipes. Three 1000 mℓ water samples were extracted from each sampling site during each visit.

Climatic parameters

Ambient and water temperatures were recorded throughout the study with an Extech EasyView™ Hygro-Thermometer EA25 (SKC) and an infrared Extech Thermometer (SKC, Johannesburg) respectively.

Microbiological analysis

Total aerobic colony counts, fungi and yeast counts as well as coliform/*E. coli* counts were determined as described previously for water used to irrigate parsley (section 4.2.1).

Molecular methods

Determination of presence of *E. coli* O157:H7 and *S.* Typhimurium – DNA was extracted from each of the enriched water samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using pathogen specific primers and the extracted DNA as template (Cebula *et al*., 1995; Standing *et al*., 2013).

Results

Microbiological analysis

Aerobic colony counts (ACC) – The ACC counts of irrigation water on the farm ranged between a minimum of 2.8 log CFU/mℓ (channel) and a maximum of 3.28 log CFU/mℓ (storage dam) (Table 48).

Fungi and yeast – The fungi and yeast counts were zero to 0.6 log CFU/mℓ (Table 48).

Coliforms and E. coli – Coliform counts for the channel water were zero and 3.5 log CFU/100 mℓ for water from the storage dam as well as irrigation pipes (Table 48).
E. coli counts were zero for all water samples (Table 48).

E. coli O157:H7 – None detected using multiplex PCR analysis.
S. Typhimurium – none detected

Table 48 Summary of results obtained for microbiological analysis of water used to irrigate basil

Irrigation water sources	Average microbial counts log CFU/g				
	Bacteria	**Fungi**	**Yeast**	**Coliform**	***E. coli***
Channel	4.55 (0.25)	2.5 (0.06)	3.01 (0.4)	4.93 (0.21)	0.00
Storage dam	3.31 (0.56)	2.23 (0)	2.55 (0.44)	3.1 (0.71)	0.00
Overhead sprinklers	4.15 (0.33)	1.98 (0.1)	2.85 (0.39)	3 (0.42)	0.00

Physiological parameters of irrigation water (temperature, relative humidity pH) are summarised in Figure 57.

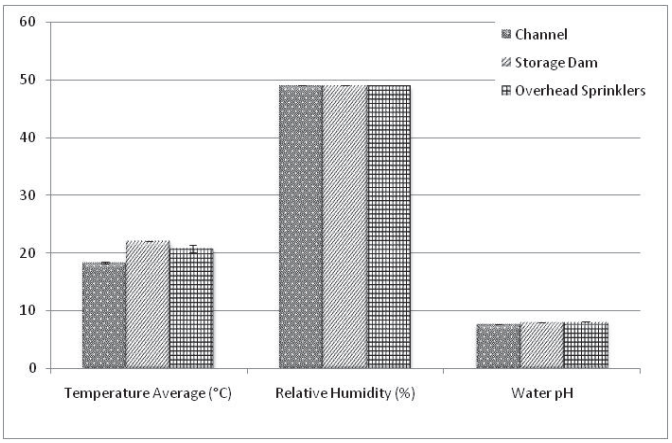


Figure 57 Physiological parameters (water temperature, relative humidity pH) of basil irrigation water samples.

Conclusion
Since the *E. coli* counts of the irrigation water were zero it is suitable for irrigation of any crops according to the DWAF guidelines (1996).

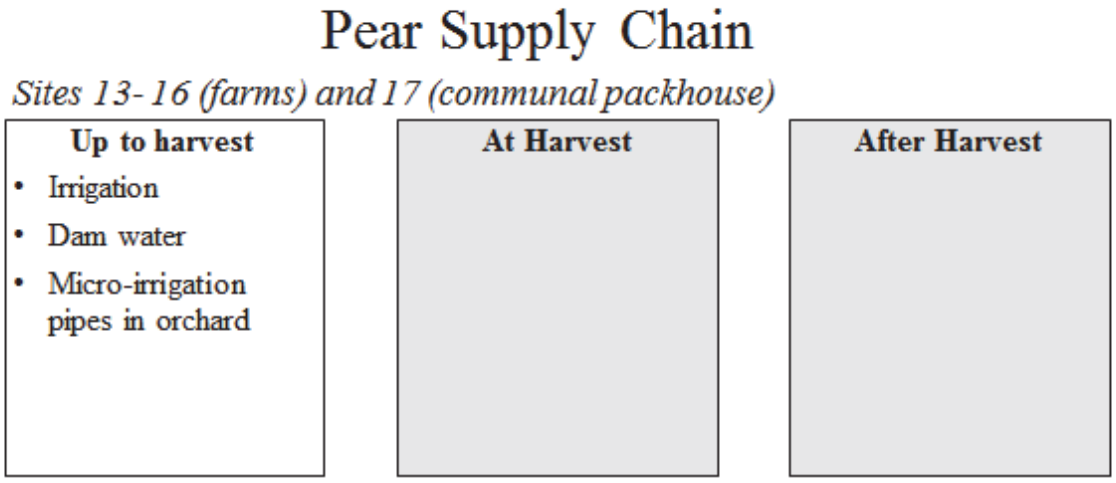
4.2.13 WESTERN CAPE PROVINCE – Microbiological quality of water used to irrigate pears from four commercial farms

Specific aim

The aim of this study was to evaluate the microbiological quality of water used to irrigate pears on commercial pear production farms.

Experimental sites

- Four commercial pear production farms in the Western Cape
- Pears (*Pyrus communis* cv Packham's Triumph) sent to a communal packhouse from four commercial farms located in the Western Cape, South Africa, were sampled.



Refer to Chapter 1, section 1.2 of this volume

Process flow

After harvesting in the orchards, the pears were transported in crates on trucks to a central packhouse. At the packhouse the pears were drenched once with water containing 75 ppm chlorine. After drenching, the pears were stored in controlled atmosphere (CA) for 14 weeks, at oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5% (consistency held with the aid of scrubbers) and temperature set at -0.5°C (with variation at front and back of the room 0.2-minus 0.7°C), where after the pears are exported.

Experimental procedures

Sample collection and processing

Irrigation water samples were collected from dams and micro-irrigation pipes in the orchards on each of the four commercial pear farms in the Western Cape Province. Water used for drenching the pears upon arrival at the communal packhouse was also analysed.

Microbiological analysis

Each of the filter membranes were cut into small pieces, added to 9 mℓ peptone buffered water [PWB] (3M, Merck, South Africa), vortexed and serially diluted from 10^{-1} to 10^{-6} in 9 mℓ tryptone soy broth (TSB). Total viable bacterial, coliform/*E. coli* and fungi/yeast counts were determined as described in section 4.2.8 for onion irrigation water analysis.

Each of the filtered water samples were enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) and *Salmonella* by incubation of the 9 mℓ 3M PBW with the filter membranes at 37°C for 24 hours. Additionally, a millilitre of the 3M PBW broth was transferred into a 9 mℓ tube of 3M *Listeria* selective broth for enrichment purposes and incubated at 37°C for 24 hours.

Subsequently, a loopful of each of the water samples (3M PBW enriched) were streaked onto eosin methylene blue differential medium (EMB) (Benton, Dickson and Company) for detection of typical *E. coli* colonies (black colonies with green metallic sheen). For detection of typical *Salmonella* colonies the enrichment broth was streaked onto *Salmonella* brilliance medium (Oxoid) agar (purple colonies). Typical colonies were purified by restreaking onto selective media. Typical *Listeria* colonies (black-grey with black precipitate surrounding the colony) were detected by streaking a loopful of the *Listeria* enrichment broths onto *Listeria* media.

Identification of presumptive pathogens using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF)

Single presumptive pathogen colonies isolated from the selective chromogenic media after selective enrichment were identified using Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF) as described in section 4.2.1.

Molecular analysis

Escherichia coli O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium presence was determined using multiplex PCR analysis as described by Standing *et al*. (2013).

Molecular Detection System (MDS) analysis

The 24h 3M PBW and 3M *Listeria* specific enrichment broths were used to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* 0157, including H7, (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

Results

Microbiological analysis of irrigation water

Aerobic colony counts (ACC) – The counts ranged between a minimum of 2.2 CFU/ml and a maximum of 4.09 log CFU/ml for the irrigation dams and micro-irrigation pipes.

Coliforms and *E. coli* – *E. coli* counts were zero for farms 1 and 2 for storage dams and in the micro-irrigation pipes. Farms 2 and 3 *E. coli* counts ranged between 1 log CFU/100 mℓ to 2.3 log CFU/100 mℓ (Fig. 58). *E. coli* counts for drench water was 0.4 log CFU/100 mℓ.

S. aureus – counts were below 0.5 log CFU/mℓ and was present in irrigation water from farm 3 only and at 0.4 log CFU/100 mℓ in drench water.

E. coli O157 (including H7) – detected in 2/57 water using 3M Molecular Detection System (MDS)

Salmonella spp.-detected in 2/57 water samples using 3M Molecular Detection System (MDS)

Listeria spp. – detected in 6/57 water samples using 3M MDS

Identification of bacteria, fungi and yeasts isolated from water

Drench water: Bacteria isolated included *Bacillus badius* and *Dothideomycetes* spp.

Micro irrigation water: Bacteria included *Chryseobacterium* spp., *Curtobacterium flaccumfaciens, Frigoribacterium* spp. *and Lodobacter* spp.. Fungi included *Cephalo thecasulfurea* and *Paecilomyces lilacinus;* Yeasts included *Ryptococcus laurentii* and *Rhodotorula glutinis.*

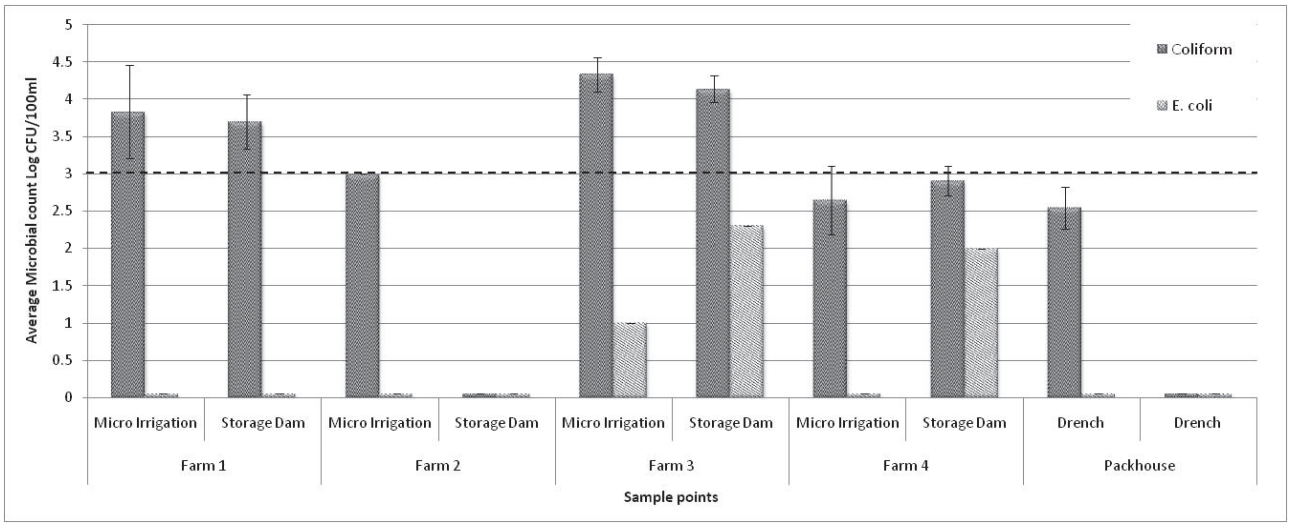


Figure 58 Total coliform and *E. coli* counts measured for irrigation water from four commercial farms in the Western Cape.

Physico-chemical analysis

The pH of the dam water from the four farms ranged from 6.8 to 7.4. The pH of the water in the micro-irrigation pipes varied between 6.8 and 7. Turbidity ranged from < 5-20 for the irrigation water samples.

Discussion and Conclusion

The *E. coli* counts in the irrigation water from the four commercial farms ranged from zero to 2.3 log CFU/100 ml, which is within the DWAF and WHO irrigation water guidelines for produce not to be eaten raw. *E. coli* O157 (including H7) and *Salmonella* spp. were detected in 2/57 water samples, and detected in 2/57 water samples, and *Listeria* spp.in 6/57 water samples. The pH of the irrigation water samples were within the guidelines set by DWAF's Target Water Quality Range (TWQR) of pH 6-pH 9 (DWAF, 1996).

4.2.14 MOLECULAR CHARACTERISATION OF HEPATITIS A VIRUS STRAINS FROM WATER SOURCES IN SOUTH AFRICA

Specific aim

The aim of this study was to characterise Hepatitis A virus (HAV) strains found in selected South African (SA) surface water to establish what HAV types are circulating in the environment, thus reflecting prevalence in the surrounding communities.

Experimental procedures

Surface water sample collection. From January 2012 to August 2012, 21 water samples (10ℓ) were collected monthly (except in February and July) from six sites near Polokwane, as part of the SA Water Research Commission (WRC) project K5/1875//4. These samples originate from rivers and dams used to irrigate fresh produce on a large commercial farm. The samples were transported in cooler bags with cold packs to the Department of Medical Virology, Faculty of Health Sciences, University of Pretoria. The temperature and pH of each sample were recorded upon arrival. The samples were either processed immediately or stored at 4°C before being processed within 24 hours of receipt. The sites from which samples were collected were: Bloed River (site 1) (two samples); River water drawn from below the Polokwane sewage works before the Sand River (site 5) (two samples); River on fresh produce farm (site 7) (five samples); Dam A at pump site on fresh produce farm (site 16B) (two samples); Dam B at pump site on fresh produce farm (site 17B) (five samples); River water from below the Seshego sewage works in the township (site 23) (two samples). Water was also collected from an irrigation pivot at the farm, at two points (three samples). Each sample was given a unique code comprising site number and the date at which it was taken.

Water sampling at wastewater treatment plants (WWTW samples). From August 2010 to December 2011, 51 WWTW samples (500 mℓ) were collected from the outflow of wastewater treatment plants in three provinces: Gauteng (GP, nine samples), Mpumalanga (MP, 20 samples) and North West (NW, 16 samples). In addition, sludge was collected in a treatment plant in KwaZulu-Natal (KZN, two samples) and in the Western Cape (WC, two samples), two samples were of unknown origin. All the samples had thermo tolerant (faecal) counts greater than 10^6 colony forming units (CFU) per 100 mℓ.

***Escherichia coli* enumeration**

The *Escherichia coli* count, expressed as CFU/100 mℓ was determined for the surface water samples, using the membrane filtration technique and m-ColiBlue24® broth (Millipore Corp., Billerica, MA) as per manufacturer's instructions.

Virus recovery and concentration

Surface water samples. Prior to recovery, 100 µℓ of mengovirus (5 x 10^4 copies/ 10 µℓ) was added to each sample as a process control to monitor the efficiency of viral recovery and downstream nucleic acid extraction. Viruses were recovered from the 10 ℓ water samples using the glass wool adsorption-elution method based on the method of Vilaginès *et al*. (1993) as described by Mans *et al*. (2013). After elution, viruses are further concentrated to a volume of 10 mℓ in phosphate-buffered saline pH 7.4 (PBS; Sigma-Aldrich Co., St. Louis, MO, USA), using polyethylene glycol (Merck Schuchardt OHC, Hohenbrunn, Germany) and sodium chloride (Merck) (PEG8000/NaCl) precipitation. The recovered virus concentrates were stored at -20°C.

Wastewater treatment plant samples. Viruses were concentrated from 75 mℓ or 100 mℓ WWTW samples to a volume of 2 mℓ in PBS pH 7.4, using the PEG8000/NaCl precipitation. No prior viral recovery was performed as the sample volumes were too small. Virus concentrates were also stored at -20°C.

Nucleic acid extraction

Total nucleic acid was extracted from 1 mℓ of virus concentrates using the automated platform of the MagNA Pure LC instrument (Roche Diagnostics GmbH, Mannheim, Germany) and the semi-automated platform of the NucliSENS® EasyMAG® instrument (BioMérieux, Marcy l'Etoile, France). The MagNA Pure LC Total Nucleic Acid isolation Kit (Large volume) was used for viral extraction on the MagNA Pure LC System (Roche Diagnostics). Extraction on the MagNA Pure LC System (Roche Diagnostics) was done for virus concentrates of the WWTW samples, while the NucliSENS® EasyMAG® instrument (BioMérieux) was used for the surface water samples. In either method, extracted nucleic acid was eluted in 100 µℓ of elution buffer and stored at -70°C.

Mengovirus detection

Nucleic acid extracted from surface water samples were tested for mengovirus using a one-step real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay. The detection was performed using mengo@ceeramTools™ Kit (Ceeram s.a.s, La Chappelle-Sur-Erdre, France). The detection was performed on 5 µℓ of RNA extracts and the conditions of the assay were as follows: 45°C for 10 min, 95°C for 10 min and 45 cycles of 95°C for 15 s, 60°C for 45 s. The primers (Table 49) included in the kit to amplify a 99 bp fragment within the 5'noncoding region (5'NCR) of the mengovirus genome which is similar to the 5'NCR of HAV (Pintó *et al*., 2009).

Table 49 Sequences of primers and probe used in this study.

PCR	Region	Primer/Probe name	Nucleotide Sequence (5'→3')	Position[a]	Reference
Real time – Mengo		Mengo110-F	GCG GGT CCT GCC GAA AGT	—	Pintó *et al.* 2009
		Mengo209-R	GAA GTA ACA TAT AGA CAG ACG CAC AC	—	Pintó *et al.* 2009
		Mengo147-probe	FAM-ATC ACA TTA CTG GCC GAA GC-TAMRA	—	Pintó *et al.* 2009
Real time – HAV	5'NCR	HAV68-F	TCA CCG TTT GCG TAG	68-85	Costafreda *et al.*, 2006
		HAV240-R	GGA GAG CCC TGG AAG AAA G	240-222	
		HAV150-probe	FAM-TTA ATT CCT GCA GGT TCA GG-TAMRA	150-169	
First round PCR	VP1	HAV1-F	GTT TTG CTC TTT ATC ATG CTA TG	2167-2192	Costa-Mattioli *et al.*, 2002
		HAV2-R	AGT CAC ACC TCT CCA GGA AAA CTT	3308-3285	
	VP1/P2B	2870P-F	GAC AGA TTC TAC ATT TGG ATT GGT	2870-2893	Nainan *et al.*, 2006
		3381N-R	CCA TTT CAA GAG TCC ACA CAC T	3381-3360	
Second round PCR	VP1	2172P-F	GCT CCT CTT TAT CAT GCT ATG GAT	2172-2195	Costa Mattioli *et al.*, 2002 ; Nainan *et al.*, 2006
		3125N-R	CCT GCA TTC TAT ATG ACT CT	3125-3106	
	VP1/P2B	2896P-F	CTA TTC AGA TTG CAA ATA CAA T	2896-2918	Nainan *et al.*, 2006
		3289N-R	AAC TTC ATT TCA TGC TCC T	3289-3268	

[a]Positions are relative to the genome of the HAV strain HM175 (accession number M14707)

Hepatitis A virus detection

Nucleic acid (5 µℓ) from each sample (surface water and WWTW) was tested for HAV using a one-step real-time RT-PCR assay. Detection of HAV in surface water samples was performed using a hepatitisA@ceeramTools™ Detection Kit (Ceeram s.a.s) while detection in WWTW samples was performed using a RNA Ultrasense™ One-step qRT-PCR system kit (Invitrogen, Carlsbad, CA). Both assays were carried out in a LightCycler 2.0 (Roche Diagnostics) and used primers which amplified a 172 bp fragment within the most conserved region of the HAV genome, the 5'NCR (Table 49). The hepatitisA@ceeramTools™ Detection Kit (Ceeram s.a.s) includes an internal control to monitor the amplification process. The RNA Ultrasense™ One-step qRT-PCR system kit (Invitrogen) does not include an internal control, but a positive control was added to each run to monitor the amplification process. The conditions for the assay were different for each kit. For the hepatitisA@ceeramTools™ Detection Kit (Ceeram s.a.s) the conditions of the assay were as follows: 50°C for 1 hour for the reverse transcription reaction, followed by a hot start of 95°C for 10 min and 45 cycles of 95°C for 15 s for denaturation, 60°C for 1 min for annealing and 70°C for 1 min for extension (Costafreda *et al*., 2006). For the RNA Ultrasense™ One-step qRT-PCR system kit (Invitrogen) the conditions of the assay were as follows: 50°C for 45 min for the reverse transcription reaction, followed by a hot start of 95°C for 15 min and 50 cycles of 95°C for 15 s for denaturation, 60°C for 1 min for annealing and 65°C for 1 min for extension (Netshikweta, 2011).

Amplification of the VP1 region. The full length VP1 region (900 bp) was amplified from cDNA (5 µℓ or 1 µℓ) by conventional PCR, using primers published by Costa-Mattioli *et al*. (2002) (Table 49). The total volume of the reaction mix was 25 µℓ and contained the following: 5 x KAPATaq™ HotStart buffer (Kapa Biosystems, Cape Town, South Africa), 10 mM dNTPs, and 10 µM each of the forward and reverse primers respectively, 25 mM MgCl2, and 1.25 units of KAPATaq™ HotStart DNA polymerase (Kapa Biosystems). The conditions under which the VP1 region was amplified were: pre-denaturation at 94°C for 3 min, follow by 45 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 30 s, and final extension at 72°C for 5 min. If no positive amplicons were obtained after the first round of PCR, a second PCR was performed and the VP1 gene was amplified using internal primers (Costa-Mattioli *et al*., 2002; Nainan *et al*., 2006) and 1 µℓ of the first PCR reaction as template. The assay conditions for the second round of PCR are essentially the same, except for the annealing temperature that decreased from 50°C to 48°C and the number of cycles that went from 45 to 35 cycles.

Amplification of the VP1/P2B junction. The VP1/P2B junction (~ 350 bp) was amplified from the same cDNA (5 µℓ) used to amplify the VP1 region using primers published by Nainan *et al.* (2006).The total volume of the reaction mix was 25 µℓ and contained the following: 5 x KAPATaq™ HotStart buffer (Kapa Biosystems), 10 mM dNTPs, and 10 µM each of the forward and reverse primers respectively, 25 mM MgCl2, and 1.25 units of KAPATaq™ HotStart DNA polymerase (Kapa Biosystems). The conditions under which the VP1/P2B junction was amplified were: pre-denaturation at 94°C for 3 min, follow by 35 cycles of 94°C for 30 s, 45°C for 45 s, 72°C for 1 min, and, final extension at 72°C for 5 min. If no PCR products were visible after the first round of PCR, a nested PCR was performed using internal primers (Nainan *et al*., 2006) (Table 28) and 1 µℓ of the first PCR reaction as template. The

conditions for the second round of VP1/P2B amplification are similar to the ones used for the first round PCR, except for the annealing temperature that increased from 45°C to 48°C.

Sequencing and cloning. Products from the PCR were run through a 2% agarose gel by electrophoresis. The gel was stained with ethidium bromide for visualization. Positive amplicons were purified directly or from the gel using the Zymogen DNA Clean & Concentrator-25™ Kit or the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA) respectively. The primers used to amplify the different regions were used to sequence positive amplicons directly after amplification, in both the forward and reverse direction, using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit on an ABI 3130 automated analyser (Applied Biosystems), as per manufacturer's instructions. In cases where more than one strain were detected in a sample, PCR products were cloned using the cloneJET™ PCR cloning kit (Fermentas, Canada). After colony PCR, at least 10 clones were randomly selected for each sample and sequenced using the pJet 1.2/blunt specific primers.

Nucleotide sequence analyses. The sequences obtained were analysed and edited with Sequencer™ v4.10.1 and BioEdit v6.0.5. A BLAST search (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/) was performed to verify the identity of the output sequences. Thereafter the edited sequences were compared to each other and to reference sequences retrieved from Genbank by pairwise comparison. The sequences obtained along with reference sequences for HAV and closely matched sequences from the output BLAST search were aligned in MAFFT version 6 (http://0-mafft.cbrc.jp.innopac.up.ac.za/alignment/server/index.html).

Detection of novel or predicted mutation. After alignment, the sequences obtained were compared to reference sequences using the software BioEdit v6.0.5, to check for novel or predicted mutations at nucleotide and protein levels. Positions at which nucleotide mutations cause amino acid change potentially resulting in vaccine escape mutants (Pérez-Sautu *et al*., 2011b; Pintó *et al*., 2012) were carefully analysed.

Phylogenetic analysis

Phylogenetic analyses were performed in MEGA 5 using the two-parameter model of Kimura from the neighbor-joining method. Two trees were generated (one for VP1 and one for VP1/P2B) and assessed by bootstrap analysis (1000 pseudo replicates). The genotype of each sequenced strain was determined by how well it clusters with reference sequences on the phylogenetic tree.

Phylogenetic analysis

Phylogenetic analyses were performed in MEGA 5 using the two-parameter model of Kimura from the neighbor-joining method. Two trees were generated (one for VP1 and one for VP1/P2B) and assessed by bootstrap analysis (1000 pseudo replicates). The genotype of each sequenced strain was determined by how well it clusters with reference sequences on the phylogenetic tree.

Results and discussion

Hepatitis A virus detection

Hepatitis A virus was detected in 76% (16/21) of the surface water samples and in only 37% (19/51) of the WWTW samples. Mengovirus was detected in samples where no HAV was detected indicating that the processing and nucleic acid extraction procedures were adequate and negative HAV result is therefore a true negative. Furthermore, the *E. coli* counts of the surface water showed that nine of the samples had a count between 1 and 1000 CFU/100 mℓ and six of the samples had a count greater than 1000 CFU/100 mℓ. According to the South African water quality guidelines using water with an *E. coli* count ranging from 1 to 1000 CFU/100 mℓ to irrigate vegetables and crops eaten raw, increases the chance of contaminating the produce and consequently transmitting human pathogens (Department of Water Affairs and Forestry, 1996). Furthermore, if the count is higher than 1000 CFU/100 mℓ water should not be used to irrigate produce that are minimally processed. Finding such high counts in the water used to irrigate fresh produce implies that there is a risk of produce contamination at that specific farm. Hepatitis A virus is environmentally stable and a minimum of 10 infectious particles is enough to induce infection (Bosch *et al*., 2011). Therefore, even the slightest contamination of produce could pose a risk of infection.

Phylogenetic analysis

Hepatitis A virus was detected in 35 samples in total. Of the 35 samples, HAV strains could be genotyped from 32 samples: 15 surface water samples and 17 WWTW samples. The junction VP1/P2B could be amplified from 29 of the samples (13 surface water samples and 16 WWTW samples), while the VP1 region could only be amplified from 17 samples (11 surface water samples and six WWTW samples). Based on the phylogenetic analysis of both the VP1 genomic region and the VP1/P2B junction all the strains typed in this study clustered within genotype IB (Figure 59 and 60, respectively). In 1997, it was established that genotype IB predominated in SA (Taylor, 1997). None of the strains sequenced in this study grouped within a genotype other than IB, suggesting that since 1997 genotype IB still predominates in SA. However, additional HAV strains will have to be characterised before a conclusive statement in this regard can be made.

The strains sequenced in this study form a unique SA cluster within genotype IB (Figure 59: South African cluster; Figure 60: South African cluster A). The clusters are supported by high bootstrap values (99% on Figure 59 and 97% on Figure 60). However, few strains grouped outside the unique cluster on Figure 60 (South African cluster B; sample 10/1010 indicated with ■) but were still within genotype IB. All the samples that grouped outside the unique cluster were either collected from the effluents of sewage treatment plants or from rivers into which sewage effluents are discharged.

When analyzing samples from the environment, multiple strains are expected to be characterised from a single sample. During amplification and sequencing of the VP1/P2B junction, no more than one strain was detected in each sample except for one. On the other hand, multiple strains were detected in 15 out of the 17 samples from which the VP1 region could be amplified. Although HAV is antigenically stable, some regions of its genome are more variable than others and therefore more informative. It is mainly for that reason that

genotyping of the different HAV strains using nucleotide sequence analyses of the full VP1 region, instead of other genomic regions commonly used, was proposed (Costa-Mattioli *et al*., 2002). Furthermore, some amino acids encoded from the VP1 region contribute to the immunodominant antigenic site targeted by most antibodies that are directed against HAV (Costa-Mattioli *et al*., 2003; Knowles *et al*., 2012) and these residues are prone to mutation (Pérez-Sautu *et al*., 2011b; Pintó *et al*., 2012). The predicted amino acid sequence of the strains characterised in this study showed great similarity (> 98% in amino acid identity) to that of the type strain HM175. However, minor amino acid changes were detected. These changes did not occur on the residues that contribute to the immunodominant antigenic site. In addition, the mutations detected among the strains were not consistent, except one: a R298K (Position is relative to the genome of the HAV strain HM175, accession number M14707). Even though the mutation occurred at the end of the protein sequence, it was consistent in all strains for which the VP1 region could be amplified. In addition an arginine (R) to lysine (K) mutation in the amino acid sequence has little effect on the final conformation of the VP1 protein. Despite a small structural difference on the side chain, both amino acids are positively charged. Therefore the strains bearing this mutation will still be antigenically stable. Although no mutants were detected in this study, the presence of multiple strains of HAV that differ in the VP1 region suggest the importance of implementing a surveillance system in order to monitor the possible emergence of new variants. This would not only help in molecular source tracking in the event of an outbreak, but in implementing vaccine if necessary.

In 2009, a study was published on the epidemiology of hepatitis A in Germany, with special emphasis on imported and non-imported HAV infections (Faber *et al*., 2009). The study detected and characterised HAV in tourists that got infected while visiting SA. These strains formed a distinct cluster when compared to strains detected in individuals who got infected either in Germany or in other regions of the world. In figure 60, the strains isolated from the tourists were included (indicated with Δ on the tree). Phylogenetic analysis clustered the German strains within SA cluster A. The high bootstrap value at the node leading to the cluster indicates close relationship with strains characterised in this study. This further confirms the distinct geographical distribution of HAV, a fact that can be exploited when investigating the source of an outbreak.

Hepatitis A virus strains were previously detected on tomatoes and lettuce at the point of retail in the Tshwane district (Netshikweta, 2011). The detected strains (indicated with ▲ in figure 60) also group within SA cluster A. The high bootstrap value at the node indicates that strains isolated from the water samples are similar to those isolated from the fresh produce. Furthermore, the strain isolated from the lettuce is identical to a strain detected in a WWTW sample (bootstrap value of 97%). The sample in question was collected from Mpumalanga (data not shown). Fresh produce can be contaminated at various points throughout the chain of production. Irrigation with surface water contaminated with faeces containing HAV and agrichemicals mixed with contaminated water are possible sources of contamination. The lack of proper washing facilities for food handlers on the farm, at commercial outlets or during preparation, are additional sources of contamination. In the present case the exact source of contamination of the produce could not be defined, but the similarity with a strain detected in

sewage effluent suggests that sewage treatment in the region of the WWTW sample does not remove all HAV particles.

Conclusions

Hepatitis A virus strains of genotype IB are still prevalent in SA as no other genotypes were detected in the water samples. The presence of genotype IB in the surface water sources used to irrigate fresh produce indicates human faecal contamination of irrigated fresh produce in SA. However, it would be essential to characterize HAV from infected individuals in order to assess whether or not the strains detected in the environment are clinically relevant.

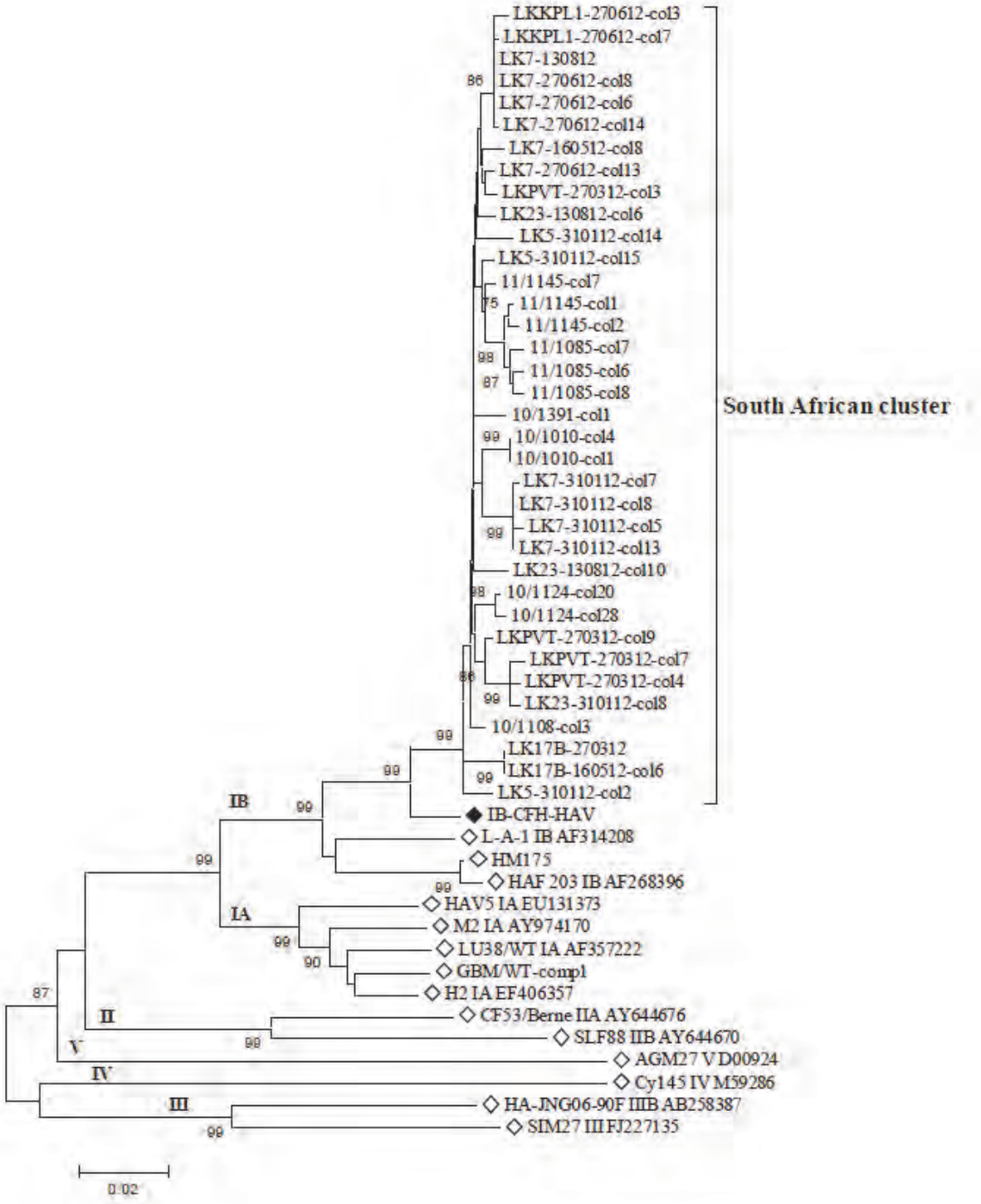


Figure 59 Phylogenetic tree based on the sequence of the full VP1 region (900 bp) of strains characterised in this study. The tree was constructed using the Kimura two-parameter model of the neighbour-joining method, with 1000 bootstrap replicates; bootstrap values ≥ 75% are indicated. Strains indicated with ◊ or ♦ are reference strains or strains from previous studies that were retrieved from Genbank. Strains with 'col' and a number at the end represent clones from strains characterised in this study.

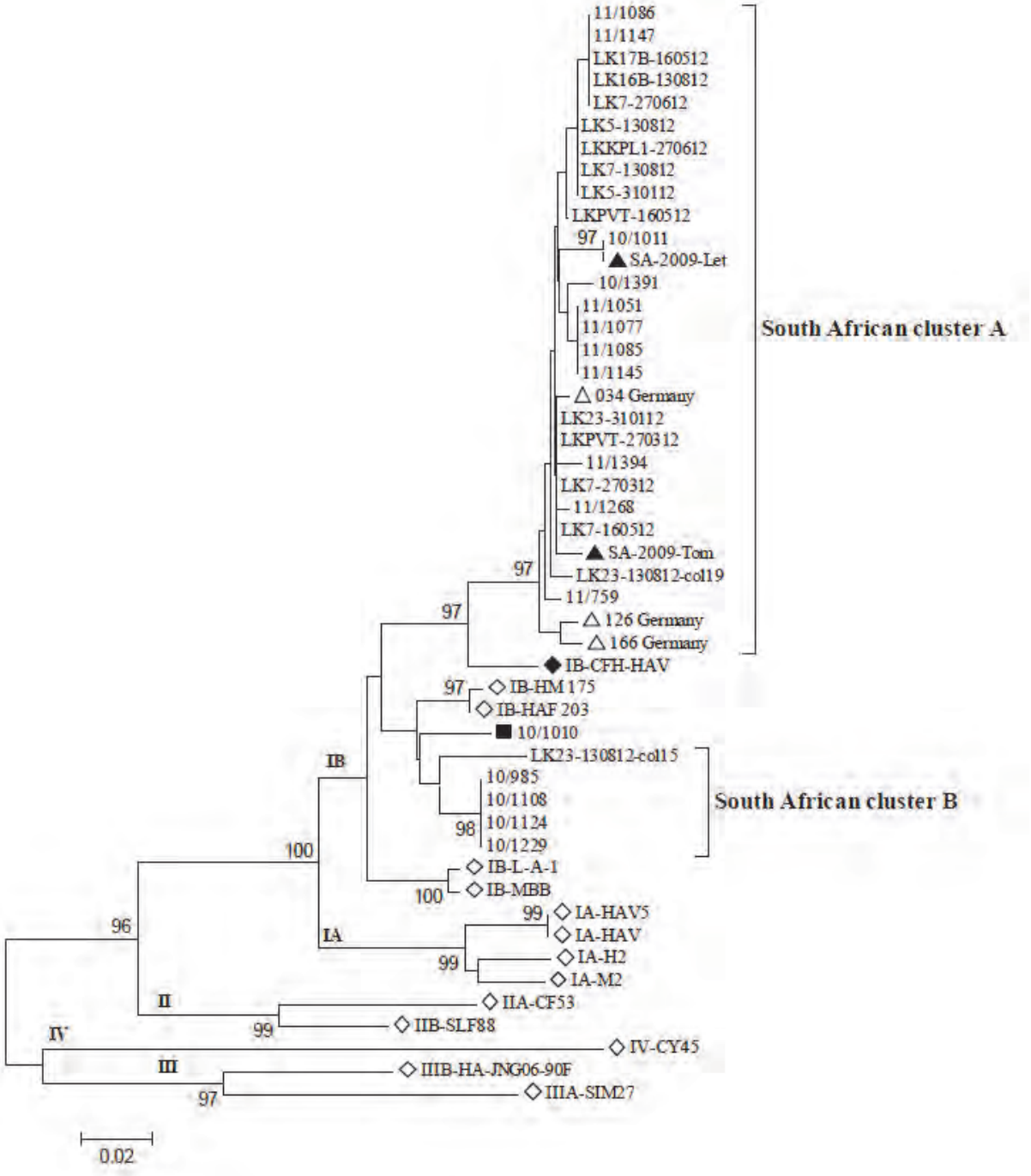


Figure 60 Phylogenetic tree based on the sequence of the VP1/P2B junction (~ 350 bp) of strains characterised in this study. The tree was constructed using the Kimura two-parameter model of the neighbour-joining method, with 1000 bootstrap replicates; bootstrap values ≥ 75% are indicated. Strains indicated with ◊ or ♦, and, ▲ or Δ are reference strains or strains from previous studies that were retrieved from Genbank. Strains with 'col' and a number at the end represent clones from strains characterised in this study.

4.3 SUMMARY OF RESEARCH FINDINGS

GAUTENG PROVINCE – Microbiological quality of water (including virological analysis) used to irrigate parsley from a farm in the Elandsdrift area

According to the South African irrigation water quality guidelines the *Escherichia coli* count for safe consumption of crops eaten raw should be ≤1 (CFU/100 mℓ) (DWAF, 1996). The World Health Organization recommended that the faecal coliform limit be relaxed to 1 000 faecal coliforms/100 mℓ for vegetables eaten raw (WHO, 1989). If *E. coli* counts are 1-1000 (log 3) CFU/100 mℓ, the water is suitable for irrigation of crops not intended to be eaten raw and allowed to dry before harvest. If the *E. coli* counts are >1000 CFU/100 mℓ no contact is allowed to take place with humans and water can only be used in irrigation for the production of fodder, tree plantations, nurseries, parks, etc. (DWAF, 1996). However, according to the WHO irrigation water with faecal counts of 1000 CFU/100 mℓ could be used for unrestricted irrigation of all crops (eaten both raw and cooked) without known ill effects (WHO, 1989).

The *E. coli* levels in the irrigation water (borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery) were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). None of the human pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium and viruses (Norovirus GI [NoV GI], Norovirus GII [NoV II] and hepatitis A virus [HAV]) tested for, were detected in any of the water samples.

GAUTENG PROVINCE – Biofilm formation in irrigation pipes and potential impact on the microbiological quality and safety of lettuce produced on a farm in the Elandsdrift area

This study investigated the potential for contamination by *E. coli* associated with heterotrophic biofilms in pipe-based irrigation water delivery systems and the microbiological quality and safety of lettuce before and after harvest. Three different water sources on the farm were tested for quality monitoring purposes: (1) the dam water, (2) the pump house water, and (3) water from the irrigation sprinklers on the plot where lettuce samples were collected. The *E. coli* levels in the irrigation water were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996).

E. coli was detected through PCR in all the water sources at some point throughout the study. On most occasions, if the dam water showed a positive result for *E. coli*, then the pump house water and the irrigation pipe water would also be positive. *E. coli* O157:H7 was not detected in any of the samples that tested positive for the presence of general *E. coli.*

GAUTENG PROVINCE – Virological analysis and indicator organism levels of water used to irrigate strawberries on a farm in the Pyramid district, Tshwane Metropolitan area

From September 2010 to August 2011 water samples used to irrigate strawberries were collected on a monthly basis from an irrigation canal flowing from the Bon Accord Dam, Pretoria. The detection of ≥ 200 (2.3 log) *E. coli* CFU/100 mℓ in the irrigation water indicated that vegetables and other crops irrigated with this water, which are eaten raw, could result in the transmission of human pathogens (DWAF, 1996). Multiplex *rt* RT-PCR assays for the detection of NoV GI, NoV GII, sapovirus, hepatitis A virus, human astrovirus, human rotavirus and mengovirus were optimised. This made the gathering of new data of the role of irrigation water as a source of contamination of irrigated berry fruits in SA possible.

The HAV virus was not detected in any of the irrigation water samples. Norovirus GI and NoV GII could be detected in 9.1% (1/11) and 36.4% (4/11) of irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR assays NoV GI and NoV GII were detected in 27.3% (3/11) and 27.3% (3/11) of the irrigation water samples. Human AstV, HRV and SaV were detected in 36.4% (4/11), 18% (2/11) and 9.1% (1/11) of the irrigation water samples collected. Human Norovirus GII.7 was detected in irrigation water samples.

GAUTENG PROVINCE and KWAZULU-NATAL PROVINCE – The extent of virological contamination and indicator organism levels in water used to irrigate cabbages on two farms (small scale and commercial)

E. coli was not isolated from the borehole sample from the small scale farm in Gauteng, but the *E. coli* levels in the Gauteng and KZN river water samples were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). However, the values were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting (DWAF, 1996). None of the viruses tested for (NoV GI, NoV GII and HAV) were detected in the river and borehole samples from the small scale farm in Gauteng. The river water sample from the KZN farm tested positive for HAV and negative for NoV GI and NoV GII. The HAV strain was characterised by neighbour-joining phylogenetic tree analysis and grouped within HAV genotype V. The latter finding suggests the strain to be associated with non-human primates, i.e. simian species.

KWAZULU-NATAL PROVINCE – Microbiological quality of water used to irrigate raw produce (broccoli, bell pepper, cabbage, Chinese cabbage, red cabbage, cauliflower, Crisphead lettuce, jam tomatoes, parsley and spinach) from three farms near Cato-Ridge, Camperdown and Richmond respectively

During the twelve month sampling period the *E. coli* counts of river water samples from farm A ranged between 1-1000 CFU/100 mℓ and were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could potentially result in the transmission of human pathogens (DWAF, 1996). *E. coli* counts of

borehole water samples exceeded the 1000 CFU/100 mℓ DWAF and WHO guideline limit only once, which indicated a potential contamination event of the groundwater. *E. coli* counts of the irrigation water sources on farm B (dam and borehole water mixture) as well as from farm C (dam water) were zero. Potential pathogens isolated from the irrigation water included *L. monocytogenes, Salmonella* spp., *Shigella* spp. and *Campylobacter* spp.

Presumptive *L. monocytogenes* was not present in the irrigation water from farms B and C and only once during October 2009 for the borehole water sample (A2, farm A). Presumptive *Salmonella* spp. was observed once for each of the following samples: river water (A1, farm A), borehole water (A2, Farm A) as well as mixed dam and borehole water (B, farm B) during the twelve month sampling period. Presumptive *Shigella* spp. were observed for water samples A1, A2 and B and not for C. Presumptive *Campylobacter* spp. was only detected in water sample C (dam water), and the population increased when the irrigation water was not in use, and the dam was allowed to be stagnate. This allowed for the growth and survival of *Campylobacter* spp., being a microaerophile, since stagnant water limit the entrance of oxygen.

KWAZULU-NATAL PROVINCE – Microbiological quality of water (including agrichemical spray water) used to irrigate spinach and lettuce on a farm near Cato-Ridge

E. coli counts of irrigation water and agrichemical spray water sampled from a farm in KZN for a period of three months (August-October 2013) were higher than the DWAF and WHO maximum limit of 1000 *E. coli* CFU/100 mℓ. Typical growth of *L. monocytogenes*, *Salmonella* spp. and *Shigella* spp. were observed. In this study the ACC and *E. coli* numbers increased in irrigation water mixed with pesticides when compared to the levels in irrigation water alone. This result is in agreement with literature reports that some pesticides have the ability to promote growth of specific microorganisms when mixed with irrigation water. Presumptive *L. monocytogenes*, *Salmonella* spp. and *Shigella* spp. were not found in the agrichemical spray water samples.

KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of fresh produce (cabbage and lettuce) from two farms near Camperdown

Irrigation water and agricultural spray water samples were collected from two farms in KwaZulu-Natal for a period of three months (July-September 2014). The *E. coli* counts were higher than DWAF and WHO maximum limit of 1000 *E. coli* CFU/100 mℓ water used for irrigation of fresh produce to be eaten raw. *L. monocytogenes* and *Salmonella* were isolated from the irrigation water samples and could therefore pose a potential health risk if transferred onto fresh produce during irrigation.

The mean turbidity levels in the irrigation water exceeded the international turbidity of 0-1 NTU standard for water (DWAF, 1996). The high turbidity levels of the irrigation water could be the result of soil erosion and runoff. There was a positive correlation between the BOD, temperature and pH, and the turbidity levels on farm A. There was a positive correlation

between the TDS and COD levels in the irrigation water from both farms. *E. coli* counts appeared to be influenced by TDS and COD on farm B. Microbial growth could have been supported by the presence of organic matter in the irrigation water.

The BOD levels were lower than the COD levels which is expected since BOD only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels on both farms. COD and TDS values were the only two parameters of water that seemed to have influenced the survival of the THB in the irrigation water on farm A. TDS seemed to have also influenced the survival of *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. There was a significant positive correlation between the COD levels and the THB and *E. coli* counts in the water samples. South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by WHO of 10 mg/l. This is an indication that the water contains organic pollutants which could help promote the growth of certain microbes in the water. There was a positive correlation between the THB and *E. coli* in the water on farm A while there was a negative correlation between the two in water from farm B. THB counts were also positively correlated with the *Salmonella* counts.

This study also showed that pesticide solutions used for agricultural spray purposes to control pests and insects can provide a suitable environment for the survival and growth of pathogenic microbes, such as *L. monocytogenes*, *E. coli*, *Salmonella* and *Shigella* spp., whereas others are inhibitory to these microorganisms. Microbe growth was favoured mainly by the pesticides with organophosphates and carbamates as the active ingredients. Organophosphate pesticides contain diazinon and chlorpyrifos. Other active ingredients were abamectin (16-membered macrocyclic lactone derivative), phthalic acid diamide and poly-1-p-methene (insect growth regulator).

LIMPOPO PROVINCE – Microbiological quality (including virological analysis) of water used to irrigate onions and tomatoes

Although the *E. coli* counts measured for the primary irrigation water source (river) on the farm exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ during the summer months, the *E. coli* counts in the river, dam and irrigation pivot point water during the winter months (during the onion production cycle) were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting, i.e. 1-1000 CFU/100 mℓ (DWAF, 1996).

No *E. coli* O157:H7 and *Listeria* spp. were isolated, however *Salmonella* was isolated from 22% of the water samples tested and included *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The MALDI-TOF MS was applied successfully as an alternative method for identification of *E. coli*, *Salmonella* and *L. monocytogenes* as well as other enterobacteriaceae from environmental sources. Noroviruses (NoV GI and NoVGII) as well as the Hepatitis A virus (HAV) were detected in the Sand River, dam and irrigation pivot point water samples. Noroviruses are the most common cause of foodborne illness and outbreaks of NoV gastroenteritis associated with the ingestion of contaminated fresh produce are well documented.

The electrical conductivity (EC) of the water samples ranged from 70.9 m Siemens/m to 115.2 mS/m which can be regarded as good and tolerable respectively according to the DWAF irrigation water guidelines (DWAF, 1996). Nitrogen occurs predominantly as nitrate in irrigation water. The ammonium form is usually a result of contamination with wastewater. The highest level of ammonium (25.5 mg/ℓ) was indeed recorded for the water sampled from the dam 3 outflow, just below the Polokwane waterworks. The waterworks often overflow after rain and subsequently lead to contamination of the river water downstream which is used for irrigation purposes on the farm. The pH of all the water samples tested were within the recommended pH range for irrigation water in South Africa, i.e. from pH 6.5 to pH 9.5 (DWAF, 1996). However, this range also promotes growth of enteric pathogens.

LIMPOPO PROVINCE – The microbiological quality of water used to irrigate peaches from a commercial peach farm near Mookgophong

The *E. coli* counts ranged between 3.2 log CFU/100 mℓ and 4 log CFU/100 mℓ. The *E. coli* levels in the irrigation water (Sterk River, dam, micro irrigation pipes in the orchard and filters in the irrigation pipes) exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ. Total average bacterial, coliform and yeast counts were higher for samples taken from irrigation pipes after filters as well as in micro-irrigation pipes in the orchard when compared to river and dam water counts. This could indicate an accumulation of microorganisms as opposed to improving the water quality by installing filters in the irrigation pipes in the peach orchard due to the filters not being replaced regularly. None of the human pathogenic bacteria tested for (*E. coli* O157 (including H7), *Salmonella* spp. and *Listeria* spp.) were isolated from the water samples.

LIMPOPO PROVINCE and NORTH WEST PROVINCE – Microbiological quality of water from the Loskop Dam Canal and the Skeerpoort River used for lettuce irrigation

The study aimed to compare the bacteriological quality of urban and rural irrigation water sources. Bacterial counts, characterisation, identification and diversity of aerobic bacteria were determined. Irrigation water samples were collected monthly from the Skeerpoort River for 10 months from January to October 2011. During the ten month sampling period the faecal coliform counts exceeded the DWAF guideline limit of *E. coli* (1000 CFU/100 mℓ) only once. The pH ranged from 7.9 to 9.2 in the Skeerpoort River and was acceptable according to the South African national guidelines (DWAF, 1996). The river water temperature ranged from 10.7 to 26.0˚C with the lowest and highest noted in May and January respectively.

E. coli isolated from both sites was subjected to antibiotic susceptibility testing, virulence gene (*Stx1*/*Stx2*&*eae*) determination and $(GTG)_5$ Rep-PCR fingerprinting. Low mean monthly counts for aerobic spore formers, anaerobic spore formers and *S. aureus* were noted although occasional spikes were observed. The most prevalent bacterial species at both sites were *Bacillus* spp, *E. coli* and *Enterobacter* spp.. *E. coli* and *Bacillus* spp. were most prevalent in winter and summer respectively. Resistance to at least one antibiotic was 84% (rural) and 83% (urban). Highest resistance at both sites was to cephalothin and ampicillin. Prevalence of *E. coli* possessing at least one virulence gene (*Stx1*/*Stx2* and *eae*) was 15% (rural) and 42%

(urban). All (rural) and 80% (urban) of *E. coli* possessing virulence genes showed antibiotic resistance. Complete genetic relatedness (100%) was shown by 47% of rural and 67% of urban *E. coli* isolates. Results from this study showed that surface irrigation water sources regardless of geographical location and surrounding land-use practices, can be reservoirs of similar bacterial pathogens.

The pH ranged from 5.4 to 9.9 and 7.9 to 9.2 in the Loskop canal and the Skeerpoort River respectively. The pH in the Loskop Canal exceeded South African national guidelines (DWAF, 1996) for irrigation water twice during the 10 month study. Water temperature in the Loskop canal ranged from 8.5 to 20.5°C with the lowest and highest noted in September and February respectively. Temperature in the Skeerpoort River ranged from 10.7 to 26.0°C with the lowest and highest noted in May and January respectively.

NORTH WEST PROVINCE – Microbiological analysis of water from an irrigation channel (fed by water from the Crocodile River and Hartbeespoort Dam) used for overhead irrigation of lettuce

In this study the faecal coliform counts ranged between 2.8 log CFU/mℓ and 4.1 log CFU/mℓ and often exceeded the upper limit (≤ log 3 CFU/100 mℓ) suggested for total faecal coliform counts in irrigation water used for irrigation of vegetables to be eaten uncooked (DWAF, 1996). Intestinal enterococci counts ranged from zero to 1.9 log CFU/mℓ. *Listeria* spp. was not detected in irrigation water samples. *Salmonella* spp. was isolated from the irrigation water and could pose a health risk when used to irrigate lettuce to be eaten raw.

NORTH WEST PROVINCE – Microbiological quality of water used to irrigate basil from a farm near Brits

Since the *E. coli* counts of the irrigation water was zero it is suitable for irrigation of any crops according to the DWAF guidelines (1996). *E. coli* O157: H7 and *Salmonella* spp. were not detected in any of the irrigation water sources. The dominant coliforms present in the water were *Klebsiella pneumoniae* spp. *pneumoniae* and *Enterobacter aerogenes*. The water temperature was between 18°C and 22 °C. Average pH of irrigation water sources was pH7.

WESTERN CAPE PROVINCE – Microbiological quality of water used to irrigate pears from four commercial farms

The *E. coli* counts in the irrigation water from water storage dams and micro irrigation pipes in the orchards from the four commercial farms ranged from zero to 2.3 log CFU/100 mℓ and were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). *E. coli* O157 (including H7) was not detected in any of the water samples. However, *S.* Typhimurium and *L. monocytogenes* were detected in 3.5% of the samples. The pH of the irrigation water samples were within the guidelines set by DWAF's Target Water Quality Range (TWQR) of pH 6-pH 9 (DWAF, 1996). The temperatures of the dam water from the four farms ranged between 9.3°C and 30.8°C. The turbidity of two of the dams ranged between 15 and 20 Turbidity Units. The other two dams were less turbid with

values < 5-7.5 T.U. Drench water was sampled twice during field trips: pH 7.4 and pH 8.4 respectively. Turbidity was 7 T.U. and 20 T.U. respectively.

The *E. coli* isolates from the water samples from the four commercial pear farms were mostly sensitive to the antibiotics (14 in total) tested. However, resistance to single antibiotics were detected for some *E. coli* isolates and included either Aztreonam (ATM), Ceftazimide (CAZ), which both belong to the Penicillin class of antibiotics or Chloramphenicol (C), which belong to the Phenicol antibiotic class. Only one isolate was resistant to two antibiotics, which included ATM and CAZ.

None of the virulence genes (*stx1, stx2 and eae*) were detected in any of the *E. coli* isolates (18 in total) from the irrigation water on the four commercial pear farms in the single pear *E. coli* isolate. Cluster analysis of ERIC-PCR DNA fingerprints showed that two *E. coli* isolates from the dam water and two isolates from the micro-irrigation pipes in the orchard on one of the farms formed a distinct cluster at an 80% similarity value. These results confirmed that the water from the storage dam was the source of the strain isolated from the micro-irrigation pipes.

Molecular characterization of hepatitis A virus strains from water sources in South Africa

Hepatitis A virus (HAV) strains found in selected South African (SA) surface water were characterised to establish which HAV types are circulating in the environment, thus reflecting prevalence in the surrounding communities. Surface water samples used for irrigation or domestic purposes, and water samples from outflow of wastewater plants were collected from six provinces. Hepatitis A virus strains were genotyped by nucleotide sequence analysis of the capsid gene VP1 and the VP1/P2B junction. Hepatitis A viruses were detected in 76% (16/21) of the surface water samples and in 37% (19/51) of the samples from the wastewater plants. Strains were characterised from 32 of the 35 samples and classified within genotype IB. The presence of genotype IB in the water sources confirms human faecal contamination. Hence these faecally-contaminated water sources may be a potential transmission route of HAV infection and potential source of contamination of irrigated fresh produce in SA.

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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

One of the aims of this project was to evaluate the extent and types of contamination found in irrigation, processing and agricultural spray water. Microbial analysis demonstrated that the levels of *Escherichia coli* in the irrigation water (five rivers, ten dams) at the fourteen study sites exceeded the DWAF (1996) irrigation water guidelines of ≤1000 CFU/100 mℓ for crops that are eaten raw. However, irrigation canal water used at two of the sites tested complied with the DWAF (1996) guidelines for irrigation water for crops to be eaten raw. Although the *E. coli* counts of the primary irrigation sources (rivers and dams) were unacceptably high, a reduction in numbers was observed for dam water and for water collected from the micro-irrigation pipes, drip-irrigation and overhead irrigation pivots.

Salmonella was isolated from up to 22% of irrigation water samples tested from a selected site. However, no *E. coli* O157:H7 or *Listeria* spp. were detected in these samples (Du Plessis *et al*., 2015). The presence of *E. coli*, hepatitis A virus (HAV), sapovirus (SaV) and norovirus (NoV) suggested that the contamination was most likely of human origin, possibly from the upstream sewage works. These pathogens pose a potential health risk to the end-user of such fresh produce.

The mean turbidity levels in irrigation water from a site in KwaZulu-Natal often exceeded the international turbidity standard of 0-1 NTU's for water (DWAF, 1996). The high turbidity levels of the irrigation water could be the result of soil erosion and run-off. In this study it was found that there was a positive correlation between the biological oxygen demand (BOD), temperature and pH, and the turbidity levels in the water. The values for total dissolved solids (TDS) recorded were within the recommended range of 1000 mg/l for drinking water and 40 mg/ml for irrigation water as specified by the World Health Organisation (WHO, 1996). There was a positive correlation between the TDS and the chemical oxygen demand (COD) levels in the irrigation water from two farms in KwaZulu-Natal. It was concluded that the *E. coli* counts could have been influenced by TDS and COD in irrigation water from one of the farms.

The BOD levels of the irrigation water sources from selected farms were lower than the COD levels which can be expected since the former only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels. The COD and TDS values were the only two parameters of water that influenced the survival of total heterotrophic bacteria (THB), *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. There was a significant positive correlation between the COD levels, the THB, *E. coli* and *Salmonella* counts in the water samples. It is important to note that South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by the World Health Organization (WHO, 1996) of 10 mg/l.

An additional aim of the study was to determine the impact of environmental parameters on the growth kinetics of microbial contaminants in irrigation water and on fresh produce. Environmental conditions such as exposure to high temperatures and UV as well as minimal

processing (chlorine washing, hydrogen peroxide treatment, cooking, blanching, microwaving and freezing) steps contributed to a reduction in microorganisms, including human pathogenic bacteria tested for. The effectivity of minimal processing steps to reduce microbial surface contamination was determined to be crop dependant due to specific characteristics, i.e. smooth versus hairy and/or rough surfaces. Surface inoculation studies confirmed the ability of pathogens to attach, survive, internalise, increase and spread in plant tissue. Once internalised the pathogens are protected against the effect of minimal processing steps employed to promote food safety in the supply chain. Agricultural chemicals can be used in the supply chain to reduce foodborne-pathogen-associated hazards, however some of the chemicals can support pathogen growth.

The effect of irrigation methods (drip, sprinkler and flood) on the surface contamination of fresh produce was found to be crop dependant. The findings of this study demonstrated that irrigation methods that minimize direct contact of the contaminated water with the edible part of the plant lowered the risk of contamination to the fresh produce.

Results obtained during the five-year investigation confirmed previous findings that water sources are seriously compromised and has the potential to negatively affect the health and well-being of the country's people. Some of the most important reasons for the contamination include the presence of informal settlements with inadequate sanitation and lack of access to potable water, inadequate municipal waste water management services, increased pressure on waterworks due to lack of capacity, accelerated urbanisation rate and increased population. Continued regular monitoring of irrigation water sources must be implemented with a view to developing a database of information and an early warning system. Currently, a national water monitoring system exists using accredited laboratories. However, this information is not centrally managed, and neither are forecasts made nor early warning systems deployed.

Revision of current microbiological guidelines for irrigation water and fresh produce based on scientific data (including actual natural microbial levels and pathogen presence/absence) is needed. The World Health Organization (WHO) recommended that the faecal coliform limit be relaxed to 1000 CFU/100 mℓ for irrigating agricultural crops and for vegetables eaten raw (WHO, 1989) which is a more realistic count taking results of the current study into consideration.

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