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Water quality of citrus dip tanks and the impact of *Salmonella* species in citrus export chain.

by

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Declaration

I declare that the thesis herby submitted to the University of Pretoria for the degree Magister Microbiology, has not been previously submitted by me for a degree at any other University.

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Contents

Summary

Water quality of citrus dip tanks and the impact of *Salmonella* species in citrus export chain.

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Citrus is grown in almost all countries with a sub-tropical or tropical climate. Africa produces about 7% of global citrus production representing 67 362 564 tonnes in the 2003-04 season. Of these countries, South Africa is the most important citrus producer with the bulk of its fruit being exported to mainly European countries. Although South Africa is the world's fourteenth biggest producer, it is currently ranked third in terms of global export volumes. An increase in the number of foodborne disease outbreaks over the past few decades has been related to the shift towards consumption of fresh produce and growing world trade. Other factors include changing demographic profiles, changing farm practises, extensive distribution and handling networks, increased consumption of unprocessed products, emergence of new pathogens and more effective detection methods. *Shigella* spp., *Listeria monocytogenes*, Hepatitis virus, Caliciviridae virus, *Salmonella* spp., *Escherichia coli* and *Vibrio cholera* are some of the microorganisms implicated in disease outbreaks caused by the consumption of fresh produce. This dissertation focuses on citrus fruit safety and a selected foodborne pathogen, *Salmonella enterica* serovar Typhimurium. *Salmonella* has been implicated in several outbreaks associated with the consumption of unpasteurized orange juice, mangoes, cantaloupes, sprouts and tomatoes. *S. enterica* sv. Typhimurium is also a water-borne pathogen, and its importance in agricultural irrigation and packhouse wash water was also studied. The presence of *S. enterica* sv. Typhimurium in biofilms in packhouse water and on fruit was finally investigated since biofilms are known to harbour a number of these pathogens. Biofilm formation was monitored in the warm water dip tank using a photo acoustic monitoring device and confirmed by scanning

electron microscopy and enumeration. Water sources, i.e. bathroom, handwash stations, irrigation water and warm water baths had high total viable bacterial counts and faecal-associated contamination. Biofilms also formed within the packhouse dip tank at an increased rate. The Polymerase Chain Reaction (PCR) was used to investigate the presence of *Salmonella* in the citrus export chain. No *Salmonella* spp. could be detected. *S. enterica* sv. Typhimurium survived for up to four weeks on the surface of citrus fruit under simulated export conditions. However, *Salmonella* is not able to survive uninterrupted cold storage conditions for a period longer than two or three weeks. Scanning Electron Micrographs showed that *Salmonella* is capable of attaching to and colonizing the citrus fruit surface and is able to be incorporated into existing biofilms in warm water tanks of packhouses. This study showed that *Salmonella* can thrive in packhouse water and potentially be a continuous source of contamination for fruit moving through the infected warm water dip tank. Everyone participating in fruit production, storage and post-harvest handling of fresh fruit should be involved in ensuring quality and safety, since events in the orchard can affect storage life, and incorrect handling during marketing may nullify all previous efforts of quality maintenance. To minimize the occurrence of these pathogens in the fruit chain from production to consumption, several basic good agricultural practices and food safety systems needs to be incorporated and managed correctly.

Chapter 1: General Introduction

1.1. Introduction

Citrus (*Citrus sinensis*) species originated in China (Hume, 1926) as early as 1 B.C. Today it is grown in most countries in the world with a tropical or subtropical climate. Two varieties of sweet orange, the Washington Navel and Valencia make up 70% of fruit produced in Commonwealth countries (Bowman, 1956). About 67 362 564 tonnes of oranges are produced annually of which 7% is produced in Africa (PPECB, 2004 &2006). During 2002 and 2003, 50 million cartons were exported from South Africa to other countries, while in 2004 and 2005 closer to 55 million cartons were exported (PPECB, 2004 & 2006). To ensure best quality fruit for export, careful handling is required to prevent damage or bruising(Bowman, 1956). During the harvest season fruit is picked from the trees and transported to the packhouse in field boxes. When the fruit arrives at the packhouse it undergoes a series of minimal processing steps before being packed, cooled to 7 ºC and exported by sea to mainly European countries.

Over the past few decades an increase in reported foodborne disease outbreaks, which has been linked to the increased consumption of fresh produce have been documented (www.who.int, 2004). Millions of people are annually affected by these diseases resulting in the significant loss of manpower and severely impacting on the economy and general trust in the supply chain. Data from the Centre for Disease Control for the period between 1973 and 1992 suggest at least a doubling in the annual number of reported diseases associated with the consumption of fresh produce with an estimated 76 million cases (De Roever, 1998; Brackett, 1999). According to the World Health Organisation the increase in foodborne outbreaks can be contributed to a number of factors. These include changing demographic profiles, changing farm practises, extensive distribution and handling networks, increased consumption of unprocessed products, emergence of new pathogens and more effective detection methods (De Roever, 1998; Brackett, 1999). Another possible reason for the increased consumption of fresh produce could be that research has

shown that a diet low in fat and fibre can be protective against cancer and heart disease (De Roever, 1998; Meng & Doyle, 2002).

Shigella spp., *Listeria monocytogenes*, Hepatitis virus, Caliciviridae virus, *Salmonella* spp., *Escherichia coli* and *Vibrio cholera* are just some of the microorganisms implicated in disease outbreaks caused by the consumption of fresh produce (De Roever, 1998). *Salmonella* spp. and *E. coli* are two of the organisms most frequently implicated in foodborne diseases caused by the consumption of fresh produce (De Roever, 1998). *Salmonella* cells can be transmitted via contaminated food or water and have been isolated from foodstuffs like orange juice, tomatoes, cantaloupes, sprouts, watermelon and mangos (De Roever, 1998). The most commonly used detection methods include viable plate counts or most probable number techniques. These techniques can be used to quantify active cells in environmental samples. Other detection methods include enzyme linked immunosorbent assays (Towner & Cockayne, 1993; Kroll *et al*., 1993; Huang *et al*., 1999), hybridization (Olsen *et al*., 1995), polymerase chain reaction (Bhagwat, 2004), and labelling with green fluorescent protein (Burke, 2005).

Each habitat in which microorganisms can be found has certain physical, chemical and biological parameters determining which microbial population will thrive in this environment (Atlas & Bartha, 1998). Most aquatic organisms can grow at very low nutrient levels and are motile (Atlas & Bartha, 1998). Because water can be a potential source of contaminating fruit, it is important to ensure the safety and quality of the water used in terms of fruit, whether it is irrigation or wash water (Brackett, 1999).

Biofilms are microbial colonies or aggregates that can be found on almost any surface with sufficient nutrients and water (Wimpenny, 2000). Biofilms are normally composed of a number of different species (Walker & Marsh, 2004). Life inside a biofilm offers many advantages (Schmid *et al*, 2004). Biofilm formation is a linear process (Hall-Stoodley & Stoodley, 2002) split into four stages (Busscher & van der Mei, 2000), transport to the surface, reversible attachment, colonisation and irreversible attachment. According to Wimpenny (2000), biofilm formation is a combination of intrinsic (genetic) and extrinsic

(environmental) factors. It is in the irreversible stage that the extracellular polymer substrates (EPS) are formed. The EPS are important in the survival of microorganisms in a biofilm in natural environments (Anwar & Costerton, 1992). According to a study by Rayner *et al.* (2004), biofilms are present on everyday household products such as tomatoes, carrots, cutting boards, kitchen sponges and dishcloths. Because pathogens like *Salmonella* can be sequestered in these biofilms, it is important to control biofilms. If a biofilm includes human pathogens and it is not removed or killed, it can provide a continuous source of contamination.

The most popular parameters to monitor biofilm formation include: light density, heat transport, electric conductivity, torque and pressure drop (Lewandowski & Beyenal, 2003). Bacterial biofilms can be cultivated using either a batch or continuous culturing method. The batch culturing method is convenient as it involves suspending a sterile surface, such as a glass slide, into an inoculated medium. After incubation, the slide can be investigated under a microscope or the cells can be removed by sonication, cultured and counted. Flow cells are often used to culture biofilms using the continuous culturing method. Here fresh medium is pumped through the system and waste leaves the system. This system mimics the natural conditions in the environment.

The purpose of this study was to monitor the biofilm formation in citrus packhouse dip tanks, and determine the level of contamination of water used in the orchard or packhouse and whether this can represent a possible source of contamination. The study also focuses on monitoring the occurrence, importance and survival of *Salmonella* spp. in biofilms and on citrus fruit while in the export chain.

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Chapter 2: Literature review

2.1. Citrus

The suggested origin of true citrus fruit is south-east Asia, including south China, north eastern India and Burma (Spiegel-Roy & Goldsmith, 1996). Although many authors claimed the origin of citrus fruit to be the aforementioned regions, it was in China where the fruit was first cultivated and several species probably started (Spiegel-Roy & Goldsmith, 1996). True citrus trees belong to the family Rutaceae, subfamily Aurantioideae (Spiegel-Roy & Goldsmith, 1996). This subfamily can further be divided into tribes and subtribes. The genus *Citrus* falls under the tribe Citreae and subtribe Citrinae (Spiegel-Roy & Goldsmith, 1996).

There are six genera of true citrus trees that are of commercial importance (Saunt, 1990). These include sweet oranges, mandarins, grapefruit, pummelo, lemon, sour lime, citron and sour orange (Saunt, 1990). Sweet oranges can further be divided into four groups, namely, navel oranges, common oranges, pigmented oranges and acidless sugar oranges. Two varieties of sweet orange (*Citrus sinensis*), i.e. Washington Navel and Valencia, make up about 70% of total global citrus production (Bowman, 1956). The Washington Navel is a standard early or winter orange while Valencia is the best-known variety (Bowman, 1956).

The evergreen citrus tree grows and produces fruit under varied tropical or subtropical climatic conditions, ranging in latitude from over 40° north to almost 40° south (Spiegel-Roy & Goldsmith, 1996). Citrus is a worldwide industry, with Brazil being the largest producer of oranges (19.7%), followed by the USA (13.4%), China, Spain, Mexico, Italy, India and Egypt (Siegel-Roy & Goldsmith, 1996). An average 67 362 564 tonnes of oranges are produced annually of which Africa produces only 7% (4 645 030 tonnes) (PPECB, 2004). In 2003, 50 million cartons have been exported from South Africa to mainly European countries while in 2005 it increased to 55 million (PPECB, 2004; 2006).

Citrus is the second biggest agricultural industry in South Africa after deciduous fruit and is mostly produced near river valleys to ensure adequate water for irrigation (Barry, 1996). The variety in climatic conditions within South Africa allows for a broader range of

cultivars that can be planted. However, the quality of cultivars planted may vary between production regions and reflect different cultivation practices (Barry, 1996).

Harvesting of citrus fruit is manually done in such a way as to avoid injury and postharvest infections (Mukhopadhyay, 2004). Harvested fruit is transported to packhouses in trucks or trolleys where it is usually packed within a day. Fruit entering the packhouse is first washed in a receive tank with disinfectant sprays. Chlorine is also usually added to the receive tank, but is mostly ineffectively managed. As fruit moves through the packhouse and is graded and sorted, it moves through a hot water tank (45 - 50 ºC) with added fungicides.

2.2. Food safety

Foodborne illnesses affect millions of people worldwide each year. According to the World Health Organisation (WHO), foodborne illnesses caused by bacteria and other microorganisms are a growing concern (www.who.int/foodsafety/micro/general/en.html, 2004). Most countries with surveillance systems have reported an increase in the incidence of foodborne disease outbreaks over the past few decades. These diseases are mainly caused by *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, and *Jejuni* spp. In 1998, 1.8 million children died of diseases caused by microbiologically contaminated food or water (www.who.int/foodsafety/micro/general/en.html, 2004). In the United States an estimated 76 million cases of foodborne illnesses occur annually (www.who.int/foodsafety/micro/general/en.html, 2004). The same can be said for Australia and the United Kingdom and the overall cost of these illnesses annually is billions of dollars.

Data from the Centre for Disease Control (CDC) for the period between 1973 and 1992 suggested a doubling in the annual number of disease outbreaks associated with fresh produce (De Roever, 1998; Brackett, 1999). According to the WHO, the increased incidence of foodborne diseases can be contributed to multiple factors. Some of these include changing demographic profiles, changes in farming practises, extensive food distribution systems, agronomic processing, preservation, packaging, distribution and

marketing technologies worldwide and the increased consumption of meat, poultry and unprocessed food products (Beuchat, 2002). Also the emergence of new pathogens and more effective detection methods of pathogens not previously associated with food, contributed to the reported increase (De Roever, 1998; Brackett, 1999). In addition, campaigns by governments to encourage healthy eating habits and increased intake of fresh produce have contributed to global changes in eating habits.

Global distribution and marketing technologies allow fresh produce to be available year round (Beuchat, 2002). However new technologies such as the use of manure instead of chemical fertilizers, as well as untreated sewage or irrigation water allows for the increased risk of human illnesses associated with fresh produce (Beuchat, 2002). Many of these factors thought to contribute to the epidemiological development of foodborne pathogens are directly or indirectly linked to environmental conditions affecting the safety of fresh produce (Beuchat, 2002). A variety of factors can affect the ecological behaviour of microorganisms , particular foodborne pathogens, accidentally introduced onto fresh produce resulting in increased risk of illnesses and disease outbreaks (Beuchat, 2002).

Virtually every type of fresh food product has the potential to become contaminated with foodborne pathogens during production, handling or distribution (Brackett, 1999). However, the mere presence of pathogens does not constitute an outbreak of disease (Brackett, 1999). Contamination of fresh produce can occur at any point in the fresh produce chain and represent the following: preharvest contamination may be due to manure used as fertilizer, faecal contamination from animals and employees, contaminated irrigation water, and unhygienic human handling (De Roever, 1998; Brackett, 1999). At the postharvest level, contamination may occur due to soiled wash water, human handling, equipment or unhygienic transportation vehicles, cross- contamination between fruit and improper storage conditions (De Roever, 1998). It is, therefore, both the responsibility of the producer and role players along the supply chain to ensure that they do everything possible to maintain the safety of the product (Brackett, 1999).

Many questions are asked as to how foodborne pathogens are transferred from their potential reservoirs to fresh fruits and vegetables. It is, however, important to keep in mind that each fruit and vegetable has a unique composition and physical characteristic and these characteristics must be taken into consideration when potential microbiological hazards and controls are being considered (De Roever, 1998).

2.3. Microorganisms associated with fresh produce

The microflora of fresh fruits and vegetables are diverse but predominantly Gram- negative (De Roever, 1998; Brackett, 1999). Microbial contamination of fresh produce is largely associated with the contamination of the surfaces of the fresh produce, while the inner surfaces are considered free of human pathogens. It is for this reason that leafy vegetables, which have a greater surface area, also have a higher probability to harbour more bacteria. It has been proved by Samish and Etinger-Tulczynska that if bacteria are applied to the surface of fruits they will penetrate over time (De Roever, 1998).

Anything in the production environment that comes into contact with the plant has the potential to contaminate the product. This includes irrigation water, animals, farm implements, workers and the soil (De Roever, 1998). During handling and distribution, fresh produce can also become contaminated due to unhygienic handling or poor facility hygiene and contaminated wash water. All fruits and vegetables receive some degree of minimal processing before commercial distribution in the form of washing, grading, waxing and packing.

The list of microorganisms responsible for foodborne illnesses is continuously growing (McCabe-Sellers & Beattie, 2004). Microorganisms of public health concern can survive for long periods of time on the surfaces of fruits or vegetables and in some cases even increase in numbers (De Roever, 1998). Outbreaks of several *E. coli* serovars have been linked to drinking water, apple cider and juice, leaf lettuce, iceberg lettuce, radish sprouts, alfalfa sprouts and carrots (De Roever, 1998). In the case of apple cider and juice it was found that because 90% of apples are picked from the ground, contamination might have

occurred before or during harvesting (De Roever, 1998). Table 2.1 gives a summary of some of the organisms implicated in foodborne diseases caused by consuming fresh produce or fresh produce products of the tested pathogens. *Salmonella* has been the most commonly reported.

Salmonella is an endemic public health concern worldwide (Mead *et al.,* 1999). It is a primary agent in gastroenteritis in both humans and animals. In the United States of America, *Salmonella* causes about 1.4 million cases of salmonellosis among humans annually (Mead *et al.,* 1999) and is usually transmitted by food (D'Aoust *et al*., 1997) or water (Harrey *et al*., 1967). Although the eradication of *Salmonella* is not possible, the provision of safe water, food, and waste treatment is necessary to reduce the outbreaks of *Salmonella* infections (National Academy of Sciences, 1969).

2.3.1. *Salmonella* species

2.3.1.1. The genus

The genus *Salmonella* is a group of highly adaptive Gram-negative bacilli belonging to the Family Enterobacteriacea, containing a number of closely related serotypes (Yan *et al.,* 2003). Many of these serotypes are potential pathogens to humans and animals. *Salmonella,* like most Enterobacteriacea, are motile, non-spore forming, facultative anaerobes, nitrate reducing, glucose fermenting and oxidase negative organisms. Most

Table 2.1. A summary of pathogens causing outbreaks associated with fresh produce and fresh produce products (adapted from De Roever, 1999).

Pathogen	Fresh Produce or Product	Reference
Shigella spp.	Lettuce	Kapperud *et al.,* 1995; CDC, 2003b
	Parsley	CDC 1999a; Naimi *et al.,* 2003
	Honeydew melon	CDC, 2003b
Salmonella spp.	Sliced Tomatoes	CDC 1993; Cummings *et al.,* 2001; CDC 2002a; CDC, 2003b; CDC, 2004; CDC 2005
	Sprouts	Mahon *et al.,* 1997; CDC 1997; CDC 2001; Van Duynhoven *et al.,* 2002; CDC, 2003b; CDC, 2004
	Sliced Watermelon	CDC 1979; Blostein 1993
	Honeydew Melon	CDC, 2003b
	Sliced Cantaloupe	CDC 1991b; CDC 2002b; CDC, 2003b; CDC, 2005b
	Unpasteurized orange juice	Parish 1997; Bates 1999; CDC 1999b; CDC, 2005b
	Various salads	CDC, 2003b; CDC, 2004; CDC, 2005b
	Mangos	CDC, 2003b; Penteado *et al.,* 2004
	Onions	CDC, 2005b
	Lettuce varieties	CDC, 2003b; CDC, 2004; CDC, 2005b
	Carrots	CDC, 2005b
	Strawberries	CDC, 2003b
	Sweet potato	CDC, 2005b
	Beans	CDC, 2004
Escherichia coli	Unpasteurized apple juice	CDC 1996; CDC 1997; CDC, 2004

Table 2.1. Continued

	Sprouts	Breuer *et al.,* 2001
	Lettuce varieties	CDC 1995, CDC, 2003b; CDC, 2005b
	Parsley	Naimi *et al.,* 2003; CDC, 2005b
	Various salads	CDC, 2003b; CDC, 2005b
	Spinach	CDC, 2003b; CDC 2006a
	Cabbage	CDC, 2003b
	Coleslaw	CDC, 2003b
	Alfalfa sprouts	CDC 1997; CDC,2004
	Carrots	CDC 1993
	Cucumber	CDC, 2004
Vibrio cholerae	Coconut milk	CDC 1991a
Hepatitis A virus	Lettuce	Rosenblum *et al.,* 1990
	Frozen Strawberries	CDC 1997
	Green onions	CDC 2003
	Sliced Tomatoes	CDC, 2005b
	Cabbage	CDC, 2003b
Cyclospora cayetanensis	Raspberries	CDC 1996; CDC 1997
	Mesculn lettuce	CDC 1997
	Basil/Basil containing products	CDC 1997; CDC, 2005b
Cryptosporidium spp.	Apple cider	CDC 1997; CDC, 2003b; CDC, 2004; Blackburn *et al.,* 2006
	Beans	CDC, 2005b
Campylobacter jejuni	Green peas	CDC, 2005b
	Various salads	CDC,2004; CDC, 2005b
	Baked beans	CDC, 2005b

Table 2.1. Continued

	Onions	CDC, 2005b
	Peppers	CDC, 2005b

	Tomatoes	CDC,2004;
Norovirus	Various salads	CDC, 2003b; CDC, 2004; CDC, 2005b
	Banana	CDC, 2003b; CDC, 2005b
	Tomato	CDC, 2004; CDC, 2005b
	Strawberries	CDC, 2005b
	Grapes	CDC, 2005b
	Melons	CDC, 2004; CDC, 2005b
	Broccoli	CDC, 2003b; CDC, 2005b
	Spinach	CDC, 2005b
	Squash	CDC, 2005b
	Lettuce varieties	CDC, 2004; CDC, 2005b
	Onions	CDC, 2005b
	Carrots	CDC, 2003b
	Cantaloupe	CDC, 2003b
	Pineapple	CDC, 2003b
	Cucumber	CDC, 2003b
	Beets	CDC, 2004

Table 2.1. Continued

	Green beans	CDC, 2004
	Coleslaw	CDC, 2004

Salmonella infections are of zoonosis origin but *S. enterica* sv. Typhi and *S.* enterica sv. Paratyphi only colonize humans (Yan *et al.,* 2003). The genus *Salmonella* is currently composed of over 2 500 different serotypes. Identification of *Salmonella* at the genus level is not very difficult because *Salmonella* members share certain common biochemical reaction profiles and have a high level of genetic similarity (Yan *et al.,* 2003).

2.3.1.2. The Species

Historically *Salmonella* classification was based on epidemiology, host range, clinical manifestation, biochemical reactions and surface antigen patterns (Yan *et al.,* 2003). Because of close genetic relatedness within a single species, *Salmonella choleraesuis*, was proposed (Crosa *et al.,* 1973; Yan *et al.,* 2003). In 1999 it was proposed to replace *S. choleraesuis* by *S. enterica*, but has not yet been accepted (Euzeby, 1999). *Salmonella bongori* and *S. enterica* are the two species currently recognised in the genus (Yan *et al.,* 2003). Each of these species has many serovars, e.g. *S. enterica* sv. Thyphimurium.

2.3.1.3. Epidemiology

According to Mead *et al.* (1999), there are an estimated 1.5 million cases of nontyphoidal *Salmonella* infections annually. The most common symptoms are gastroenteritis with nausea, vomiting, and diarrhoea without a fever. A small percentage of these patients develop extra gastrointestinal infections. Invasive *Salmonella* infections can be caused by many *Salmonella* serovars. *Salmonella* is capable of invading phagocytic cells of the intestines and cause gastroenteritis by multiplying in these cells (Yan *et al.,* 2003). *Salmonella* has the capability to escape host defence systems by activating certain pathways (Yan *et al.,* 2003). This trait is thought to have been acquired by horizontal gene transfer and its integration into the chromosome (Yan *et al.,* 2003).

Treatment with appropriate antimicrobial agents is important in cases of severe infections (Yan *et al.,* 2003). However, the emergence of antimicrobial-resistant *Salmonella* strains has resulted in treatment failure (Yan *et al.,* 2003). Surveillance showed an increase in

Salmonella resistance to at least one antimicrobial drug, i.e. fluorquinolone, ampicillin, trimethoprim, sulphonamides, streptomycin and nalidixic acid (Butaye *et al.,* 2006). Resistance is acquired through mutations or the acquisition of resistance genes. Although there are many serovars of *Salmonella*, in this study only the two serovars most commonly associated with disease outbreaks will be discussed, *S. enterica* sv. Enteritidis and *S. enterica* sv. Typhimurium.

Since 1963 there was an increase in the number of diseases caused by *S. enterica* sv. Enteritidis associated with poultry products (Rabsch *et al.,* 2001). During the 1980s, a dramatic increase in *S. enterica* sv. Enteritidis infections was seen. This made it the most common isolated serovar (Rabsch *et al.,* 2001). During 1993, it became the most isolated serovar in England, Wales, and Germany, other parts of Europe, North and South America (Meng & Doyle, 1998; Rabsch *et al.,* 2001). According to Leanderas *et al.* (1998), the increased consumption of fast foods and the international food trade also played an important role in spreading *S. enterica* sv. Enteritidis (Said, 2005). *Salmonella* can also spread throughout the environment through faecal contamination of water sources by humans and animals (Okafo *et al.,* 2003).

Rodents are an important vector or reservoir of *S. enterica* sv. Enteritidis (Rabsch *et al.,* 2001) and can introduce the pathogen into poultry farms. It is believed that *S. enterica* sv. Enteritidis was introduced to rodents through the use of rodenticides in 1985 during an outbreak of *Yersinia pestis* (Rabsch *et al.,* 2001). *S. enterica* sv. Enteritidis was used to control the rodent population in San Francisco (Rabsch *et al.,* 2001).

According to Rabsch *et al.* (2001), there are two major evolutionary lineages of *S. enterica* sv. Enteritidis. This is based on the phage types (PT); the one lineage contains PT4 and PT1 while the other lineage contains PT8 and PT13a. In the current epidemic in Europe, the bulk of human isolates belong to PT4 (Rabsch *et al.,* 2001). It is, therefore, suggested that PT4 may have acquired the ability to enter and persist in the poultry population, and that introductions of breeding lines through international trade in the 1980s may have lead to the epidemic spread (Rabsch *et al.,* 2001).

In the United Sates of America PT8, 13a and 13 are the most commonly associated with humans (Rychlik *et al.,* 2000). PT8 is most commonly found in Canada, while PT4 is most frequently isolated in Germany (Rabsch *et al.,* 2001). According to Liebana *et al.* (2000) cases of salmonellosis caused by *S. enterica* sv. Enteritidis have started to decrease in the UK and many other countries.

According to Stephen *et al.* (2003), there was a high incidence of multiresistant *S. enterica* sv. Typhimurium in the UK, Europe and North America during the 1990s (Said, 2005). The phage type was identified using two characteristics, i.e. resistance to multiple antimicrobial drugs (ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline) and the definitive phage type (DT) 104 (Duijkeren *et al.,* 2002). *S. enterica* sv. Typhimurium first emerged in 1984 in cattle in England and Wales then spread very rapidly through Europe, Asia and North America (Stephen *et al.,* 2003 as cited in Said, 2005). The incidence of DT104 continues to increase and spread through parts of Europe (Meng & Doyle, 1998; Rabsch *et al.,* 2001).

During investigations of the 46 outbreaks of DT104 in the UK from 1992 to 1996, the studies showed that 78% of these outbreaks were linked to foodborne transmissions (Said, 2005). Contact with infected animals and pets may also have been a source of transmission (Meng & Doyle, 1998). Gastroenteritis cuased by *Salmonella* is contracted by travellers in developing countries and then brought or taken back to the travellers' home country. These *Salmonella* infections are more likely to be multi drug resistant than domestically acquired infections (Said, 2005). It is suggested that the rapid distribution of DT104 is more consistent with human travel than with movement of domesticated animals (Said, 2005).

2.3.1.4. *Salmonella* in food safety

Salmonella spp. have been implicated in outbreaks of foodborne illnesses originating form a wide variety of different food products. In 1955, 1979 and 1991 outbreaks of *Salmonella* infections were traced back to pre-cut watermelons (De Roever, 1998). After *Salmonella* was isolated from melon skins, the Food and Drug Administration (FDA) recommended that melons be washed before cutting and then kept at temperatures lower than 7 °C. The retail display of the cut watermelons must also be limited to no more than 4 hours.

In 1990 and 1993 the outbreak of *Salmonella* was linked to the consumption of fresh tomatoes (De Roever, 1998). Studies following these outbreaks showed that *Salmonella* spp. can grow and survive on mature intact tomatoes held at ambient temperatures. It also showed that growth is rapid on ripe tomatoes kept at ambient temperature (De Roever, 1998). Although the contaminated tomatoes were washed before eating, people were infected with the pathogen. In these studies it has been shown that *Salmonella* spp. can be introduced into the tomato during slicing and will subsequently increase in numbers.

According to Penteado *et al.* (2004), *Salmonella* spp. can even internalize into fresh produce, in particular fresh mangoes. During an outbreak in December 1999, they found that water used in the packhouse to wash the fruit was contaminated with an environmental source of *Salmonella*. Because the water was not managed correctly, the *Salmonella* survived and internalized into the mangoes after hot water (46.1 ºC) and then cold water (22 ºC) treatment. The internalisation was triggered by the cooler environments and gases inside the fruit contracts and subsequently draws bacteria into the fruit.

In 1995, a *Salmonella* outbreak was linked to drinking unpasteurized orange juice (Bates, 1999; CDC 1999b). Pao and Davis (2001) demonstrated that surface contaminants of fruit could be carried over to the juice during the juicing process. In this particular case (1995), the specific *Salmonella* sp. was also isolated from a toad. Amphibians were found in the proximity of the processing building. The building was also open to the environment. Because the bacteria could be isolated from the patients, product and processing environment, the cause of the outbreak could be determined. It was also established that the facility lacked efficient cleaning and sanitizing programmes.

2.3.1.5. Detection methods

2.3.1.5.1. Most Probable Number (MPN)

Viable plate counts or most probable number (MPN) techniques are frequently used to quantify active cells in environmental samples. This method allows for an estimate of the original number of *Salmonella* cells present in a sample. Because *Salmonella* cells can be damaged or stressed during processing or environmental factors, non-selective and selective enrichment is necessary. After the pre enrichment or non-selective enrichment, using buffered peptone water (BPW), two other selective enrichment processes takes place. The first employs a selective broth, Rappaport. The second employs a selective medium, normally Xylose Lysine Deoxicolate agar (XLD) (Oragui *et al*., 1993). After incubation, colonies can be examined and further characterised with biochemical tests.

2.3.1.5.2. Enzyme Linked Immunosorbent Assay (ELISA)

Another detection method is the Enzyme Linked Immunosorbent Assay or ELISA. This method employs labelled antibodies that will then conjugate to the appropriate antigen and thus allow detection (Towner & Cockayne, 1993). There are several ELISA techniques that can be used, i.e. direct, indirect or sandwich ELISA. In most cases, polyclonal antibodies are used (Kroll *et al.,* 1993). ELISA methods, however, turned out to be less efficient with low numbers of *Salmonella* present ($<10^5$ cells/ml) (Blackburn, 1993).

2.3.1.5.3. Hybridization

Hybridization has also been widely used in the detection and identification of bacterial species (Olsen *et al.,* 1995). Probes can be labelled which in turn will make detection easier. Although a lot of research has been done on probes, sensitivity still remains a problem (Olsen *et al*., 1995). Studies indicated that hybridization requires 10^4 - 10^5 or even as high as 10^8 organisms to create a signal clearly different from the background (Olsen *et al*., 1995). Probe techniques may be combined with other methods to facilitate surveillance (Olsen *et al*., 1995).

2.3.1.5.4. Polymerase Chain Reaction (PCR)

Conventional detection methods may take up to three or four days to give a result. To overcome this delay, several alternative methods have been developed. The polymerase chain reaction (PCR) has been one of the most promising new methods used in the detection and identification of low numbers of microbes in a sample (Bhagwat, 2004). This method is sensitive to the extent that it can detect unculturable cells. PCR uses a thermo stable polymerase to exponentially amplify a specific piece of the chromosomal DNA in a three-step process. Several PCR methods have already been published, including some for the detection of *Salmonella* spp. (Aabo *et al*., 1993; Soumet *et al*., 1999a). Although most of the focus was on clinical and medical microbiology, a commercial PCR test for food safety has recently been introduced (Hines, 2000 as cited in Bhagwat, 2004). PCR generally can detect signal with 1 - 10 organisms (Olsen *et al*., 1995). A disadvantage of the PCR reactions is that all unnecessary cell debris and potential environmental inhibiters needs to be removed before the reaction can take place. Because only small samples can be analysed, the sensitivity of the PCR in food samples is low. Another disadvantage is that the method cannot discriminate between active and inactive organisms. Multiplex PCR allows the multiplication of more than one target gene by mixing multiple pairs of primers with different specificities (Zarlenga & Higgins, 2001).

Real time PCR has improved the identification of several bacterial species, since amplification and analysis of the target sequence can be done within a single apparatus by adding fluorescent probes or dyes (Wittwer *et al.,* 1997; Whitcombe *et al.,* 1999). Bhagwat (2004) developed a real time PCR that detected *Salmonella* in vegetable washing water within 12 hours.

2.3.1.5.5. Biomarkers

Biomarkers can also be a method of detection of tagged cells. These biomarkers or marker genes can be defined as a DNA sequence introduced into an organism, which confers a distinct genotype of phenotype (Jansson & de Bruijn, 1999). This allows monitoring of the organism in a given environment. Luciferase and green fluorescent protein (GFP) are both examples of biomarkers. These cultures can be differentiated from other cultures using a fluorescent microscope. It can also help distinguish between viable and nonviable cells.

GFP is a protein synthesized by the jellyfish *Aequorea victoria*. It has proved to be a very useful probe as it can be inserted into almost any genome with no obvious side effects (Wimpenny, 2000). It has been useful in studying host parasite interactions (Valdivia *et al.,* 1996). GFP has a few advantages which distinguish it from other types of fluorescent markers. It is a cytoplasmic protein with low toxicity, it can be continuously synthesized and it is easy imaged and quantitated (Chalfie *et al.,* 1994). It is a good choice in non-destructive studies of bacterial systems that require live cells.

The wild type GFP must undergo a series of self-modifications in order to become fluorescent (Tsien, 1998). Wild type GFP is usually resistant to proteolysis (Tsien, 1998), which means that once GFP is made, it will persist in a cell after the promoter is shut down (Leveau & Lindow, 2001). GFP variants, with significantly reduced maturation times, have been created (Cormack *et al*., 1996; Patterson *et al*., 1997). Bacteria tagged with GFP have been used to study the distribution and dynamics of bacterial populations in soil, aqueous systems and biofilms (Möller *et al*., 1998; Skillman *et al*., 1998; Stretton *et al*., 1998; Tresse *et al*., 1998). There are several methods to tag cells with GFP and include plasmids, allelic exchange and mini transposons.

2.4. Water

Water from the tap should be pathogen free, but not sterile (Walker & Marsh. 2004). The number of bacteria present in water is regulated by a country's own authorities and must adhere to set standards. The authorities will determine a level that is most suitable for that country and its conditions. In Europe the level of colony-forming units (cfu) is regulated by the European Union (EU) and should be less than 100 cfu/ml (Walker & Marsh. 2004). In South Africa the limits for acceptable drinking water is 1 count/100 ml for *E. coli* and 10 counts/100 ml for coliform bacteria per 1% of samples tested over a one year period (SABS, 2006). Although the bacterial numbers are low they are still present and under favourable conditions they could start multiplying and cause infections once in contact with

humans (Walker & Marsh. 2004). Because water is a potential source of contamination, it is important not to irrigate with contaminated water (Brackett, 1999).

During 2001 and 2002 a total of 31 outbreaks associated with water were reported in the USA (Kuusi *et al.,* 2004). These 31 outbreaks caused illness amongst 1 020 people and were linked to seven deaths (Kuusi *et al.,* 2004). The source of these infections is 87.5% from groundwater and 12.5% from surface water (CDC, 2006b). Of all reported water-borne diseases gastroenteritis makes up 67.7%. Of this, 28.6% is caused by bacterial species, 4.8% by viruses, 4.8% by parasites, 28.6% by chemical agents, 9.5% by mixed agents and 23.8% unidentified (CDC, 2006b). Organisms most commonly isolated include *Legionella pneumophila*, *Salmonella enterica* sv. Typhimurium, Norovirus, *E. coli* and *Campylobacter* spp. (CDC, 2006b). In 2003 and 2004 *Salmonella enterica* sv. Typhimurium was implicated in 70 cases of water-borne disease in the USA (CDC, 2006b).

2.4.1. Biofilms

2.4.1.1. What is a biofilm?

Biofilms are microbial colonies or aggregates found in almost any environment or on any surface with sufficient nutrients and water (Wimpenny, 2000). According to Walker & Marsh (2004), a biofilm is a three-dimensional functional consortia of microbial cells, bound to, and growing at an interface (e.g. solid:liquid, solid:air, liquid:air, liquid:liquid) enveloped within extracellular polysaccharides (EPS). Environmental biofilms are generally composed of a number of different species of microorganism, which develops a functional consortium (Walker & Marsh. 2004). The advantages for a colony of microorganisms to attach to a surface are that they can use both the nutrients from the water and the substratum to their benefit (Davies, 2000).

Existence inside a biofilm offers many advantages for bacteria and protozoa (Schmid *et al.,* 2004). The degradation of complexed substrates can be performed more efficiently than if the cells were in suspension (Schmid *et al.,* 2004). The EPS matrix can absorb nutrients

and can also act as a protective barrier against sanitizers, predators or changes in the physiochemical environment. (Schmid *et al.,* 2004). Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants and antimicrobials more effectively than planktonic cells (Jefferson, 2004).

Biofilms will attach to almost any surface with sufficient nutrients and water to maintain the colony; within these colonies there is a constant exchange of substrates, products, inhibitors, signal molecules etc. (Wimpenny, 2000). These colonies normally enclose themselves with an extracellular polysaccharide or slime matrix (EPS) (Wimpenny, 2000). This complex aggregate of cells and polysaccharides represent the biofilm community. Biofilm development is most rapid in flowing systems with adequate water and nutrients. The development of multicellular biofilm communities involves specific cell-cell interactions, allowing microbial populations to co-exist in environments where individually they cannot.

2.4.1.2. Biofilm formation

Biofilm formation is a linear process, which commences when free-floating cells attach to a surface (Hall-Stoodley & Stoodley, 2002). Biofilm formation can be grouped into four phases (Busscher & van der Mei, 2000). Biofilm formation was traditionally said to begin with a mass transport of microorganisms towards a surface after the adsorption of a conditioning film (Busscher & van der Mei, 2000). The first stage of attachment of biofilms to a surface is reversible; where primary colonizers colonize the surface. During this phase bacteria attach themselves to the surface with weak van der Waals forces (Wimpenny, 2000).

In the environment more than one organism can colonize a surface (Busscher & van der Mei, 2000). This is called co-adhesion. Once the primary colonizers have adhered, secondary colonizers will co-adhere, forming a multispecies biofilm. The third phase is characterised by irreversible attachment with much stronger bonds (Lawrence *et al.,* 1991). Some of these stronger bonds are achieved by the expression of EPS (Wimpenny, 2000;

Marshall, 1992). The EPS is a slime-like matrix which holds the microcolonies together (Hall-Stoodley & Stoodley, 2002). The chemistry of the EPS is complex but include molecules such as polysaccharides like glucose, galactose, mannose, fructose, rhamnose, nucleic acids and proteins (Hall-Stoodley & Stoodley, 2002). Once the cells are irreversibly attached they start to grow and produce more EPS. During biofilm formation a succession of different species will flourish, influenced by changes in the environment (Wimpenny, 2000). At a certain point pieces of the biofilm may detach and recolonize.

According to Wimpenny (2000), the formation of the detailed structure of a bacterial colony is a combination of intrinsic and extrinsic factors. Intrinsic factors are products of the genetics of the cell (Hall-Stoodley & Stoodley, 2002) and extrinsic factors include the prevailing external environment. The primary colonizers play a significant role in attaching the biofilm to the surface. If they cannot withstand the shear forces, the entire biofilm may detach from the surface (Busscher & van der Mei, 2000). During the adherence process microorganisms undergo phenotypic changes in response to the surface (Walker & Marsh. 2004). Microorganisms exhibit specific changes in response to the surface environment. These changes are morphological changes and represent altered gene expression (Lawrence *et al.,* 1991; Walker & Marsh, 2004).

Microbial attachment to a surface is a complex process and can be influenced by a number of variables such as flow rate, surface roughness, hydrophobicity and the presence and properties of conditioning films (Walker & Marsh. 2004). Once the microbes have attached themselves to a surface they start growing rapidly. The growth is logarithmic, forming micro colonies (Walker & Marsh. 2004). At this stage, as the micro colonies are dispersed over the surface they begin to grow horizontally and then into the liquid phase, where the three-dimensional, multi-species consortia biofilm begins to develop (Walker & Marsh. 2004).

The EPS are crucial for the life of the biofilm. EPS can account for 50 – 90% of the total organic carbon of the biofilm and is highly hydrated, since it can incorporate large amounts of water into its structure by hydrogen bonding (Walker & Marsh, 2004). EPS can

improve biofilm nutrition, interfere with host defence systems and aid in surface attachment and aggregation (Anwar & Costerton, 1992; Gristina, 1987) and enable biofilms to survive in environments where planktonic cells cannot.

In natural habitats biofilms are in constant flux (Atlas & Bartha, 1998). Populations are moving within the biofilm, where new communities constantly replace older ones. This succession is based on a sequence of physical and biological events, starting with the adsorption of organic films and closely followed by surface colonization (Atlas & Bartha, 1998).

When a biofilm community reaches a climax, or when the nutrients in the system are depleted, individual cells or part of the biofilm may detach from the surface (Walker & Marsh. 2004). Detachment can be of newly formed daughter cells or due to physical forces such as erosion and shear. Detachment can be a method which biofilms use to spread from one place to another.

2.4.1.3. Biofilm monitoring and culturing

The type of equipment, methods or techniques required to investigate biofilm formation depends largely on the questions asked. The most popular parameters to monitor or measure biofilms are light density, heat transport resistance, electrical conductivity, torque, and pressure drop (Lewandowski & Beyenal, 2003). When measuring biofilm on the basis of these parameters it is important to take into account that they are also influenced by other factors and not just biofilm formation (Lewandowski & Beyenal, 2003). There are several methods in which biofilms growth and formation can be monitored. Some of these methods include using fibre optical devices, photo acoustic spectroscopy, confocal laser scanning, infrared monitors, electrochemical probes, etc. Biofilms can be measured using physical parameters such as thickness, activity, and density. These measurements can then be used to generate a hypothesis about specific functions of these biofilms (Lewandowski & Beyenal, 2003). The parameters used to measure biofilm can roughly be divided into four categories: (1) microbial, physiology, ecology, and genetics; (2) biofilm morphology;

(3) mass transport and flow velocity; and (4) biofilm chemistry (Lewandowski & Beyenal, 2003). Photo acoustic monitoring is a nondestructive and one of the most used biofilm monitoring methods (Schmid *et al.,* 2004). It works on the principle of spectroscopy where the amount of light that is scattered or absorbed is measured (Schmid *et al.,* 2004).

Bacterial biofilms can be cultivated by batch or continuous culture (O'Toole *et al.,* 2001). In the batch culture method a sterile glass microscope slide or cover slip is suspended into an inoculated medium. After an incubation period the slide can be examined under a microscope. This makes it a very convenient method to use. Disadvantages of this method include the accumulation of metabolites in the culture vessel. This may alter or inhibit biofilm formation. Flow cells are often used to culture biofilms using the continuous culturing method. In this method fresh medium is pumped through the system and waste leaves the system, so nutrients are not recycled. This system allows mimicking of the natural conditions in an environment (Davey & O'Toole, 2000).

Firstly, biofilm formation was measured using the principle of photo acoustic monitoring specifically light refraction. The amount of light that is refracted from a membrane inside the apparatus is measured. As the biofilm grows on the membrane, less light will be refracted from the membrane, decreasing the refraction readings. Thus the more biofilm forms, the lower the readings will be (Cloete & Maluleke, 2005).

The machine also contains a compartment with microscope slides, or a modified Peterson device. As the water is circulated through the compartment, biofilm will develop on the slides. The formation of biofilms can thus be confirmed by viewing the slides under a Scanning Electron Microscope (SEM). Biofilm was also enumerated by growing it on microscope slides in the same method as mentioned previously. The slides were washed in an ultrasonic bath to remove the cells. A serial dilution was performed and the number of cells determined.

2.4.1.4. Biofilm management and control

The undesired growth of biofilms on a surface is often termed biofouling (Schmid *et al.,* 2004). When these biofilms form on medical devices it could hold a serious health threats to patients (Vickery *et al.,* 2004). It is not only in the medical field where biofilms may cause serious problems, but also in the food or drinking water industries. Established biofilms can tolerate antimicrobial agents in concentrations of 10 10 000 times higher than needed to kill planktonic cells (Jefferson, 2004). Detachment of biofilms can lead to the clogging of pipes, tubes, valves and nozzles. Biofilm growth on heat exchangers can also lead to a serious decrease in heat transfer efficiency (Schmid, 2004). Due to the decrease in water quality, it is very important to control biofouling in facilities such as drinking water reservoirs and distribution systems and food processing facilities (Schmid, 2004).

Organisms like *Staphylococcus aureus, Enterococcus faecalis, Streptococcus viridans, Escherichia coli, Pseudomonas aeruginosa*, *Proteus mirabilis, Salmonella* spp. and, *Staphylococcus epidermidis*, have been found present in biofilms (Hume *et al*, 2004). Although some of these organisms show very low pathogenic potential in normal circumstances, they can be highly pathogenic in immunocompromised people (Hume *et al*, 2004). It is therefore important to control the growth of these biofilms.

There are various approaches to control biofilm growth in different environments. Biofilms can either be controlled by physically (ultrasound or mechanically) or chemical methods (Vickery *et al.,* 2004). Although physical methods have been proven to be more successful than chemical methods, it is difficult to supervise the effectiveness of these methods. Chandy (2001) suggested removing the nutrients that biofilms need to grow, thus preventing regrowth and formation. To be able to do this, it is important to determine which nutrients are responsible for the regrowth of organisms or biofilm formation.

Chemical methods, on the other hand, are easier to supervise but less efficient due to resistance of the biofilms to the chemicals. The ideal cleaning agent should remove all biological soil and microorganisms in the biofilm without additional physical cleaning (Vickery *et al.,* 2004). Biological soil of the biofilm compromises the cleaning efficiency of the chemicals, resulting in inadequate disinfection of the surface (Vickery *et al.,* 2004).

Normally two kinds of detergents are recommended for the removal of biofilm from a surface, detergents with enzymes and detergents without enzymes (Vickery *et al.,* 2004). The detergents containing high levels of enzymes remove bacterial cells more effectively from the EPS, than the detergents containing no enzymes. They also found based on SEM studies that the detergent removed the biofilm as a sheet and that the bacteria left behind by the nonenzyme containing detergents did not grow again, suggesting that the bacteria have been killed.

Inadequate removal of soil can lead to selective disinfection; which may enhance selection of biocide resistant bacteria. This may also lead to the selection of antibiotic resistant bacteria (Vickery *et al.,* 2004). Mechanisms that lead to biocide resistance are similar to those that cause antibiotic resistance (Vickery *et al.,* 2004). These mechanisms include changes in the cell wall or membrane, development of efflux pumps, enzymatic changes, etc. It has been suggested that the widespread use of antibiotics may induce the spread of antibiotic resistance. However the amount of detergents used in laboratories to induce detergent resistance is much less than the amount of detergents used in practice (Vickery *et al.,* 2004).

The presence of biological soil can influence disinfectant failure by preventing penetration of germicide and by inactivating disinfectants. Some microorganisms may be protected from disinfectant action by the creation of localized microenvironments of reduced disinfectant concentration. This is mainly due to organic matter acting as a sink for the antimicrobial agents. Biofilms can also result in decrease disinfectant action similar to the activity described for biological soils. However, the resistance of biofilm bacteria to chemical inactivation is enhanced by their sessile (low metabolic) state, slower growth rate, and EPS production (Pajkos *et al.,* 2004).

Conventional countermeasures against biofouling are hampered by five mistakes (Flemming, 2004): firstly biofilm monitoring is performed by process performance or product quality with no early warning system. Secondly, biofouling detection is performed

in many cases by the exclusion of other problems. Thirdly, disinfection is misunderstood for cleaning. After disinfection of the system, the dead biomass will remain and needs to be removed. Fourthly, nutrients are not limited and in non-sterile systems, microorganisms are always present, but nutrients are at very low levels. Thus biodegradable substances must be considered as potential biomass. Disinfection, however, rarely reduces the amount of nutrients present in the system. Fifthly, assessment of the efficacy of the countermeasures is measured by process performance, not by the analysis of the biomass remaining on surfaces that represent further biofouling potential (Flemming, 2004).

2.4.1.5. Biofilms role in the food safety industry and in our everyday lives

Literature relating to biofilms in domestic, food production and processing environments, is limited (Rayner *et al.,* 2004). Because of the importance of foodborne disease outbreaks it is important to recognize how these pathogens survive (Rayner *et al.,* 2004). Consumer concerns have shifted from a chemical to a microbiological focus where food safety is concerned (Bruhn, 1997), with the focal point being mainly on fish, meat and poultry products.

According to Carmichael *et al.* as cited in Beuchat (2002), the colonization of spoilage and non-spoilage organisms on fruits and vegetables and post harvest contact surfaces can provide a protective environment for pathogens thus reducing the effectiveness of sanitizers. These biofilms may offer protected colonization sites for pathogens such as *Salmonella* and *E. coli* (Beuchat, 2002).

A range of antimicrobial products is now commercially available. These products, however, should be tested under conditions that will realistically determine their effectiveness (Rayner *et al.,* 2004). According to a study by Rayner *et al.* (2004), biofilms were present on tomatoes, carrots, mushrooms, cutting boards, kitchen sponges, damp and dry socks and damp and dry towels. If a biofilm is present on the surface that needs to be cleaned, it is fair to say that organisms of public health concern may be present in these biofilms (Rayner *et al.,* 2004).

Because pathogens can become sequestered in pre-exiting biofilms, which offers them protection, they can prevail in such environments (Rayner *et al.,* 2004). These pathogens may then be carried to other surfaces, causing them to become contaminated with the pathogens (Rayner *et al.,* 2004). Sanitizers and detergents currently used in households are not superficially tested against biofilms, which may cause them to be inefficient in biofilm control (Rayner *et al.,* 2004).

2.4.2. *Salmonella* in biofilm

The occurrence of biofilms in water or food production systems can have severe technical and health consequences (Lee & Kim, 2003). The formation of sessile communities and their inherent resistance to disinfectants and sanitizers are potentially a significant cause of persistent deterioration of the microbiological quality of the water (Lee & Kim, 2003).

Salmonella spp. are known to form biofilms in different environments under different conditions (Solano *et al.,* 2002) and on all kinds of surfaces, plastic, metal and glass (Stepanović *et al.,* 2004). Studies showed that *S. enterica* sv. Typhimurium normally form better biofilms in nutrient-limited environments than nutrient rich environments (Stepanović *et al.,* 2004).

2.5. Conclusion

An increase in the number of foodborne disease outbreaks the past few decades due to the growing importance of fresh produce contamination has focused research on the development of systems to minimise such outbreaks. Very little research as to the occurrence of these pathogens in the fruit export chain has been done, specifically on citrus fruit. This review focuses mainly on food safety and one specific foodborne pathogen, *S. enterica* sv. Typhimurium. Because this is also a waterborne pathogen, the review includes aspects of the occurrence of this pathogen in water, biofilms and on fruit surfaces. To minimize the occurrence of these pathogens in the fruit chains, as it moves from production

to consumption, requires basic good agricultural practices and effective food safety systems to prevent product contamination and risks to the consumer.

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Chapter 3: Biofilm formation in citrus packhouse dip tanks

3.1. Abstract

The quality of agricultural water is important in ensuring product safety and is currently a major concern for the export industry. Biofilms are microbial colonies or aggregates found in almost any environment or on any surface with sufficient nutrients and water. The total viable bacterial counts and faecal-associated contamination of water sources of three citrus farms and packhouses were monitored in this study. Biofilm formation was monitored in a warm water dip tank (42 ºC) of one of the packhouses using a photo acoustic monitoring device. To determine the influence of continuous fruit throughput on the quality of packhouse water, the monitoring device was linked to the dip tank and readings taken over a two-week period. Biofilm formation was confirmed by scanning electron microscopy and enumeration. Water sources, i.e. irrigation water, bathroom, and hand wash stations and warm water dip tanks had high total viable bacterial counts and faecal-associated contamination. The chlorine and chemical tanks had the lowest total viable bacterial counts and coliform contamination throughout the study. Biofilms formed within the packhouse dip tank. Since biofilms can contain pathogens and these pathogens can represent a contamination threat, effective management of packhouse water is critical to ensure food safety.

3.2. Introduction

Modern production, distribution and marketing technologies allow fresh produce to be available year round in countries over the world (Beuchat, 2002). As a result of these technologies not being implemented correctly, increased risks of human illnesses has more recently been associated with fresh produce (Beuchat, 2002). Irrigation and wash water are some of the known sources of produce contamination with human or waterborne pathogens (Beuchat & Ryu, 1997; Solomon *et al*., 2002). Biofilms are microbial colonies or aggregates found in almost any environment or on any surface with sufficient nutrients and water (Wimpenny, 2000). According to Walker & Marsh (2004), a biofilm is a three-dimensional functional consortia of microbial cells, bound to, and growing at an interface (e.g. solid:liquid, solid:air, liquid:air, liquid:liquid) enveloped within extra cellular polymers (EPS). Environmental biofilms are generally composed of a number of different species of microorganisms which develops a functional consortium (Walker & Marsh. 2004). An advantage for microorganisms in a colony attaching to a surface is that the nutrients from the water and substratum can be utilized more effectively (Davies, 2000).

There are several ways in which the growth and formation of biofilm can be monitored. Some of these methods include using fibre optical devices, photo acoustic spectroscopy, confocal laser scanning, infrared monitors and electrochemical probes. The parameters used to measure biofilms can roughly be divided into four categories: (1) microbial, physiology, ecology, and genetics; (2) biofilm morphology; (3) mass transport and flow velocity; and (4) biofilm chemistry (Lewandowski & Beyenal, 2003). Biofilms can be measured using physical parameters such as thickness, activity and density, these measurements can then be used to generate opinions about specific functions of these biofilms (Lewandowski & Beyenal, 2003). Photo acoustic monitoring is a non-destructive method to monitor biofilm formation (Schmid *et al.,* 2004). The method works on the principle of spectroscopy where the amount of light that is scattered or absorbed is measured (Schmid *et al.,* 2004).

Because pathogens can become sequestered in pre-existing biofilms, that offers them protection, they can prevail in an environment for extended periods of time (Rayner *et al.,* 2004, Costerton *et al*., 1999). These pathogens may then be carried to other surfaces and

potentially cross contaminate products (Rayner *et al.,* 2004). According to Rayner *et al.* (2004), biofilms have been reported on a variety of fresh produce surfaces including tomatoes, carrots and mushrooms. Another study done by Lapidot *et al*. (2006), showed that biofilms were also able to form on parsley and survive several disinfectant treatments. Thus the formation of biofilms on food surfaces is likely to influence the effectiveness of cleaning and disinfection to control foodborne pathogens (Lapidot *et al*., 2006).

The aim of this study was to determine the microbiological quality of water sources used on citrus farms focusing on potential contamination points such as irrigation water used in orchards, packhouse wash water and hygiene wash stations. The aim of the study was also to determine the presence and rate of biofilm formation in citrus packhouse wash tanks.

3.3. Materials and Method

All experiments were performed in ISO 17025 accredited laboratories. All work was done in a biohazard safety cabinet and all areas exposed to the pathogen was thoroughly cleaned and disinfected afterwards. All staff and students working in the food safety laboratories have been trained in biohazard best practices and ISO principles.

3.3.1. Sample collection

One-litre water samples were collected in sterile plastic water bottles at three farms and packhouses (identity not disclosed) in the Limpopo and Mpumalanga provinces of South Africa. The bottles were sterilised by washing them with aseptic soap and rinsing with 70% ethanol. The bottles were left to air-dry overnight. Water samples at farm 2 and 3 in the Limpopo Province were taken in the orchard at several points and in the packhouses. At the packhouse in the Mpumalanga province (Farm 1), water samples were only taken in the packhouse and at the supply water source used to fill the dip tanks (Appendix K, L, M). All water samples were obtained over a week at two-day intervals and sampling was repeated three times during the citrus harvesting season between July and September 2005.

Samples from the orchard included supply water samples, including the dam, river and channel water and three irrigation points in the orchard. Samples from the packhouses included water from warm water, chemical and receiving dip tanks inside the packhouses and at four hand wash stations and four bathroom basins.

Samples were taken aseptically by dipping the bottle in the water without opening the lid. Once the bottle was covered by the water the lid was opened and the bottle allowed to fill. Before removing the bottle from the water the lid were screwed on tightly. Wherever water was collected from a tap, contact between the bottle and tap was avoided. Samples were immediately transported to Plant Pathology Laboratories in cooler boxes and stored at 4 °C for no longer than 48 h till processing. One, one-litre sample was taken at each point. Water sampling was repeated three times.

3.3.2. Total viable bacterial counts

To determine the total viable bacterial count, the water samples were filtered through 0.45 µm membrane using a filter manifold and vacuum pump. Only 250 ml of each sample were filtered through a 0.45 µm membrane, yielding four membranes per sample. The four filter membranes of each sample were suspended in 9 ml Ringer's solution (physiological saline) (Merck, Johannesburg, South Africa) and mixed vigorously using a vortex mixer to remove all microbes from the membrane. Total counts were obtained by performing a serial dilution in Ringer's solution. Dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were plated out in triplicate on Standard 1 Nutrient agar (STD1) (Biolab, Merck) using the spread plate technique. All agar plates were incubated at 37 °C for 48 hours. Total viable counts were recorded and statistically analyzed as described in section 3.3.6.

3.3.3. Colilert

To determine the microbiological quality of the water, faecal coliform and *Escherichia coli* tests was used as indicator organisms. Microbial analyses using the Colilert-18 (IDEXX

Laboratories, Inc, Maine, USA) was conducted within 24 h after samples were collected. Tests were conducted using a modification of the SANAS accredited ISO

Table 3.1. Water sampling points and a summary of management steps at the different farms and pack houses

Sampling source	Temperature	Chemicals added	Replenishment rate
Farm 1			
Source	Room temperature	Chlorine	-
Tank	42 ºC	Appendix N	Seasonally
Farm 2			
Dam water	Environmental temperature	None	-
Water channel	Environmental temperature	None	-
Orchard (Three points)	Environmental temperature	None	-
Chlorine tank	Room temperature	Chlorine (250 ppm)	Daily
Warm water tank	45 ºC	Appendix N	Per load basis
Quatrokill tank	Room temperature	None	-
Imazalil spray	Room temperature	Imazalil sulphate	Per load basis
Bathroom	Room temperature	None	-
Hand wash stations	Room temperature	None	-
Farm 3			
River	Environmental temperature	None	-
Water channel	Environmental temperature	None	-
Dam water	Environmental temperature	None	-
Orchard	Environmental temperature	None	-
Borehole	Environmental temperature	None	-
Chlorine tank	Room temperature	Chlorine (250 ppm)	Per load basis
Warm water tank	38 ºC	Appendix N	Daily
Chemical tank	Room temperature	Appendix N	Per load basis
Bathroom	Room temperature	None	-

17025 test method (PPL 006) of the Plant Pathology Laboratories. According to manufacturer's instructions one ampoule of Colilert-18 reagent was added to 100 ml water sample and allowed to dissolve. The water was carefully poured into a 98 well quanti tray, put into a rubber mould and sealed using the quanti tray sealer (IDEXX Laboratories). Trays were incubated at 37 °C for 18 to 22 h. A positive reaction was obtained if a colour

change to yellow occurred after incubation. The presence of *E. coli* was confirmed by checking the tray under ultraviolet light for fluorescence. The Most Probable Number (MPN) of coliforms and *E. coli* were determined using the standard table for the Colilert test according to the manufacturer's instructions.

3.3.4. Biofilm monitoring

Biofilm monitoring was achieved by measuring the light refraction from a membrane caused by the biofilm that formed in the apparatus through which the water of the dip tank was circulated (Fig. 3.1.) (Cloete & Maluleke, 2005). Three refraction readings were taken three times per day, i.e. early in the morning, midday and late afternoon. The average of those readings was used to determine the average growth per day. Data were plotted onto a graph to depict biofilm formation.

The water was circulated through a separate compartment in the biofilm monitoring apparatus, which contains the microscope slides. As the water circulates through the unit it also moves through the compartment while the biofilm grows on the glass slides. The slides are removed and analyzed under a Scanning Electron Microscopy (SEM). This study was only preformed at the Mpumalanga (Farm 1) packhouse on a continuous basis for a week in August 2004 and the experiment was repeated in August 2005 representing the peak citrus packing season.

The slides were prepared for analysis by suspending in a fixing solution (2.5% gluteraldehyde in 0.15 M Na/K phosphate buffer diluted to 50% with ddH_2O) (Coetzee & van der Merwe, 1994). The blocks were stored at 4 °C for seven days followed by three successive 15-min rinsing in 0.15 M Na/K phosphate buffer. The rinsing process was followed by successive 15-min dehydrations in 50, 70, and 90% ethanol and finally three times for 15 min in 100% ethanol which was left overnight before drying. Samples were dried in a Biorad drier (Biorad Polaron Division, England) under CO_2. Specimens were coated for 2.5 min with 10mÅ of gold-palladium (Polaron Equipment Ltd., England) and examined under a JEOL (JSM-840) SEM operating at 5 kV.

Two trials were performed, a laboratory and field trial. In the laboratory trial, a 24 ℓ water sample was received from the packhouse in the Mpumalanga province (Farm 1). This water was used to perform a laboratory trial to monitor the presence of biofilms in packhouse water. After the laboratory water monitoring experiment was completed a field trial was conducted. The field trial was done to determine what influence the continuous flow of fruit would have on biofilm development. This was done by linking the apparatus to the dip tank as illustrated in Figure 3.2. During the laboratory trial it was found that light from the sun and or fluorescent lights influenced the readings. To minimise this influence during the field trial a black plastic bag was used to cover the membrane.

Figure 3.1. The Biofilm monitoring apparatus using refracted light.

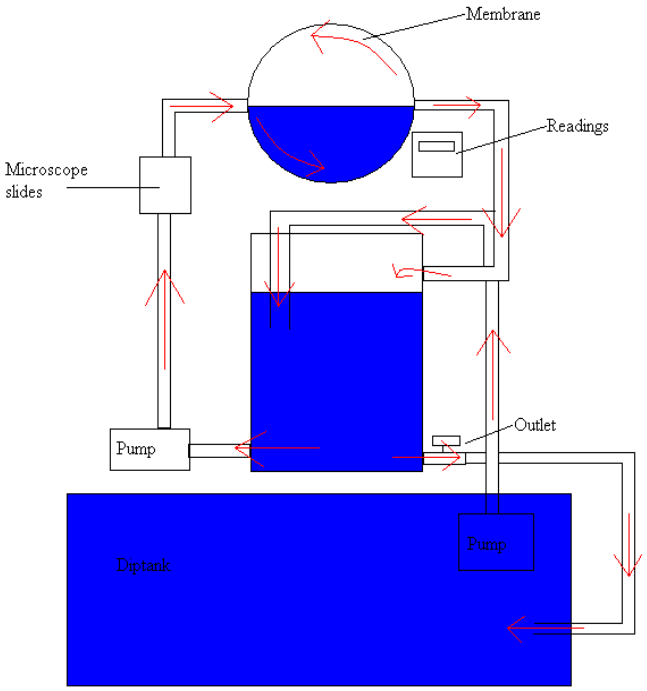


Figure 3.2. Flow line sketched to illustrate biofilm monitor linked to the dip tank system to measure the influence of continuous flow through of citrus fruit through the dip tank.

3.3.5. Biofilm enumeration

During the second field trial in August 2005 biofilm formation in the dip tanks was enumerated. Water from the dip tanks was pumped through the compartment and back into the tank. Biofilms were allowed to grow on the microscope slides. Slides were removed at 7, 24, 30, 48 and 72 h. The slides were suspended in 30 ml sterile Ringer's solution and stored at 4 °C for one week prior to analysis. The slides were sonicated and a serial dilution performed in Ringer's solution as described before.

3.3.6. Statistical analysis

The data was analyzed as for a completely randomized design (CRD). Analysis of variance (ANOVA) was used to test for differences between the sampling points. Bacterial counts are log normally distributed, thus the data was logged (base 10) to stabilize

sampling variance. Sample means were separated using Fishers' protected t-test least significant difference (LSD) at the 1% level of significance (Snedecor & Cochran, 1980). Data were analysed using a statistical programme (GenStat, 2003). For statistical purposes, bacterial plates with 300 or higher number of colonies were considered as 300.

3.4. Results

3.4.1. Total viable bacterial count

A significant difference between the original water source used in the packhouse and the dip tank water from Farm 1 (Fig. 3.3, Appendix A) was recorded. Comparing the samples from the dip tank over time reflected a significant increase in microbial load. The tank was filled up at the beginning of the season (April - May 2005) and not exchanged at time of sampling. An average of 135 tonnes of fruit move through the tank on a daily basis. The water source remained the same for the first two sampling days but increased significantly in microbial load on the fourth day after initial sampling. At Farm 2 and Farm 3 all sampling points had high numbers of total viable bacterial counts,

irrespectively of the source (Fig 3.4 & 3.6, Appendix B & D). Only the chlorine treated water tank samples showed very low numbers but had high standard deviations.

Data from the different sampling points can further be grouped according to the different places the samples was taken from, i.e. all the samples taken from the packhouse grouped under packhouse, all the source samples grouped under source etc., to assess trends.

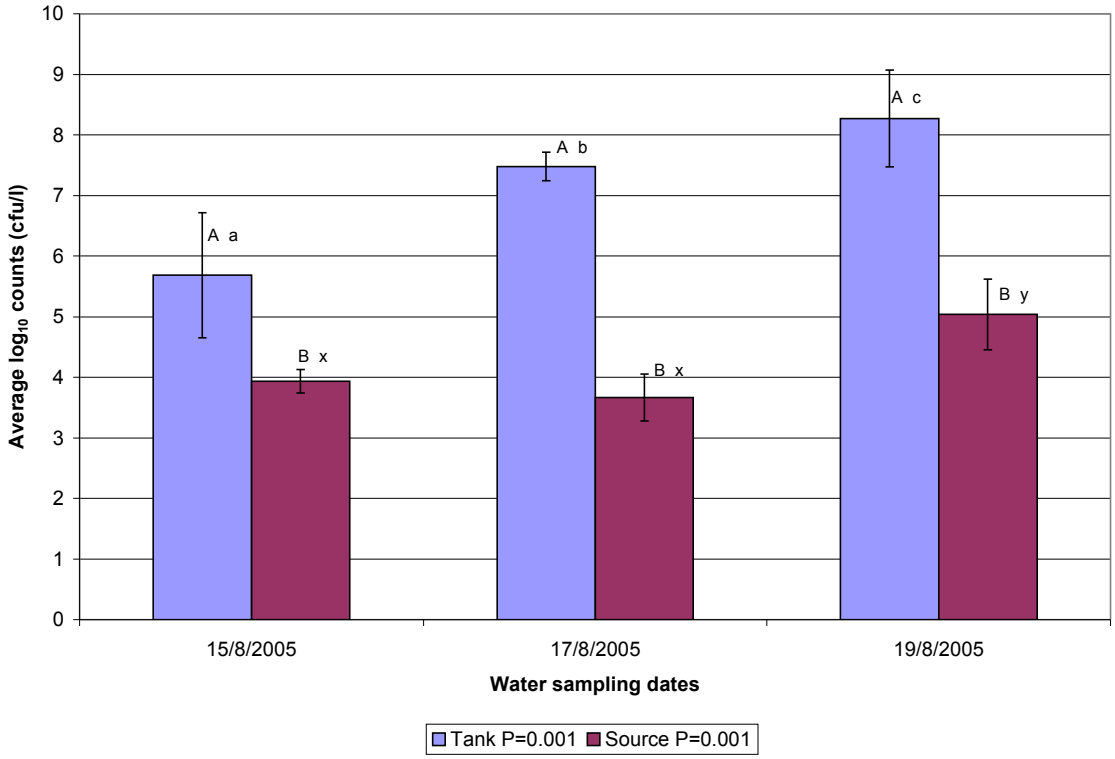


Figure 3.3. Total viable bacterial counts of water samples taken on different sampling dates at Farm 1. The source water is purified river water used to fill up the dip tank. Value points with the same capital letter did not differ significantly while small letters a to c and x to y compare sources over time at P=0.001. Average $\log_{10}$ counts refer to the average log count of the colony forming units (cfu) per litre.

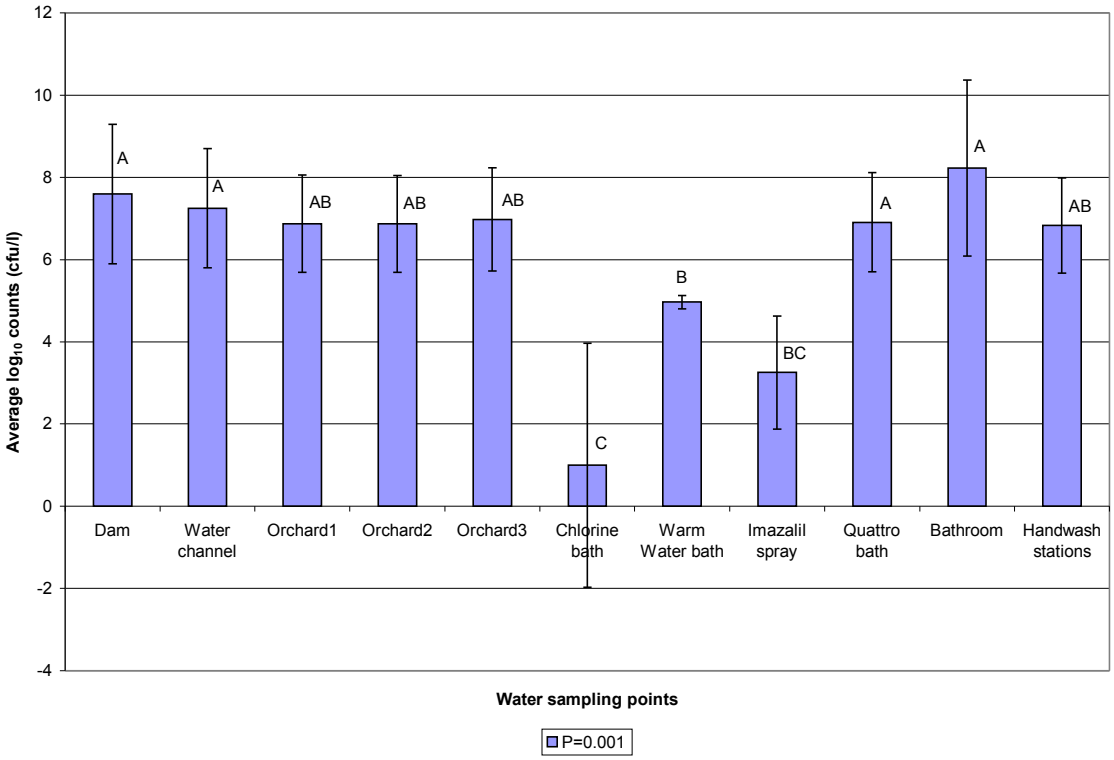


Figure 3.4. Total viable bacterial counts of water as sampled at the different points on Farm 2. Values with the same letter did not differ significantly at P=0.001. Average $\log_{10}$ counts refer to the average log count of the colony forming units (cfu) per litre of water sample analysed.

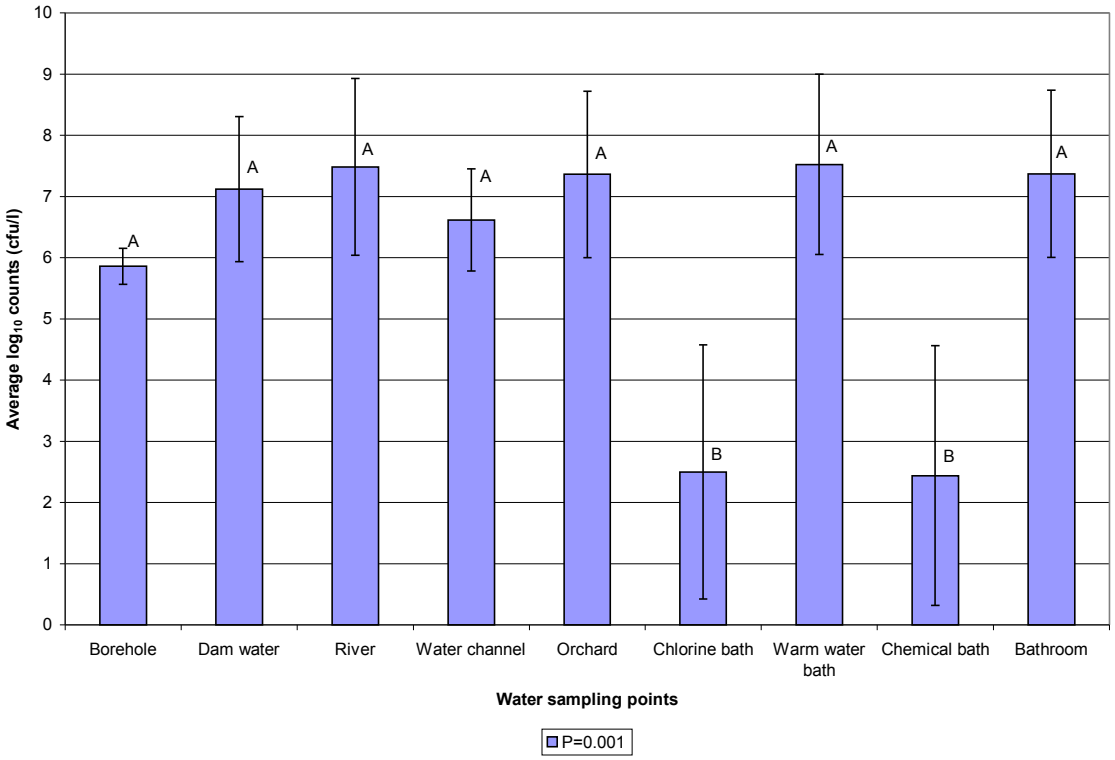


Figure 3.5. Total viable bacterial counts of water as sampled at the different points on Farm 3. Value points with the same letter did not differ significantly at P=0.001. Average $\log_{10}$ counts refer to the average log count of the colony forming units (cfu) per litre.

On Farm 2 the packhouse water fell within the acceptable limits (Fig. 3.6) as with Farm 3 (Fig. 3.7) where the packhouse water did not fall within these limits. These groups show the difference between the groups of samples sampled in and around the packhouses.

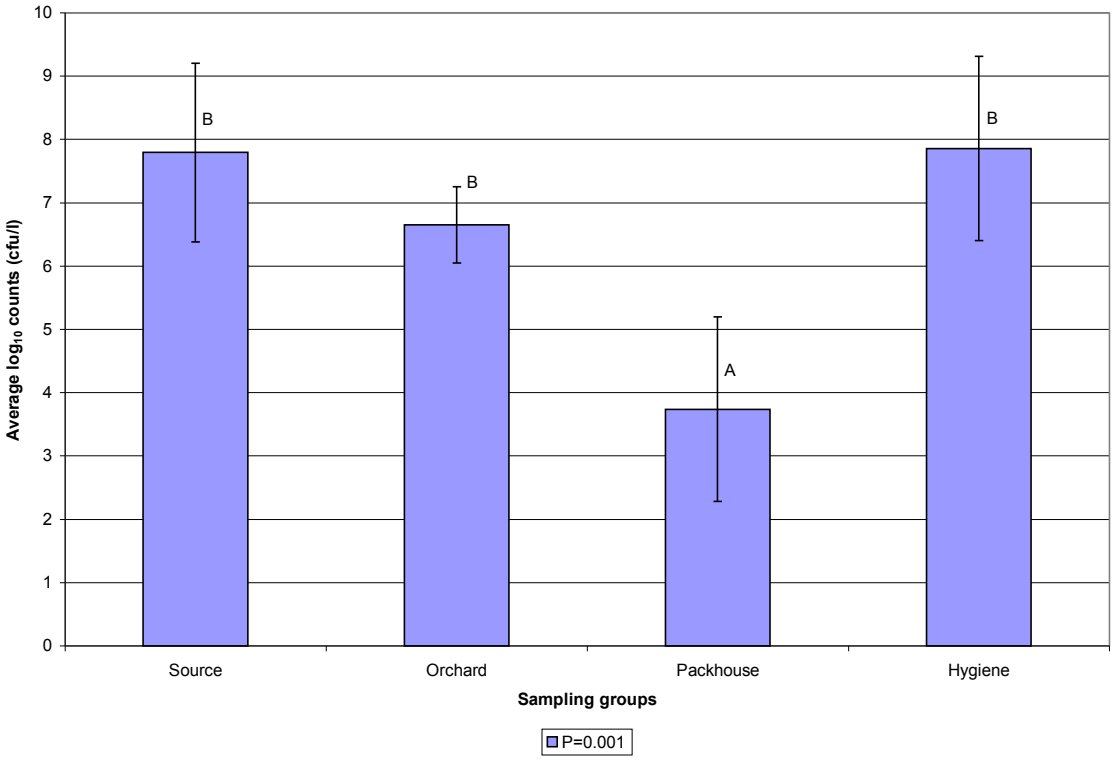


Figure 3.6. Total viable bacterial counts of sampling points grouped on Farm 2. Value points with the same letter did not differ significantly at P=0.001. Average $\log_{10}$ counts refer to the average log count of the colony forming units (cfu) per litre.

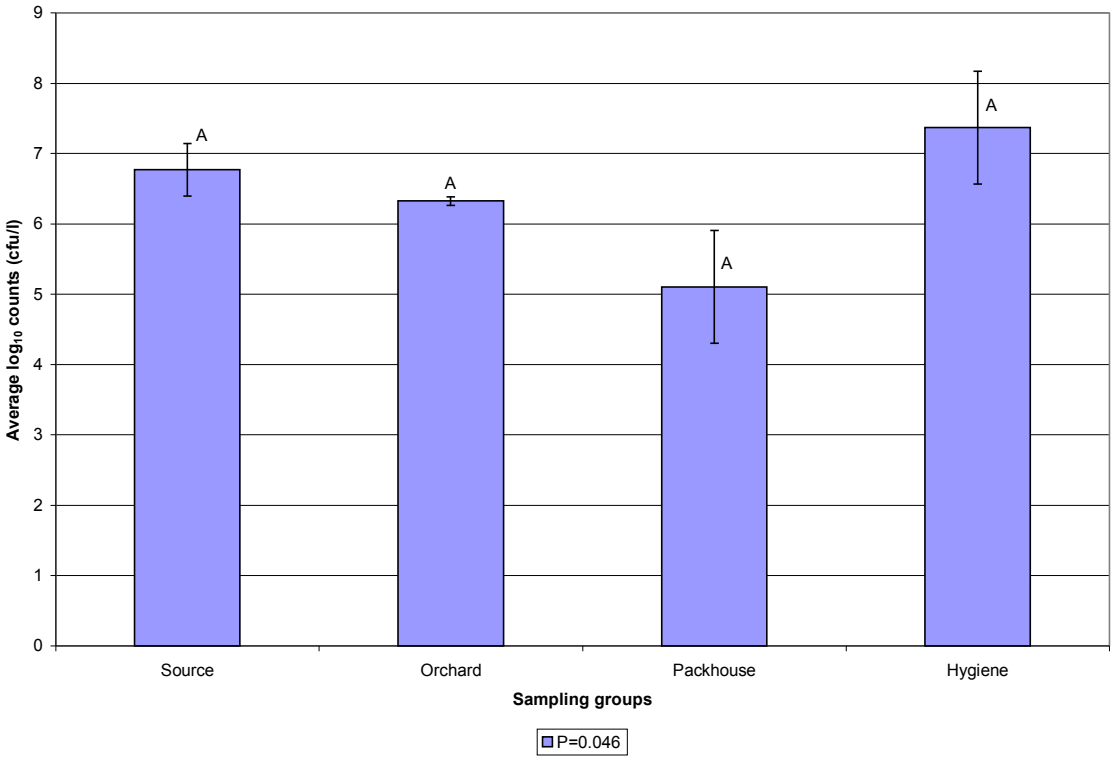


Figure 3.7. Total viable bacterial counts of the different sampling points grouped from Farm 3. Value points with the same letter did not differ significantly at P=0.046. Average $\log_{10}$ counts refer to the average log count of the colony forming units (cfu) per litre.

3.4.2. Colilert

On the 17/8/2005 both the dip tank and source water of Farm 1 far exceeded the allowable standard for potable drinking water (Table 3.1). A similar exceedence was recorded on 15/8/2005 although it did not reflect the same high levels as on 17/8/2005. Samples from Farm 2 and 3, including river, water channel water, dam water, orchard irrigation water, dip tanks, bathrooms and hand wash stations, showed very high numbers of coliforms and *E. coli* levels suggesting that the waters are highly contaminated with faecal associated organisms (Table 3.2 & 3.3).

Table 3.1. The most probable number of coliforms and *E. coli* cells present in the water samples, collected from the same point in the tank on different dates, from Farm 1

Sample	**Sampling Date**															
	6/8/2004		**30/8/2004**		**1/9/2004**		**3/9/2004**		**15/8/2005**		**17/8/2005**		**18/8/2005**		**19/8/2005**	
	MPN/100ml															
	Coliforms	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***
Tank	<1	*<1*	*<1*	*<1*	*<1*	*<1*	*<1*	*<1*	30.5	*<1*	>2419.2	*<1*	*<1*	*<1*	*<1*	*<1*
Source	-	-	-	-	-	-	-	-	*<1*	*<1*	*>2419.2*	*<1*	*<1*	*<1*	*<1*	*<1*

Table 3.2. The most probable number of coliforms and *E. coli* cells present in the water samples taken at Farm 2

Sample	Sampling Date					
	20/6/2005		11/7/2005		28/7/2005	
	MPN/100ml					
	Coliforms	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***
Dam water	-	-	2419.17	111.9	>2419.2	39.9
Water channel	-	-	>2419.2	64.4	322.3	73.8
Irrigation water 1	-	-	185	14.6	>2419.2	<1
Irrigation water 2	-	-	111.2	14.6	>2419.2	5.1
Irrigation water 3	-	-	770.1	4.1	>2419.2	3.1
Chlorine tank	<1	<1	<1	<1	-	-
Warm water tank	<1	<1	<1	<1	<1	<1
Quatrokill tank	>2419.2	<1	<1	<1	>2419.2	<1
Imazalil spray	<1	<1	<1	<1	-	-
Hand wash station	-	-	>2419.2	1	41.6	<1
Bathroom	-	-	20.3	5.2	<1	<1

Table 3.3. The most probable number of coliforms and *E. coli* cells present in the dip tank water samples taken at Farm 3

Sample	Sampling Date					
	20/6/2005		12/7/2005		28/7/2005	
	MPN/100ml					
	Coliforms	*E. coli*	Coliforms	*E. coli*	Coliforms	*E. coli*
Bore hole	-	-	3.1	<1	<1	<1
Dam water	-	-	547.5	<1	1732.87	<1
River	-	-	>2419.2	70.3	>2419.2	143.9
Water channel	-	-	816.4	26.9	381.1	29.2
Irrigation water	-	-	372.4	1	172.2	2
Chlorine tank	-	-	<1	<1	1	<1
Warm water tank	>2419.2	1	>2419.2	<1	<1	<1
Chemical tank	<1	<1	<1	<1	-	-
Bathroom	-	-	517.2	<1	>2419.2	2

On Farm 2 the quatrokill bath on the 20/6, hand wash station on the 11/7 as well as the dam water and channel water far exceeded the allowable coliform and *E. coli* limits. In addition the bathroom, irrigation water, quatrokill tank, hand wash stations, dam water and water channel all exceeded the allowable limits of coliforms on the 11/7 and 28/7. The dam water, irrigation water and water channel also exceeded the allowable standard for *E. coli* on the 28/11.

On Farm 3, the warm water tank exceeded the allowable limits for coliforms on the 20/6. In addition, the warm water tank, irrigation water, river water, bathroom, dam water, and water channel exceeded the allowable limits for coliforms on the 12/7 and the 28/7. The water channel, bathroom, river water, irrigation water exceeded the allowable limits for *E. coli* on all the sampling dates.

3.4.3. Biofilm monitoring

From the refraction readings in the laboratory trial (Fig. 3.8) no specific pattern of growth could be observed, but in the field trial (Fig. 3.9) the refraction readings indicate biofilm formation.

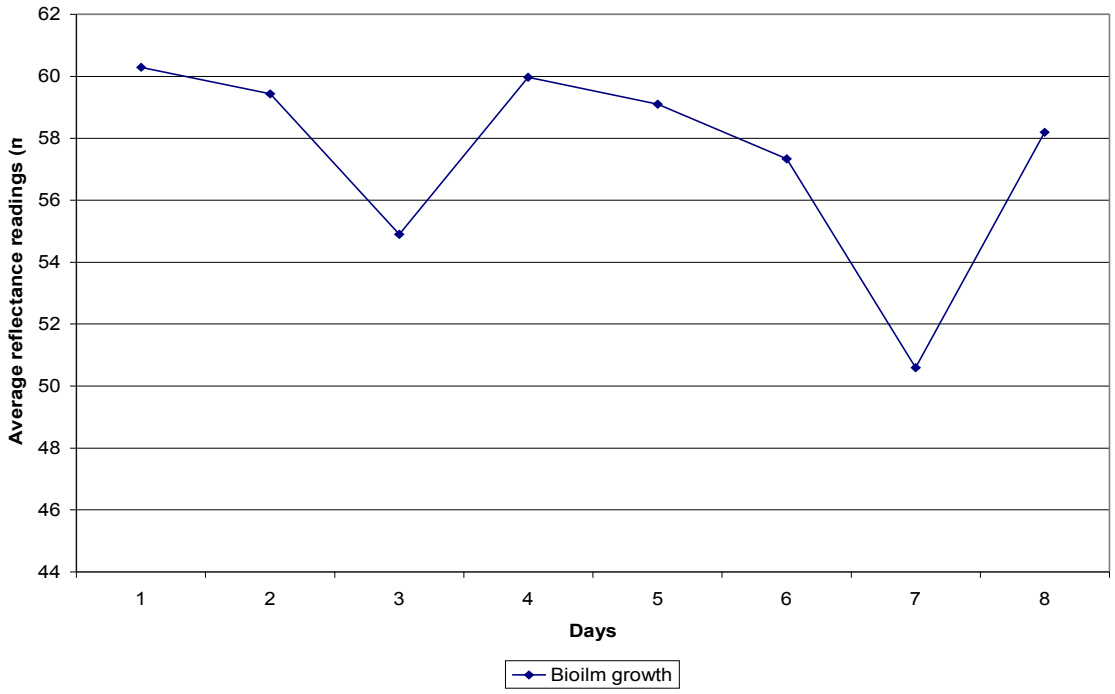


Figure 3.8. Average biofilm growth per day over a period of eight days conducted in the laboratory trial with stagnant water.

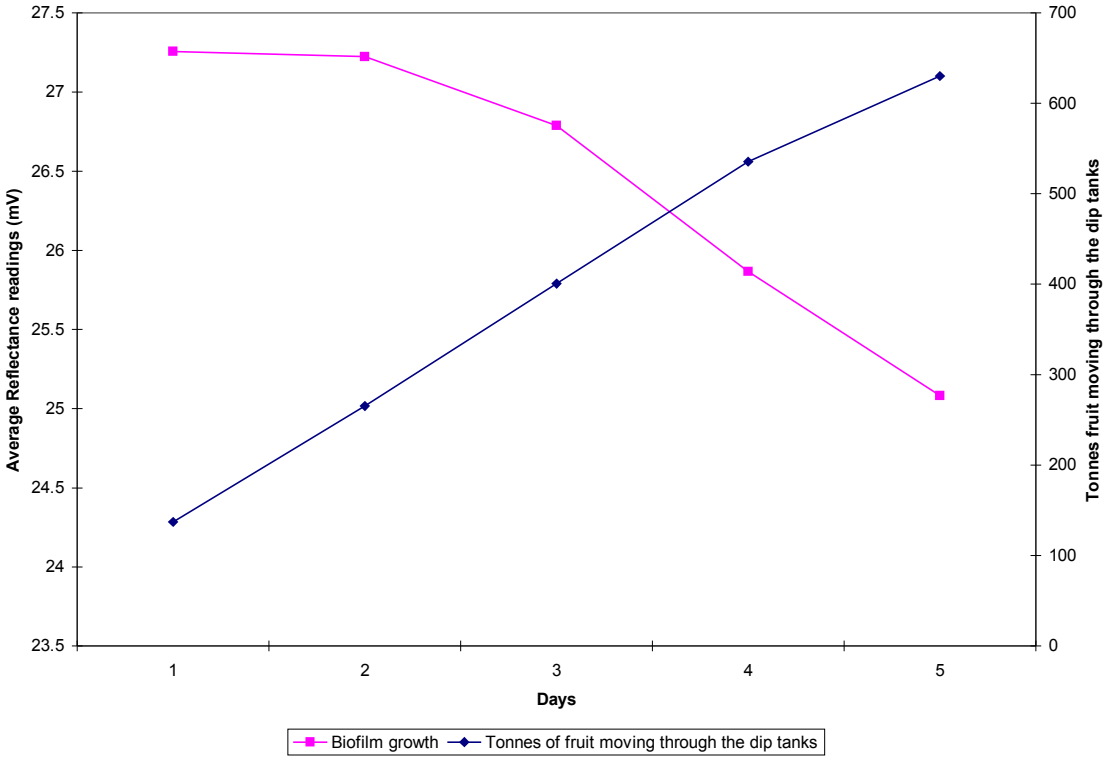


Figure 3.9. Average biofilm growth per day over a period of five days conducted in the field trial at Farm 1, compared to the total tonnes of fruit packed for the same period.

Biofilm was allowed to grow on microscope slides for a period of 72 hours. An increase in the number of cells was recorded during this period (Fig. 3.10).

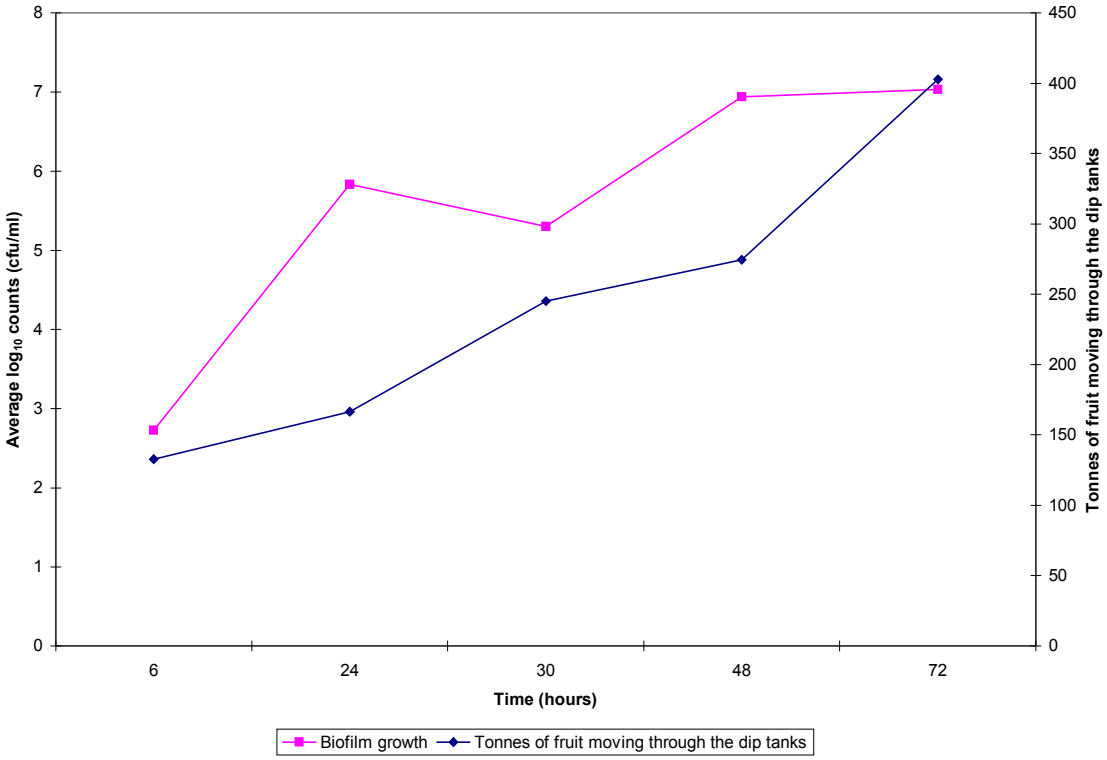
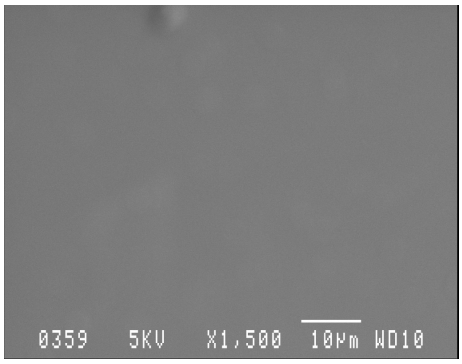
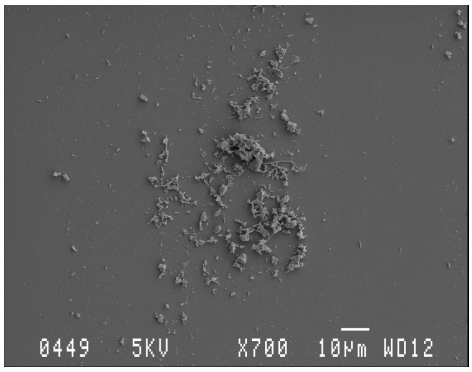


Figure 3.10. Biofilm formation in a citrus packhouse dip tank at Farm 1 as monitored over a period of 72 hours compared to the total tonnes of fruit packed for the same period.

The SEM photos showed presence of biofilm on the microscope slides, formed from water collected from Farm 1, assessed over time (Fig. 3.11 and 3.12).



Control



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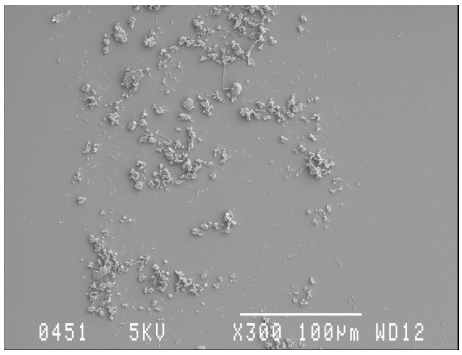
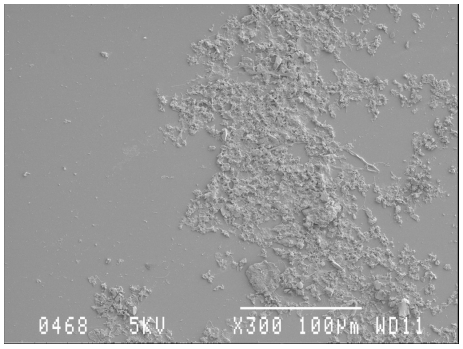
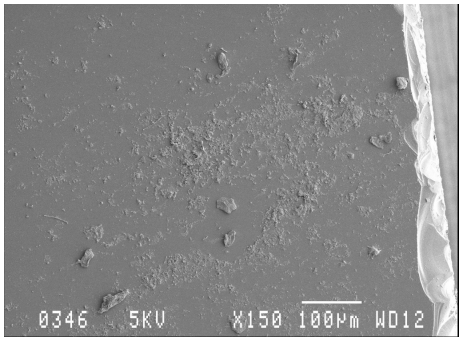


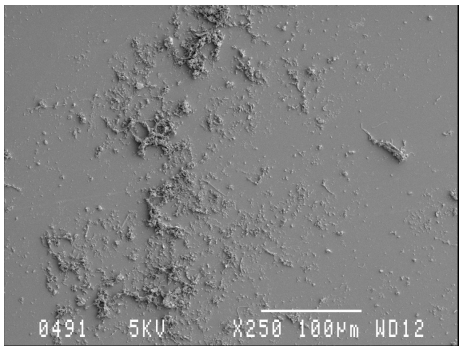
24h



48h

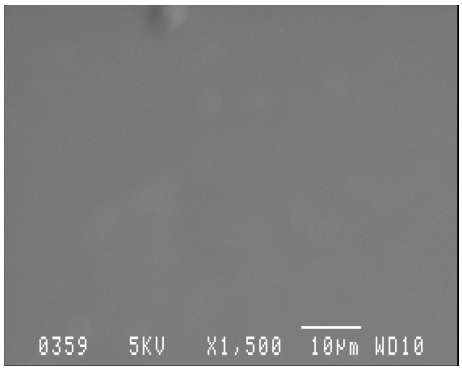


72h



96h

Figure 3.11. Scanning electron micrograph of biofilm formation in the laboratory trial taken over a period of 96 hours.



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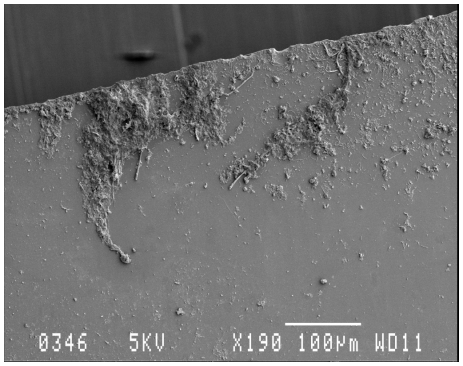

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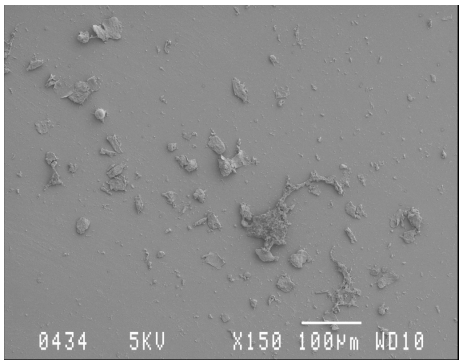

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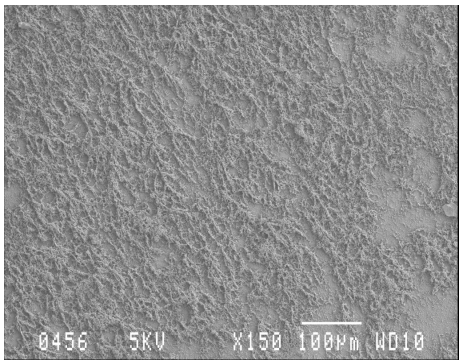
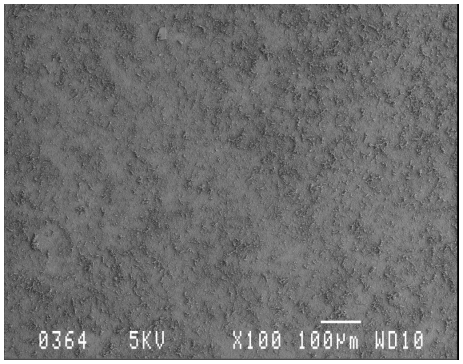


72 h



96h

Figure 3.12. Scanning electron micrograph of biofilm formation in the field trial taken over a period of 96 hours.

3.5. Discussion

This is the first study of its kind regarding biofilm formation in citrus packhouse dip tanks. Previous studies focused on the role of biofilms in drinking water and related industries (Codony *et al.,* 2005; Berry *et al*., 2006; Hallam *et al.*, 2001) but not in the fresh fruit industry. In this study it was found that biofilms formed in warm water (42 ºC) dip tanks of citrus fruit pack houses. When results were compared to the laboratory trial, a significant difference could be seen in terms of a more consistent development of a biofilm layer developing over time in a packhouse dip tank. No clear pattern of biofilm formation could be derived from the reflectance reading or SEM in the laboratory trial. It was later found that outside light from the laboratory or sun interfered with the readings (unpublished data). To minimise this interference a black plastic bag was used to cover the membrane during the field trial.

The influence that the continuous throughput of fruit has on water quality and biofilm formation could be seen from the reflectance readings, total counts and biofilm enumeration. An association could be drawn between the tonnes of fruit passing through the tank and water quality and biofilm formation. The more fruit moving through the system, the higher were the bacterial counts. The influence of fruit flow through the tanks could also be seen from the reflectance readings, the higher the volume of fruit passing through the warm water tank, the lower the reflectance readings, suggesting more biofilm formation. The fact that the water samples had high bacterial counts, increased biofilm formation and high coliform counts, that exceeded the South African standard, indicate potential contamination. Previously it was found that pathogens can incorporate into biofilms (Costerton *et al.,* 1999), although no pathogens were isolated from the biofilm, it is still potentially possible that it can happen in contaminated citrus packhouse dip tanks.

In this study a total of 56.36% of water samples tested higher than the allowable limit for coliforms and 32.73% of the water samples tested higher than the allowable amount for *E. coli* in South Africa. These samples include the water channel, river, bathrooms, hand wash stations, irrigation water, water tanks and dams. In South Africa the standard for

dinking water is 10 counts/100 ml for coliforms and 1 count/100ml for *E. coli* per 1% of samples tested over a one-year period (SABS, 2006). Coliforms are indicator organisms for faecal associated contamination. All three packhouses monitored in this study are currently EUREPGAP certified. Yet all of them had contamination levels beyond required standards. This is of some concern, since EUREPGAP require the use of only potable water in packhouses (www.eurepgap.org/fruits/documents.html 2, 2006). The fact that more than half the samples tested had higher than the allowable limits, raises certain questions as to the credibility and reliability of current standard water testing results or local interpretation of it.

Of all the samples tested only the borehole water was within the required standard of potable water. Since Farm 1 uses purified tap water obtained from a nearby river, the reliability and effectiveness of the on farm water purification system should be questioned. Farm 2 uses untreated and unfiltered dam water. Because most of the tanks within the packhouses tested contains 10 000 litres or more, it is not economically feasible to replace the water on a daily basis. The EUREPGAP standard requires reused water to be filtered (www.eurepgap.org/fruits/documents.html 2, 2006). Farm 1, therefore, installed sand filters which are replaced once a season. This study was done late in the season and the cleaning efficiency of these filters proved ineffective indicating that more frequent cleaning is required. Farm 1's warm water dip tank tested negative for coliforms, except on one day. On this day the packhouse did not pack any fruit and thus the chemicals were not replenished and the water not filtered, which could explain the increase in coliform count. When a water sample was taken the following day, after the packhouse started packing and the water was treated, the numbers of coliforms fell below the standard set by the SABS (SABS, 2006). This indicates that the effective management of the warm water tank chemicals is essential to prevent possible cross-contamination.

Although Farm 2 is the only one where contaminated source water is used, it is also the only farm where the dip tanks tested negative for coliforms and *E. coli* throughout the study. On this farm the warm water dip tank is managed by keeping the water temperature above 45 ºC and the chemicals are replenished at more regular intervals. Farm 3 used clean

borehole water, and exchanged water on a weekly basis, but the warm water dip tank tested positive for high numbers of coliforms meaning that management of the tank is not effective. At Farm 2 the warm water tank is kept at 45 °C and a number of postharvest decay control chemicals are added to the water (Appendix N). Although the farmer started with contaminated water, he can effectively sanitise it with chemicals. At Farm 1 the water is kept at a lower temperature (42 °C), but also here a number of postharvest decay control chemicals are added (Appendix N). At Farm 3, however, the warm water tank is kept at a 38 °C and nothing except for a harvest wash activator is added to the water. Here the postharvest decay chemicals are added to the chemical tank which is kept at room temperature. This tank tested negative for any coliforms or *E. coli.* The fact that no chemicals were added, as well as the temperature being kept so close to the optimal growth temperature of the indicator organisms it could explain the high number of coliforms isolated from this tank. This study highlighted the importance of correct management practices to ensure potable water is used in packhouses.

Although all the tanks that are treated with chlorine had low total viable bacterial counts and no coliforms throughout this study, it reflected an effective management point in the chain. The chlorine is used to treat the water and can also be used to disinfect the fruit, to some extent (Behrsing *et al*., 2000). If appropriate free chlorine levels are maintained and the system is effectively managed, product contamination can be avoided. This was proven by Pao and Davis (1999), who showed that populations of *E. coli* inoculated onto orange surfaces could be reduced more than 2 log cfu/cm^2 after immersion in 200 ppm chlorine at 30 °C. In their study fruit were exposed for eight min to these conditions. Fruit in this study moved through the chlorine tank within five min and then moves to the warm water and chemical treated tanks. Reductions of microflora on whole and fresh cut produce exposed to chlorine-treated water are dependent upon the type of produce and the type of natural microflora present (Parish *et al.,* 2003). In this study five min contact time proved effective.. One important point that emerged from this study was that if water tanks further down the packline are not managed at the same level of effectiveness as the first chlorine wash tank, product contamination can potentially happen.

The fact that the bathroom and hand wash stations at the farms had such high total viable bacterial counts and Colilert counts exceeding the required standards it potentially holds a serious health risk for workers and may result in transferral of potential waterborne pathogens to fruit. Several studies have previously shown that hand washing with contaminated water can result in product contamination via the hand wash water, water taps or soap (Mermel *et al.,* 1997; Weber *et al.,* 1997; Widmer, 2000; Kappstein *et al.,* 2000). In a study done by Ackers *et al.* (1997) pathogens, specifically *Vibrio cholerae*, were carried to two people via secondary transmission from an asymptomatic carrier. In previous studies it was shown that foodborne pathogens can spread from spiked gloved hands to the fruit via handling during the picking, sorting and packing process (unpublished data). Further, personal hygiene is a requirement within the EUREPGAP standard as a means of minimising product contamination by foodborne pathogens.

Everyone participating in fruit production, harvesting and post-harvest handling storage and transport of fresh fruit should be involved in ensuring safety and quality (Combrink *et al.,* 1994). Thus incorrect management of the various processes or systems within production, packing and export may compromise safety. Future studies should therefore focus on rapid identification of contamination points and detecting the presence of waterborne pathogens and the establishment of appropriate microbiological standards for packhouse hygiene and agricultural water. Studies should also focus on the best methods and products for controlling biofilms within water tanks in packhouses.

3.6. References

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Chapter 4: Monitoring *Salmonella* presence in the citrus export chain.

4.1. Abstract

Over the past decade there has been an increasing number of reported annual nontyphoidal *Salmonella* outbreaks linked to the consumption of fresh produce. Citrus for instance has been reported contaminated with *Salmonella* which was linked to unpasteurised orange juice. Early detection and regular monitoring for the presence of foodborne pathogens is important to ensure food safety compliance. Rapid and more reliable detection methods such as Polymerase Chain Reaction (PCR) has recently been developed to detect the presence of *Salmonella* spp. in the environment. In this study, the presence or absence of Salmonella spp were assessed in the citrus supply chain. Sampling included 50 water, 1 051 swab and 276 fruit samples collected from three South African citrus farms and their packhouses, six containers and three distribution centres and retailers in Europe. Two primer sets were used to test firstly for the genus *Salmonella* and secondly, for *Salmonella* T*yphimurium*. No *Salmonella* spp. could be detected in any samples.

4.2. Introduction

Salmonella is an endemic public health concern worldwide (Mead *et al.,* 1999). It is a primary agent in gastroenteritis in both humans and animals. According to Yan *et al.* (2003), there are an estimated 1.5 million cases of nontyphoidal *Salmonella* infections annually associated with the consumption of fresh produce. The most common symptoms are gastroenteritis with nausea, vomiting, and diarrhoea without a fever. A small percentage of these patients develop extra gastrointestinal infections. The genus *Salmonella* are currently composed of over 2 500 different serotypes. In the 1970s, *Salmonella enterica* sv. Enteritidis emerged as a pathogen of poultry, but only later became an important human pathogen. Invasive *Salmonella* infections can be caused by many *Salmonella* serovars (Rabsch *et al.,* 2001).

Salmonella species have been implicated in foodborne illnesses originating from a wide range of different food products. In 1955, 1979 and 1991 outbreaks of *Salmonella* infections were traced back to pre-cut watermelons (De Roever, 1998). In 1990 and 1993 the outbreak of *Salmonella* was linked to the consumption of fresh tomatoes (De Roever, 1998). Studies following these outbreaks showed that *Salmonella* spp. can survive and grow rapidly on mature intact tomatoes held at ambient temperature (De Roever, 1998). According to Penteado *et al.* (2004), *Salmonella* spp. can penetrate into fresh produce. During an outbreak in 1999 they found that water used to wash fruit, was contaminated with *Salmonella* within the direct environment. Because the water quality was not effectively managed, the pathogen survived and penetrated the fruit after hot and then cold water treatments.

Although the skin of orange fruit is not consumed, contamination by foodborne pathogens may spread to the edible part of the fruit during slicing, peeling or juicing (Martinez-Gonzales *et al.,* 2003). In 1995, a *Salmonella* outbreak was linked to drinking unpasteurised orange juice (Bates, 1999; CDC, 1999). In this particular case, the specific *Salmonella* spp. isolated was also obtained from a toad. Amphibians were found in the proximity of the processing plant that was also found to be open to the environment. Because the same bacterial species could be isolated from the patients, product and processing environment, the cause of the outbreak could be established. Thus, it was determined that the facility was lacking an efficient cleaning and sanitising programme.

Salmonella can cause disease at very low infective doses, reportedly as low as 10 cells (Harris *et al.,* 2003). Although the concept of zero tolerance is the ultimate objective in food safety systems it is often not realistic to achieve. Prevention through effective hygiene and food safety management provides an effective preventative tool but still requires sensitive detection methods in end point product inspection systems. In order to detect the pathogen at the lowest possible dose sensitive accurate and rapid methods are required (Riyaz-Ul-Hassan *et al*., 2004). Standard culturing methods are widely used for the detection of pathogenic organisms. These methods however are laborious and time

consuming. To overcome this, immunological and molecular methods have been employed (Aabo *et al*., 1993; Soumet *et al*., 1999a) such as the Polymerase Chain Reactions (PCR). The PCR combines simplicity and sensitivity in detection of foodborne pathogens (Riyaz-Ul-Hassan *et al*., 2004). In addition to the fact that PCR can give results within a day, it can also detect non-culturable cells in different substrates or the presence of antigens (Hoorfar *et al*., 1999).

The aim of this study was to assess the possible occurrence of *Salmonella* spp. in the citrus export chain from production in South Africa through picking, packing shipping and different environments such as harbours, repacking facilities, distribution centres and retail centres at export destinations. Another objective of this study was to identify presumptive *Salmonella* spp. isolated at different sampling points using molecular PCR.

4.3. Materials and Method

All experiments were performed in ISO 17025 accredited laboratories. All work was done in a biohazard safety cabinet and all areas exposed to the pathogen was thoroughly cleaned and disinfected afterwards. All staff and students working in the food safety laboratories have been trained in biohazard best practices and ISO principles.

4.3.1. Sample collection

Water, swab and fruit samples were taken at two packhouses in the Limpopo Province (Farm 2 and 3) and one in Mpumalanga. All three packhouses sampled during this study are EUREPGAP certified. Swab and fruit samples were also taken at the Ports in Cape Town and in Rotterdam (Netherlands), at three repack, -distribution and retail centres in Sweden, Germany and Belgium.

4.3.2. Water sampling

Two to three water samples (1 ℓ) were collected in sterile 1-ℓ plastic water bottles at watering points at different sites on the three farms and in the packhouses as described in Chapter 3, Table 2.1. and Appendices K, L and M. Not all watering points could be sampled three times because of seasonal changes and the fact that the citrus seasons were shorter than normal due to a drought.

Table 4.1. Water sampling points at the different farms and packhouses.

Farm 1 (Mpumalanga)	**Farm 2 (Limpopo)**	**Farm 3 (Limpopo)**
Source (purified river water from tap inside packhouse)	Dam water	River
Tank (Warm water tank kept at 42 ºC)	Water channel	Dam water
	Orchard (three points)	Water channel
	Chlorine tank (receiving tank)	Borehole
	Quatrokill tank	Orchard
	Warm water tank (45 ºC)	Chlorine tank (receiving)
	Imazalil spray	Warm water tank (38ºC)
	Bathroom	Chemical tank
	Handwash stations	Bathroom

The bottles were sterilised by washing them with disinfectant soap and rinsing with 70% ethanol. They were left to air-dry overnight under ultra violet light. The samples were taken aseptically by dipping the bottle in the water without opening the lid. Once the bottle was covered by the water, the lid was opened and the bottle was filled. Before removing the bottle from the water the lid were closed. Wherever water was collected from a tap, contact between the bottle and tap was avoided. Samples were transported to Plant Pathology Laboratories in cooler boxes and stored at 4 °C for no longer than 48 h till processing. All samples were collected during the period July to September 2005.

4.3.2.1. Water sample processing

One ml of the water samples were transferred to 9 ml quarter strength physiological saline Ringer's solution (Merck, Johannesburg) and serial dilutions of 10^{-1} to 10^{-4} performed. One ml of each of the dilutions, including the zero dilution (water sample), was transferred to three 9 ml buffered peptone water (BPW) (Biolab, Merck) tubes and incubated for 18 to 24 h at 37 °C. After this 100 µl of the BPW was then transferred to 10 ml Rappaport selective broth (Merck) and incubated at 42 °C for 18 to 24 h. After incubation a loop full was plated out on xylose lysine deoxicolate (XLD) (Biolab, Merck) agar and incubated at 37 °C for 24 h (Oragui *et al.,* 1993) and the results noted (Beliaeff & Mary, 1993).

4.3.3. Fruit sampling

Fruit samples were taken at different sites from a single consignment as the fruit moved through the export chain. Three replicates of four fruit each were collected at different sites from two Limpopo Province farms (Farms 2 & 3). At Farm 2, fruit was also taken from the top of the tree (three trees were selected per site for sampling) and from fruit hanging in the spray line of the irrigation system. Fruit were cut, using scissors disinfected with 70% ethanol, and the fruit dropped directly into the paper bags. Fruit was also taken from the transport trailers filled with harvested fruit destined for the packhouse, at the packhouse just after arrival and after the fruit was packed into boxes and palletilised. At Farm 3, fruit were randomly picked from three neighbouring trees, selected from the same orchard. At this farm fruit was only taken again after packing.

Four fruits were randomly selected from each of three boxes from the same original consignment taken at the Ports of Cape Town and Rotterdam (Netherlands). Similarly, fruit was collected after being re-packed in Belgium and Germany and at distribution and retail centrums in Belgium, Germany and Sweden.

All fruit was handled with washed ethanol sprayed hands, ensuring minimal contact and contamination. The four fruit replicates were placed together in a marked brown paper bags. Bags were placed in cooler boxes and transported to the Plant Pathology Laboratories for analysis. In South Africa fruit was directly transported to the laboratory

which arrived within 4 hrs, while fruit sampled overseas were returned to South Africa via DHL using an import permit from the National Department of Agriculture. Fruit was stored at room temperature (25 °C) for no longer than one week before processing.

4.3.3.1. Fruit sample processing

To retrieve possible *Salmonella* spp from the fruit surfaces, fruit was submerged in quarter strength physiological saline Ringer's solution and sonicated for 15 - 20 s. The presence or absence of *Salmonella* was determined as described in section 4.3.2.1.

4.3.4. Swab sampling

Transwabs (MW&E, United Kingdom) used for stabilising the inoculum and keeping cells buffered, were used throughout this study. Different points in the supply chain were sampled using three replicates per sampling site. Sampling points included picking bags (10 picking bags were randomly selected per farm), transport trailers (three trailers per farm), washroom taps (three taps per packhouse), hands of ten pickers, -sorters and -packers, three sites on the sorting bands, -metal rollers, -grading bands, -packing bins and floors in the packhouses. At Cape Town harbour the cold room (7 ºC) walls and floors and tarpaulin covering the fruit were swabbed (three sites each). Swabs were also taken from the boxes (three per consignment) in which the fruit was packed at the Cape Port cold room.. Swabs taken were immediately taken to Plant Pathology Laboratories for processing within a week.

At Rotterdam harbour (7 ºC) the walls and floors (three sites) of six containers (after offloading) and -trucks were swabbed. In the harbour, three cold rooms were selected and sampled at three sites each. Three boxes were further transported (at room temperature) to re- pack facilities in Belgium, Germany and Sweden . Boxes in which the fruit were packed were sampled after re-packing (three sites in the box). Swabs were taken at the re-pack centres in Belgium and Hamburg. Swabs were taken in the re-packing area (floors and walls, re-packing surfaces), cold storage room (7 ºC) floors and walls and also from the

repackers' hands (10). At the distribution centres, three cold rooms were selected (7 ºC) for walls and floors and at the retail centre swabs were taken from floors and walls of the retail area and the cold storage facility (three sites), display baskets (three sites) and customers hands (10).

Wet swabs were taken by placing the swab in the sterile buffering media and then swabbing the surface forming a rectangle of 5 cm x 5 cm for 20 s while rotating the swab. The swabs were replaced in the sterile buffering media and transported back to Plant Pathology Laboratories in cooler boxes and stored at 4 °C for no longer than one week. Swabs taken in Europe were returned to South Africa by DHL for processing within a week.

4.3.4.1. Swab sample processing

Swabs were processed by first incubating the swabs in 9 ml buffered peptone water (BPW) at 37 ºC for 24 - 48 h, after which a 100 µl was transferred to 10 ml Rappaport selective broth and incubated at 42 °C for 18 to 24 h. After incubation a loopful was plated on XLD agar and incubated as before at 37 °C. The presence or absence of *Salmonella* was determined as before.

4.3.5. Determination of the Gram status, shape and motility of the isolates

Environmentally isolated *S. enterica* sv. Typhimurium and *S. enterica* sv. Typhimurium (ATCC 14028) was used as reference culture. All colonies growing on XLD medium resembling the reference culture colony growth on the same medium were purified and preserved by freeze-drying (Dhingra and Sinclair, 1985). For further analysis working cultures were maintained and monitored for purity on nutrient agar plates. Gram status of the cultures was determined using the potassium hydroxide (KOH) test (Buck, 1982). All the Gram-negative isolates were examined under the microscope to determine shape and motility of the cells.

4.3.6. DNA extraction

DNA was extracted from the presumptive positive *Salmonella* isolates by resuspending a loop full of an overnight culture into 100 µl of distilled water and boiling it for 10 min to lyse the cells. After centrifuging for 30 s the supernatant containing the DNA was removed.

4.3.7. Determination of specific *Salmonella* serovars using Polymerase Chain Reaction

Two PCR assays were performed. The first primer set, ST11 and ST15 (Table 4.2.) is specific for the *Salmonella* genus and thus determines whether the organism is of this genus (Aabo *et al*., 1993). The second primer set Fli 1515 – Tym (Table 4.1.) was specific for the *Fli 15C* gene of *S. enterica* sv. Typhimurium encoding flagellin H1 (Soumet *et al*., 1999a).

Amplification reactions were carried out in a total volume of 25 µl, containing 0.3 µl (5 U) Taq polymerase (Bioline), 0.2 µl of each primer in a primer set, 0.5 µl of a 25 mM dNTP mix, 1.5 µl of a 10 X NH_4 PCR buffer, 1.5 µl (50 mM) $MgCl_2$. One µl of the bacterial DNA solutions was added. Sterile nonpyrogenic SABAX (The Scientific Group Adcock Ingram, South Africa) water was added to a final volume of 25 µl. The reaction was carried out in a Perkin Elmer 2400 Thermocycler. The cycling profile consisted of initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. The cycle was completed with a final terminal extension at 72 °C for 7 min.

Positive control reactions were also included with the ST primer set using *S. enterica* sv. Enteritidis, *S. enterica* sv. Dublin, *S. enterica* sv. Muenchen and three *S. enterica* sv. Typhimurium cultures (results not shown). Two different *S. enterica* sv. Typhimurium isolates were used in this study as positive controls. The first culture was received from the American Type Culture Collection (ATCC) and was a certified *S. enterica* subspecies *enterica* serovar Typhimurium (ATCC 14028) culture. The second culture was a wild type

S. enterica sv. Typhimurium isolated from water samples in the Free State Province of South Africa (Burke, 2005). This wild type culture was genetically modified by randomly inserting a green fluorescent protein (GFP) in the genome (Burke, 2005). This was done to simplify screening of the organisms. The unmodified wild type was used to confirm the suspicions that the GFP gene was inserted within the f*liC* region of the genome.

Table 4.2. Primers used for the detection of the genus *Salmonella* and the species *S. enterica* sv. Typhimurium.

Target sequence	Primer	Length	5'-3' primer sequence	PCR Amplicon size
Random genomic Fragment*	ST11	24	GCCAACCATTGCTAAATTGGCGCA	429
	ST15	25	GGTAGAAATTCCCAGCGGGTACTGG	
Fli 15C gene **	Fli 15	22	CGGTGTTGCCCAGGTTGGTAAT	559
	Tym	22	ACTCTTGCTGGCGGTGCGACTT	

* Aabo *et al*., 1999

** Soumet *et al.,* 1999a

4.3.8. Agarose gel electrophoresis

Amplified products (10 µl) were separated by electrophoresis on a 1.2% agarose gel run in 1X TBE buffer. The gel was stained with ethidium bromide (EtBr) and photographed under UV light. The images were visualised on a Vilber Lourmet imaging system and analysed with PhotoCapt.

4.4. Results

4.4.1. Detection of *Salmonella* species from water, fruit and swab samples

Only three of 50 water samples had colonies resembling *Salmonella* spp. on XLD plates. No fruit samples of 276 had any colonies resembling *Salmonella* spp. From the 1 051 swabs analysed, only three had colonies similar to those of *Salmonella* spp.

4.4.2. Determination of specific *Salmonella* serovars using PCR

None of the presumed positive isolates showed positive results for the PCR reaction Fig. 4.1). The identities of the unmodified wild type (Fig. 2), the genetically modified wild type (Fig. 3) and the certified (Fig. 4) *Salmonella enterica* sv. Typhimurium were verified using PCR.

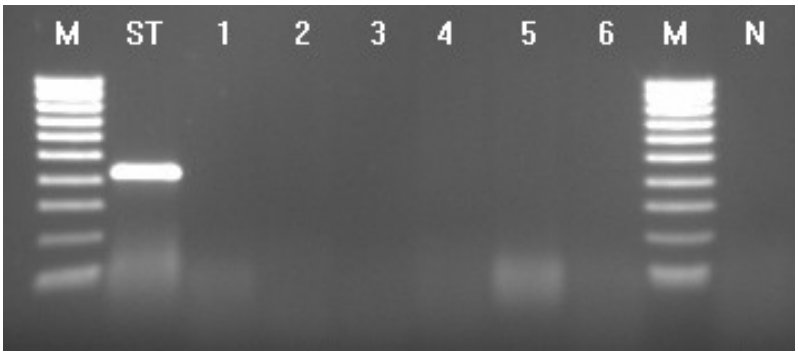

Figure 4.1. Analysis of presumptive *Salmonella* isolates isolated from water, fruit and swab samples from the Limpopo and Mpumalanga (South Africa) citrus farms using the ST11 and ST15 primer set.

(ST) A positive control of *Salmonella choleraesuis* subspecies *choleraesuis* serotype Typhimurium was included [ATCC 14028 (American Type Culture Collection, Manassas, USA)]

(1) Swab sample from sorter hands at Farm 2 (2) The chemical tank at Farm 3,

(3) The water channel at Farm 2 (4) Swab sample from the sorting bands at Farm 2,

(5) The handwash station at Farm 2 (6) Swab sample from sorter hands at Farm 2.

(M) Hyperladder IV (Bioline) (N) Negative control

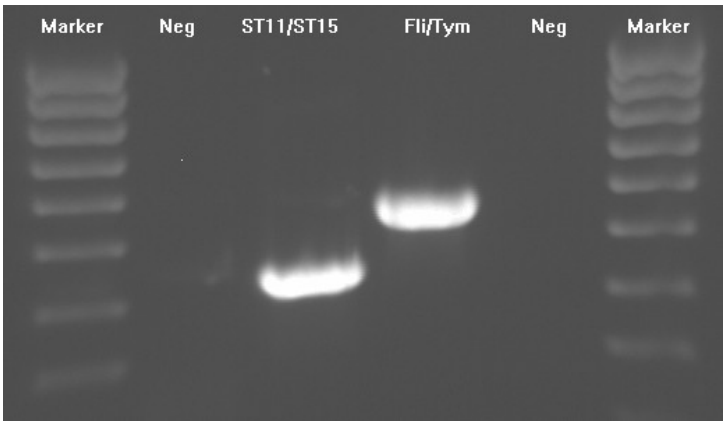

Figure 4.2. Verification of the identity of the unmodified wild type *Salmonella enterica* sv. Typhimurium used as reference culture.

ST11 and ST15 primer set specific for the genus *Salmonella* (band size 429) and Fli 15 and Tym primer set specific *Salmonella enterica* sv. Typhimurium (band size 559).

(N) Negative control (Marker) Hyperladder IV

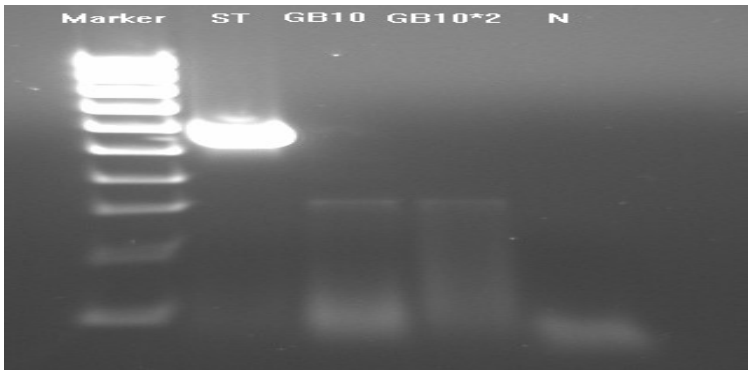

Figure 4.3. Verification of the identity of the genetically modified wild type *Salmonella enterica* sv. Typhimurium.

(ST) Certified *Salmonella enterica* sv. Typhimurium culture,

(GB10) Genetically modified *S.* enterica sv. Typhimurium culture using the Fli 15 and Tym primer set,

(GB10*2) second DNA extraction of the genetically modified *S.* enterica sv. Typhimurium culture using the Fli 15 and Tym primer set, (N) Negative control

(Marker) Hyperladder IV

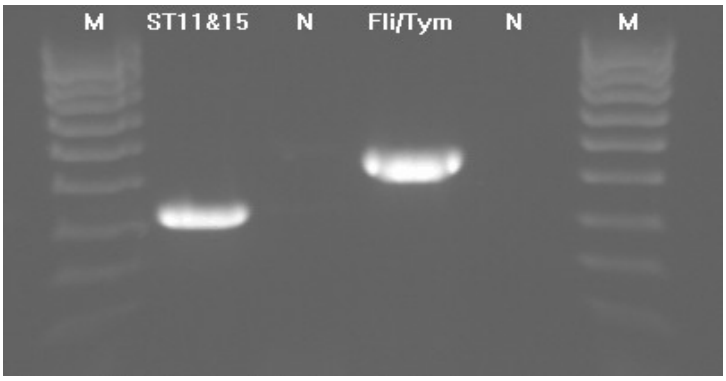

Figure 4.4. Verification of the identity of the certified *Salmonella enterica* sv. Typhimurium (ATCC 14028) strain.

ST11 and ST15 primer set specific for the genus *Salmonella* (band size 429) and Fli 15 and Tym primer set specific *Salmonella enterica* sv. Typhimurium (band size 559).
(N) Negative control (M) Hyperladder IV

4.5. Discussion

From all the swab, fruit and water samples, only six isolates represented typical *Salmonella* growth on selective medium. Potential suspect samples were, therefore, only 0.44%. In this study we could clarify the identity of the presumptive organism as not being *Salmonella enterica* sv. Typhimurium. Globally, *Salmonella* has been categorized as one of the most important agents causing foodborne diseases, with the most commonly isolated serotypes being *S. enterica* sv. Typhimurium and *S. enterica* sv. Enteritidis (CDC, 2004). Although most PCR systems have been developed to detect *Salmonella* spp. in samples from poultry or other meat and meat products after a selective pre-enrichment step (Soumet *et al*., 1999a; Soumet *et al*., 1999b; Urfer *et al*., 2000; Oliveira *et al*., 2002; Whyte *et al*., 2002; Myint *et al*., 2006; Esteves *et al.,* 2006), a few focus on fresh produce (Bhagwat, 2004; Hu Liming & Bhagwat, 2004) and water (Kong *et al.,* 2002). In this study, fresh fruit, environment and water samples could be tested for the presence of *Salmonella* spp using a selective enrichment and then a PCR confirmation approach. Bhagwat (2004), developed a PCR to detect *Salmonella* from vegetable rinse water and was able to detect numbers as low as 1 - 10 cfu/ml. Hu Liming & Bhagwat (2004) also developed a PCR to detect *Salmonella* from fruits and vegetables including alfalfa sprouts, cilantro, cantaloupes

and pre-packed mixed salads. Another PCR system was developed to detect *S. enterica* sv. Typhimurium from barley roots for a period of up to four weeks after inoculation (Kutter *et al.*, 2006).

Various PCR systems have been developed to detect *Salmonella* spp. from swab samples, after an enrichment step (Soumet *et al*., 1999a; Soumet *et al*., 1999b; Oliveira *et al*., 2002; Esteves *et al.,* 2006). However, most of these systems were developed for poultry samples. Esteves *et al.* (2006) monitored the spread of *Salmonella* through four Alheira processing plants and found 35 isolates of *Salmonella*. Four of these samples originated from water samples, five from the Alheira, one each from workers' hands and working surfaces. The majority of samples, however, were isolated from the meat products (Esteves *et al.,* 2006).

Primer set ST11 and ST15, specific for the genus *Salmonella* showed no amplified product at any of the suspected *Salmonella* cultures, obtained from the 1377 samples isolated through out the citrus chain sampled in this study. This primer set amplifies a random genomic fragment specific for the *Salmonella* genus (Aabo *et al*., 1993). The fragment was selected due to its hybridization to 396 *Salmonella* strains and not to any of the 214 non-*Salmonella* strains (Aabo *et al.,* 1993). These primer sets have successfully been used to identify cells of the *Salmonella* genus from swab samples, after enrichment, taken from poultry farms (Soumet *et al.,* 1999a; Soumet *et al.,* 1999b). In this study the primer sets could be used successfully but did not confirm the presence of any *Salmonella* spp.

Primer set Fli 15 and Tym were chosen to detect *S. enterica* sv. Typhimurium specifically. This primer set amplifies the *fliC* gene. The unmodified wild type *S. enterica* sv. Typhimurium without the GFP protein does amplify with both primer sets and amplifies the correct size bands. The primer set has been used successfully to detect *Salmonella enterica* sv.Typhimurium (Soumet *et al.,* 1999a; Soumet *et al.,* 1999b; Olivier *et al.,* 2002). The author could find no evidence that any of the primer sets have ever been used for detecting *S. enterica* sv Typhimurium from fresh produce. The PCR has been used in connection with unpasteurized citrus juice (Khan *et al.,* 2007) but not to detect *S. enterica*

sv Typhimurium from orange fruit surfaces or environments such as containers, pickers and packers' hands or bags, cold rooms and other production or transport surface.

Because all three packhouses are EUREPGAP certified, it means that they comply with certain good agricultural practices (GAP) which includes generic hazard analysis critical control point (HACCP) principles, environmental protection programmes and occupational health and safety welfare criteria (www.eurepgap.org/fruit/documents.html 1, 2006). All workers must receive basic instructions in these hygiene requirements before working with fresh produce, to help ensure the safety of the produce. Different types of surfaces were sampled during this experiment. The surfaces identified that can present possible contamination points was based on previous studies (unpublished data) and included, sorters' hands, sorting bands, chemical water tank, water channel and handwash stations. It is important to notice that three of the six samples that resembled typical *Salmonella* growth on selective medium are connected to water used in and around the packhouses.

From this study it can be concluded that *Salmonella* could not be detected in water used in irrigation, dip tanks, orange fruit or working surfaces that may come into contact with the fruit or its immediate surroundings. All of the previous studies mentioned incorporating a pre-enrichment step. Future studies should include the development of a method that excludes the pre-enrichment step. Future studies should also investigate the development of a multiplex PCR system to detect the major foodborne pathogens, i.e. *Salmonella* spp., *E. coli*, *Staphyllococcus aureus* and *Listeria monocytogenes*.

4.6. References

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Chapter 5: Monitoring the Survival and Spread of inoculated *Salmonella enterica* sv. Typhimurium in the Citrus Chain

5.1. Abstract

The number of nontyphoidal infections caused by *Salmonella,* linked to the consumption of fresh produce has increased over the past decade. This study assessed the survival of *S. enterica* sv. Typhimurium inoculated on citrus fruit under simulated export and cold storage conditions. *S. enterica* sv. Typhimurium were able to survive in numbers of up to 5 logs for up to four weeks on the surface of citrus fruit under simulated export conditions. However, *Salmonella* was not able to survive these conditions for a period longer than two to three weeks. Scanning electron micrographs showed that *Salmonella* was capable of attaching to and colonizing the citrus fruit surface. *Salmonella* cells were also able to incorporate into existing biofilms in warm water tanks of packhouses, showing that *Salmonella* can thrive and potentially be a continuous source of contamination to any fruit moving through the warm water tank. The importance of maintaining the cold chain is, therefore, critical. It is recommended that the cold chain should be maintained at all times throughout the transport and export of the fruit.

5.2. Introduction

Non-typhoidal infections associated with the consumption of fresh produce have increased over the past few decades (www.who.int/foodsafety/micro/general.html, accessed 11/2004). *Salmonella* is one of the leading foodborne pathogens causing disease, with an estimated 1.5 million cases of non-typhoidal infections annually (Yan *et al.,* 2003). *Salmonella* spp. has the capability of escaping human host defence systems by activating certain pathways (Yan *et al.,* 2003). This ability is considered to have been acquired by horizontal gene transfer and subsequent integration into the chromosome (Yan *et al.,* 2003).

Salmonella spp. has been implicated in foodborne illnesses originating from the consumption of a wide range of different food products, which include chicken products, tomatoes, sprouts, cantaloupes.. In 1955, 1979 and 1991 outbreaks of *Salmonella* infections were traced back to pre-cut watermelons (De Roever, 1998). Similar cases in 1990 and 1993 were linked to the consumption of fresh tomatoes (De Roever, 1998). Such outbreaks have also been linked to the consumption of fresh unpasteurized orange juice (Bates, 1999; CDC, 1999). According to Penteado *et al.* (2004) *Salmonella* spp. can even penetrate fresh produce, in particular fresh mangoes when transferred between hot and cold postharvest treatments

Green fluorescent protein (GFP) is synthesized by the jellyfish *Aequorea victoria*, and can be a very useful probe method to detect the presence of labelled organisms. The GFP can be inserted into a genome with no obvious side effects (Wimpenny, 2000). This approach is useful in studying host pathogen interactions (Valdivia *et al.,* 1996) and can be used to distinguish it from other types of fluorescent markers. The cells used as reference culture in this study were tagged using pUT mini-Tn5 Km transposons to insert the GFP into the chromosome of the *Salmonella* strains (Burke, 2005).

The occurrence of biofilm in a water or food production system can have severe technical and health consequences (Lee & Kim, 2003). The formation of sessile communities and their inherent resistance to disinfectants and sanitizers are potentially a significant cause of persistent deterioration of the microbiological quality of water (Lee & Kim, 2003).

Salmonella spp. are known to form biofilms in different environments under different conditions (Solano *et al.,* 2002). Studies showed that *S. enterica* sv. Typhimurium normally form better biofilms in nutrient-limited environments compared with nutrient-rich environments (Stepanović *et al.,* 2004).

Current citrus production practice in South Africa is to pick and pack citrus usually within a day, although fruit may stand over for another day or over the weekend in mid-season. Once packed fruit will be loaded and transported to the ports in Durban or Cape Town for export. This can take between two and seven days mostly at room . At the port the fruit is first cooled down to between 3.5 ºC and 11 ºC (PPECB, 2006). Once fruit pulp reached a temperature of approximately 4.5 ºC, fruit will be loaded into containers. After loading, transport to the export destinations can take an average of 16 days. On arrival fruit is placed in cold storage between 7 ºC and 14 ºC, before further distribution at room temperature, which may take up to seven days. Thus effective cold chain management does not exist in practice. The effect this may have on the presence growth and survival of microorganisms particularly if fruit potentially gets contaminated with foodborne pathogens, is essential to determine.

The aim of this study was, therefore, to monitor the attachment, colonisation and survival of *S. enterica* sv. Typhimurium if challenge inoculated onto fruit in simulated export and cold storage conditions. The study also investigated the potential incorporation of *Salmonella* in existing biofilms in packhouse dip tanks.

5.3. Materials and Methods

All experiments were performed in ISO 17025 accredited laboratories. All work was done in a biohazard safety cabinet and all areas exposed to the pathogen was thoroughly cleaned and disinfected afterwards. All staff and students working in the food safety laboratories have been trained in biohazard best practices and ISO principles.

5.3.1. *Salmonella enterica* sv. Typhimurium strain

Salmonella enterica subspecies *enterica* serovar Typhimurium isolated from fresh water sediment samples from the Venda and Free State regions of South Africa by Burke (2005) was used in this study. The strain was chromosomally tagged with green fluorescent protein (GFP) that is used to distinguish labelled organisms from others (Burke, 2005). Subcultures were stored in 30% glycerol with quarter strength Ringer's solution (Merck, Johannesburg, South Africa) at –70 °C. Identity of the organism was verified by viewing under a fluorescent microscope (Zeiss Axiovert 200 (excitation – 490 nm and emission – 510 nm), fitted with a 100**x**/1.4 Zeiss Neofluor objective.

5.3.2. Inoculum preparation

Cultures were revived on nutrient agar (Biolab, Merck) containing 100 µg/ml kanamycin (Merck). On the day before the experiment was done cultures were transferred to 500 ml sterile tryptone soy broth (Biolab) with 6% yeast extract (TSBYE) (Biolab) and incubated in a shaking incubator at 37 °C for 18 - 24 h. The 500 ml culture was then transferred into a Schott bottle containing 4.5 ℓ of sterile 0.1% buffered peptone water buffer (BPW) (Biolab) (Martinez-Gonzales *et al.,* 2003) making up a final concentration of approximately 10^7 cells/ml. This inoculum was used as the inoculum and reference culture throughout this study unless mentioned otherwise.

5.3.3. Inoculation of oranges

Twenty waxed and unwaxed early season Valencia oranges were obtained freshly picked from a packhouse in the citrus production region of the Limpopo province (South Africa). Before inoculation, oranges were sprayed with 70% ethanol and left to air- dry for 20 min (previously assessed as not harmful to surface wax structures (unpublished data). Waxed and unwaxed fruit samples were divided in two groups and immersed in 5 ℓ of culture suspension containing 10^7 cells/ml for either 3 or 5 min (Martinez-Gonzales *et al.,* 2003). The two different time intervals were used to optimise the experiment, establishing which time allows the most bacterial attachment to the orange surface. Fruit were left to air-dry

after treatments. Three untreated fruit were included in all experiments as control. This experiment was repeated twice.

5.3.4. Retrieval of *Salmonella enterica* sv. Typhimurium from oranges

After inoculation, according to the different experiments, individual fruits were washed in 400 ml 0.1% BPW for 15 – 20 s in an ultrasonic water bath (Ultrasonic Manufacturing Company, UMC-5, Krugersdorp, South Africa). The 0.1% BPW solution (1 ml) was transferred to 9 ml of quarter strength Ringer's solution and serial dilutions of 10^{-1} to 10^{-4} performed. The dilutions (1 ml of each) was transferred to three 9 ml BPW tubes and incubated for 18 to 24 h at 37 °C. After this, 100 µl of the BPW was transferred to 10 ml Rappaport selective broth (Merck,) and incubated at 42 °C for 18 to 24 h. After incubation, a loopful was plated out on xylose lysine deoxicolate (XLD) (Biolab) agar and incubated for 18 - 24 h at 37 °C (Oragui *et al.,* 1993) and the most probable number (MPN) was determined (Beliaeff & Mary, 1993). This method has a detection limit of 2 cells/cm^2.

5.3.5. Survival of *Salmonella enterica* sv. Typhimurium under simulated export conditions

To simulate the survival potential of *Salmonella* under different export conditions a spiking trial was initiated. A total of 36 untreated freshly harvested oranges from the citrus production region of the Limpopo province, were inoculated with a *Salmonella* suspension as described in 5.3.3. Eight of the oranges were washed (5.3.4) immediately after inoculation and allowed to air-dry at room temperature for a few minutes before a dilution series was done to retrieve cells. The counts (5.3.5) obtained from these oranges were used to determine the initial inoculation count of *Salmonella* on the oranges. The other 24 oranges were stored in citrus carton boxes at room temperature (24 ºC – 25 ºC) for one week. After the first week, eight oranges were individually removed aseptically with sterile gloves, washed and the average amount of *Salmonella* cells present on the eight fruit determined as described before. The rest of the fruit (16) were transferred to a cold room at

the Plant Pathology Laboratories, University of Pretoria, and stored at 4 °C for two weeks to simulate export conditions. Eight fruit were then removed and washed for total viable counts after 14 days in cold storage. The last eight fruit were placed at room temperature (24 ºC – 25 ºC) for a week, after which fruit was washed and total viable *Salmonella* counts done as described before. This experiment was repeated four times with fruit ranging from early to very late season. In the 2004 trial the oranges were early- to mid-seasonal fruit while in the first trial in 2005 mid- to late-seasonal fruit was used. In the second trial in 2005, (late-season), fruit was kept for an additional four weeks at 4 °C prior to using it in the trial. In the fourth trial, oranges used represented very early-season fruit.

5.3.6. The effect of temperature on survival of *Salmonella enterica* sv. Typhimurium

A total of 36 untreated freshly harvested fruit from the citrus production of the Limpopo province, were inoculated with a *Salmonella* suspension. Eight fruit were washed (5.3.4) immediately after inoculation to determine the initial count of *Salmonella* present on the fruit. The remaining 24 oranges were stored at 4 °C in a cold room, at the Plant Pathology Laboratories, University of Pretoria, for a period of four weeks. Oranges were removed at the same time intervals as in section 5.3.6.1. This experiment was done once in 2004 and repeated twice in 2005.

In both experiments of sections 5.3.6.1. and 5.3.6.2. controls of eight untreated oranges were included. All initial counts were taken at time interval zero.

5.3.7. Attachment of *Salmonella enterica* sv. Typhimurium to oranges moving through a dip tank in a small simulated commercial packline

This experiment was carried out at the Citrus Research Institute (CRI, Nelspruit, South Africa). A simulated commercial dip tank containing 200 ℓ of water was inoculated with *Salmonella enterica* sv Typhimurium, to a final concentration of 10^6 cells/ml in the tank. The water in the dip tank was kept at a temperature of 38 °C, as this is the most commonly

used industry practice. This temperature is also close to the optimal growth temperature for human pathogens. The ability of *Salmonella* to attach to citrus fruit surfaces was monitored over a period of four days. On day one, 10 untreated orange fruit were submerged into the spiked water into the dip tank for 5 min and put through a short simulated commercial pack line, after which fruit was stored at room temperature (2 4ºC – 25 ºC) for five days till analysis. Because this experiment was done at the CRI and no laboratory facilitiesor space in the cold room were available, the oranges had to be stored at room temperature until analysis. On day four, another 10 oranges were submerged in the spiked water in the dip tank for 5 min, removed, dried and stored at room temperature for 24 h till analysis for the presence of *Salmonella*. The pack line and all surfaces that came into contact with the *Salmonella*-inoculated fruit were clearly marked, washed and disinfected after each experiment. Controls of eight oranges were included by dipping the oranges in sterile water.

5.3.8. Monitoring the incorporation of *Salmonella enterica* sv. Typhimurium into existing biofilms

The aim of this experiment was to see whether *Salmonella* will be incorporated into an existing biofilm and therefore potentially represent a source of contamination to the fruit moving through the dip tank. In this experiment the role of *Salmonella* spp. in biofilm formation was investigated by first growing a biofilm on a microscope slide using the biofilm monitoring device. After this the device were transferred to the dip tank of the small simulated commercial pack line. The water of the dip tank was inoculated with the *Salmonella enterica* sv. Typhimurium labelled with GFP to a final concentration of 10^6 cells/ml. The *Salmonella* was allowed to grow on the microscope slides (two for each time interval), simulating natural conditions in the dip tanks. The water was kept at 38 °C for three days. The microscope slides were removed from the biofilm monitoring apparatus after 5, 24, 30, 48 and 72 h. The slides were permanently fixed using Histofluid (Marienfield, Germany). Because *Salmonella* used in this experiment contained the GFP protein, fluorescent microscopy was used to view the incorporation of the spiked

Salmonella into the biofilms. The slides were examined under an inverted Zeiss Axiovert 200 fluorescent microscope (excitation – 490 nm and emission – 510 nm), fitted with a 100x/1.4 Zeiss Neofluor objective. Images were captured using a Nikon charge-coupled (CCD) device.

5.3.9. Attachment and colonization of orange fruit surfaces by *Salmonella enterica* sv. Typhimurium

The whole fruit were sterilised with 70% ethanol and air-dried. A *Salmonella* culture was grown overnight to a concentration of 10^9 cells/ml and used to inoculate a 5 mm x 5 mm felt pen marked block on the surface of the orange. Four drops of culture were dropped onto the marked blocks and left for 1, 5, 10, 20, 30 and 60 min. Sterilised water was also dropped onto a block as control. Each marked block was rinsed with sterile water after each respective time interval. Afterwards the blocks were cut out and the sample prepared for analysis by suspending them in a fixing solution (2.5% Gluteraldehyde in 0.15 M Na/K phosphate buffer diluted to 50% with ddH_2O) (Coetzee & van der Merwe, 1994). Samples were stored at 4 °C for 24 h followed by three successive 15 min rinsing in 0.15 M Na/K phosphate buffer. The rinsing process was followed by successive 15 min dehydrations in 50, 70, and 90% ethanol and finally three times for 15 min in 100% ethanol which was left overnight before drying. Samples were dried in a Biorad drier (Biorad Polaron Division, England) under CO_2. Mounted specimens were coated for 2.5 min with 10mÅ of gold-palladium (Polaron Equipment Ltd., England) and examined under a JEOL (JSM-840) SEM operating at 5 kV.

5.3.10. Statistical analysis

All experiments were designed as a completely randomized design (CRD). Analysis of variance (ANOVA) was used to test differences between treatments. The data were acceptably normal with homogenous treatment variances. Treatments were separated using

Fisher's protected t-test least significant difference (LSD) at the 1% level of significance (Snedecor & Cochran, 1980). Data were analysed using the statistical programme GenStat (2003).

5.4. Results

5.4.1. Inoculation of oranges

No statistical difference could be found between numbers of *Salmonella* spp. retrieved from waxed or unwaxed and 3 or 5 min dipped fruit. Waxed fruit could retain higher numbers of *Salmonella* spp. compared to unwaxed fruit after a 3-min exposure although these differences were not statistically significant (Fig. 5.1., Appendix F). When repeating the experiment a similar trend was observed.

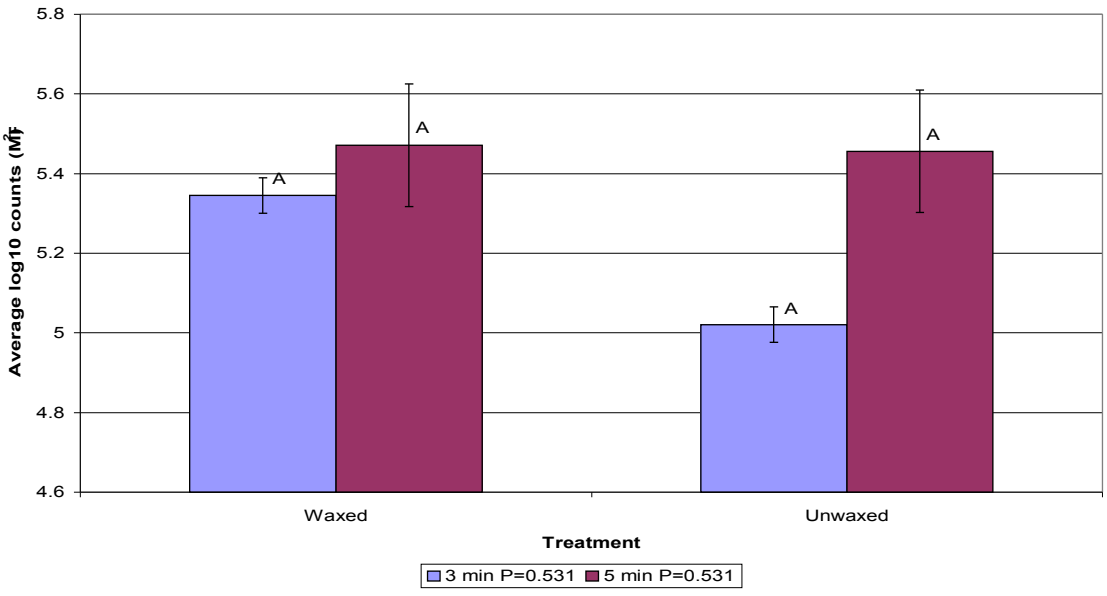


Figure 5.1. Retention of *Salmonella enterica* sv. Typhimurium from waxed and unwaxed fruit surfaces for 3 and 5 min. Value points with the same letter

did not differ significantly. Average $\log_{10}$ counts refer to the average log count of the most probable number (MPN) per cm^2.

5.4.2. Survival of *Salmonella enterica* sv. Typhimurium under simulated export conditions

The experiment to assess the survival potential of *Salmonella* on oranges under simulated export conditions showed a decrease in the numbers of viable cells over time (Fig. 5.2). . In the third and fourth trial, the *Salmonella* numbers initially increased during the first week when kept at room temperature. During the next two weeks at 4 °C and finally at room temperature, the numbers gradually decreased

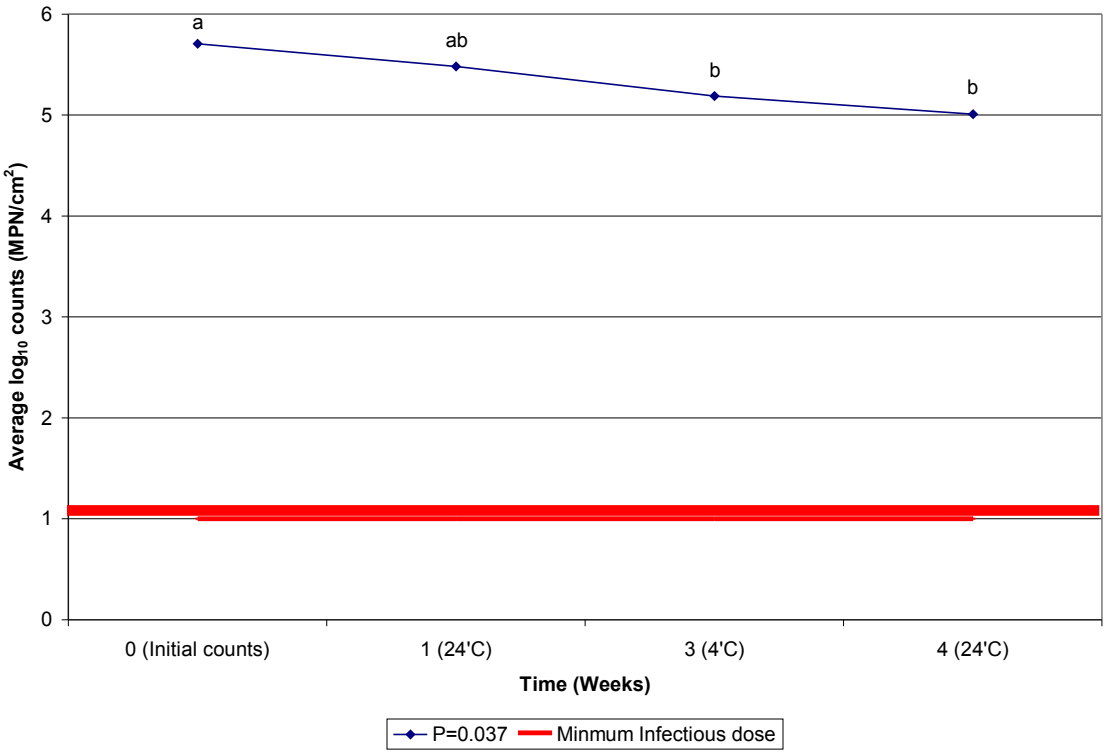


Figure 5.2. Monitoring the growth and survival of *Salmonella enterica* sv. Typhimurium under simulated export conditions (2005). Value points with

the same letter did not differ significantly (P=0.037). Average $\log_{10}$ counts refer to the average log count of the most probable number (MPN) per cm^2 of the four trials performed in 2005.

5.4.3. The effect of temperature on survival of *Salmonella enterica* sv. Typhimurium

In both trials done in 2004 and 2005, the number of *Salmonella* exposed to continuous cold storage conditions, decreased over time until none or almost no *Salmonella* could be detected from orange surfaces (Fig. 5.3).

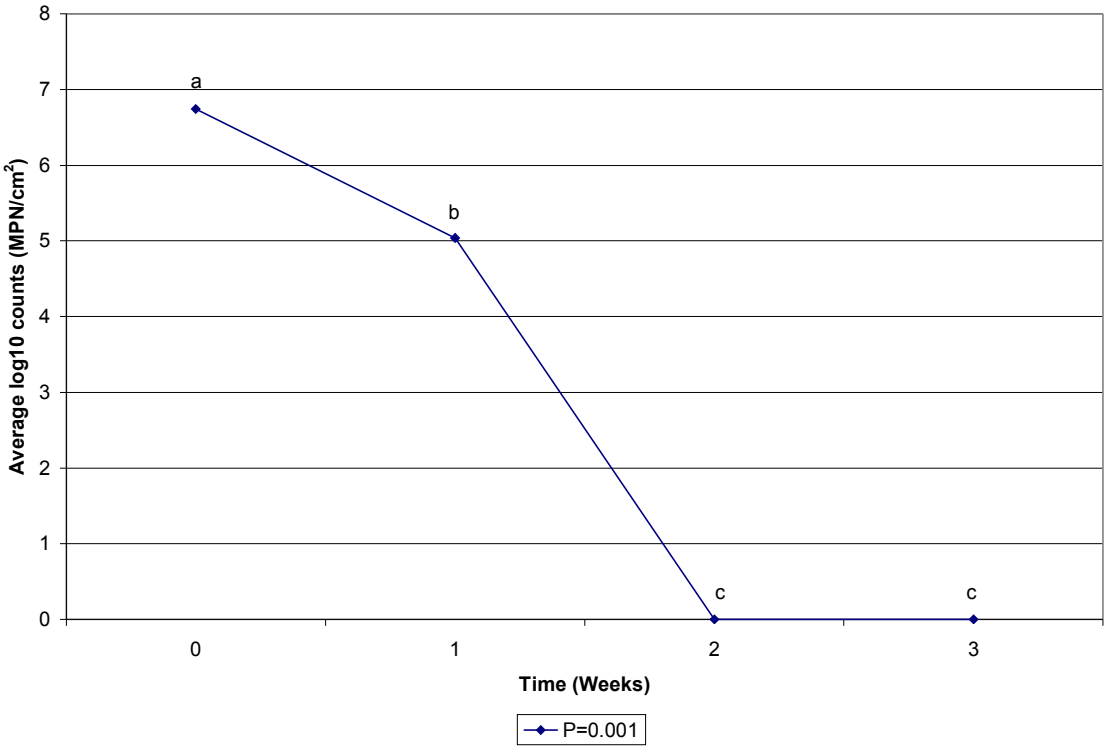


Figure 5.3. Growth and survival of *Salmonella enterica* sv. Typhimurium under cold storage conditions (4 °C) for a period of three weeks (trial 2). Value points with the same letter did not differ significantly (P=0.001). Average $\log_{10}$

counts refer to the average log count of the most probable number (MPN) per cm^2 of the trials performed in 2005.

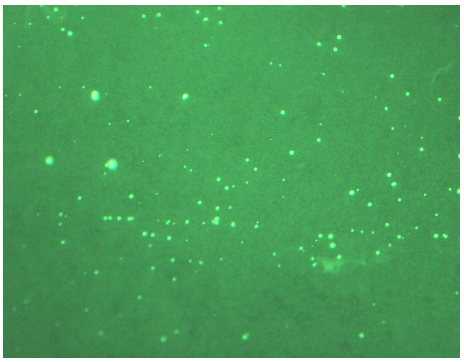
5.4.4. Incorporation of *Salmonella enterica* sv. Typhimurium into an already existing biofilm in a dip tank in a small simulated commercial packline

All oranges inoculated with 10^6 cells/ml in the semi commercial packline at the CRI showed almost no *S. enterica* sv. Typhimurium contamination. Only one of the 20 oranges, after being stored at room temperatures for four days, had detectable contamination of 5.66 x 10^3 cells/cm^2. Results indicated that *S. enterica* sv. Typhimurium, however, was incorporated into the biofilm as soon as 5 h after inoculation (Fig. 5.4.).

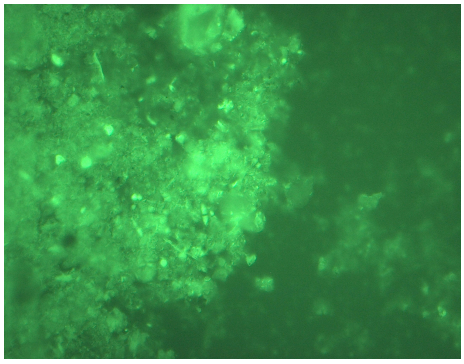
5.4.5. Attachment and colonization of orange fruit surfaces by *Salmonella enterica* sv. Typhimurium

After 1, 5, 10, 20, 30 and 60 min of inoculation, *Salmonella* could be observed on the orange fruit surface as well as its attachment structures (glycocalyx) Fig. 5.5.). Cells could also be seen replicating after 60 min time (Fig. 5.6) and forming extracellular polymer substrates (EPS) after 60 min (Fig. 5.7). The control SEM sample had no visible bacterial cells attaching. Glycocylax structures formed after one min, but cell density increased as time increased. The density of the cells were the densest after 60 min.

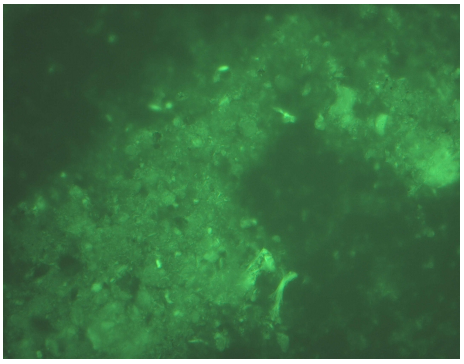
Negative control



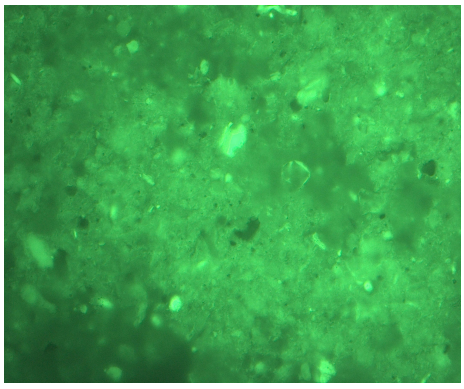
Positive control



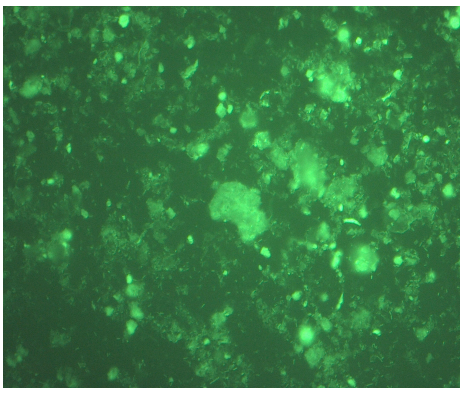
5 Hours



24 Hours

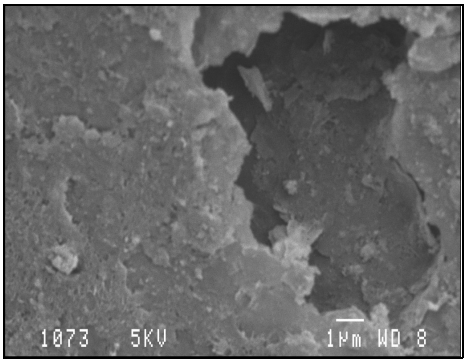


48 Hours



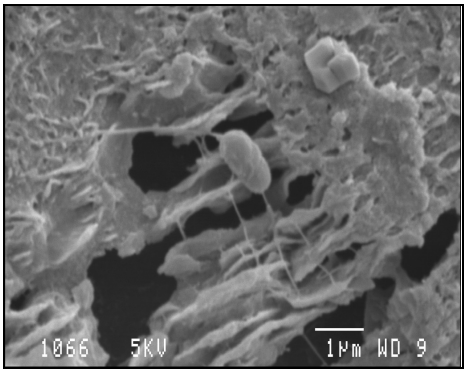
72 Hours

Figure 5.4. Incorporation of *Salmonella enterica* sv. Typhimurium into an existing biofilm over a period of 72 hours.



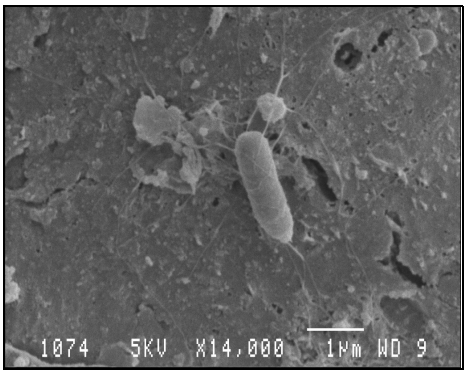
A

Control



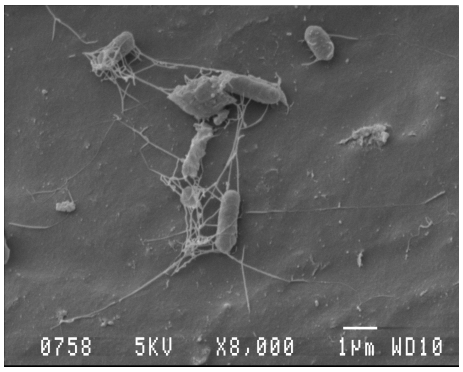
B

1 min



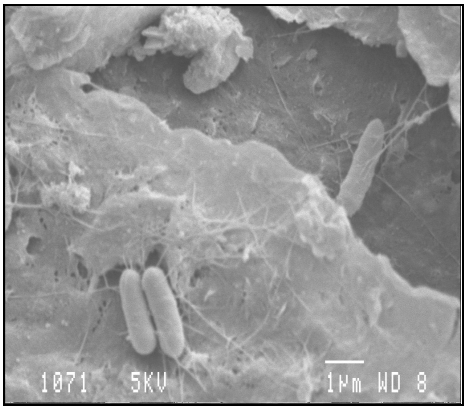
C

5 min



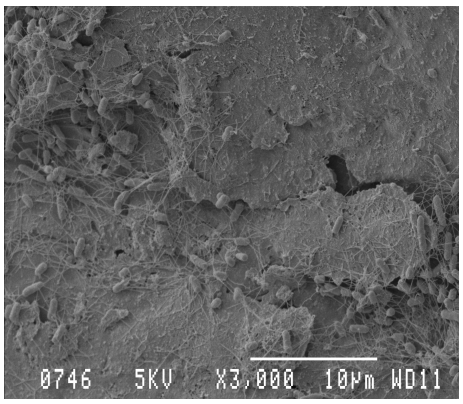
D

10 min



E

20 min



F

60 min

Figure 5.5. Scanning electron micrographs of a orange fruit surface inoculated with *Salmonella enterica* sv. Typhimurium culture with A) the negative control. B) 1, C) 5, D) 10, E) 20 and F)60 min.

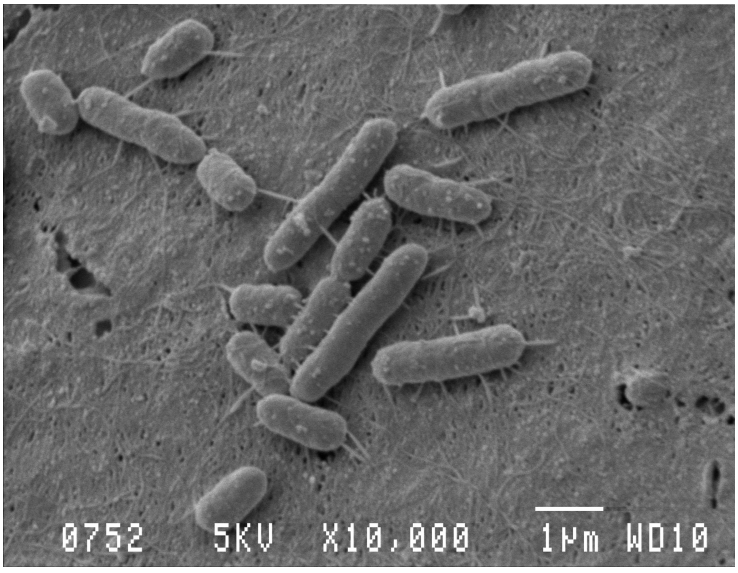


Figure 5.6. Scanning electron micrograph of *Salmonella enterica* sv. Typhimurium cells attaching to and dividing on orange fruit surface.

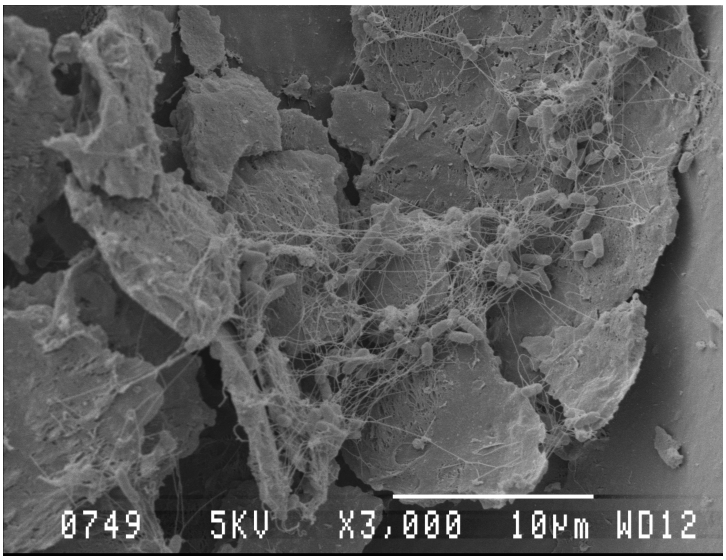


Figure 5.7. Scanning electron micrograph of *Salmonella enterica* sv. Typhimurium cells starting to form biofilm like structures on the surfaces of citrus fruit.

5.5. Discussion

This study clearly showed the ability of *Salmonella enterica* sv. Typimurium to attach to waxed or unwaxed fruit surfaces after being dipped for 3 or 5 min. Attachment of pathogens to fruit surfaces is facilitated by stomata, lenticels, bruises and cracks on the skin (Burnett & Beuchat, 2001). Decontamination treatments are also now less effective because of these protective structures (Burnett & Beuchat, 2001). In a study by Zhuang *et al,* (1995), it was found that the attachment and infiltration of *Salmonella* was due to a negative temperature difference between the tomatoes and water. This means that when water temperature is cooler than that of the produce, a higher number of cells will be taken up by the core tissue of the produce (Zhuang *et al.,* 1995). This, however, does not exclude the possibility of attachment and infiltration of cells when the fruit temperature is lower than that of the water.

The SEM study showed that S*almonella* cells were able to attach to the surface of the orange after 1 min. *Salmonella enterica* sv. Typhimurium is also able to survive for the whole period of the simulated export chain on the fruit surface. In the first trial (2004), the *Salmonella* cells multiplied and then reached a stationary phase during the room temperature period of the experiment (unpublished data). Although the number of *Salmonella* cells decreased gradually over the four-week period in the 2005 trial, under the different simulated export conditions, with a significant reduction of 0.698 logs, the reduction was not enough to eliminate the *Salmonella* contamination on the oranges. Here the importance of maintaining the cold chain by all parties involved is emphasised.

The infectious dose of *Salmonella* depends on the age and health of a human. It also depends on the strain differences within the genus. According to Harris *et al.* (2003) the

infectious dose for *Salmonella* spp. is anything between 10 and 100 000 cells. The infectious dose for humans for enteric fever is about 10^2 - 10^3 organisms, whereas the infectious dose for gastroenteritis salmonellas is 10^6 to 10^8 organisms (Lightfoot, 2003). The main reason for this is because *Salmonella* is susceptible to gastric acids (pH >4) (Lightfoot, 2003). In only one experiment, during cold storage conditions, could the level of *Salmonella* concentration be lowered to below the minimum infectious dose and that only after three weeks of cold storage conditions. During the simulated export conditions experiment, the level of *Salmonella* on the oranges stayed well above the minimum infectious dose.

Since 3 min inoculation practice has previously been shown as an optimum time to ensure adequate microbial attachment to fruit surfaces (unpublished data) and as was shown in this study, *Salmonella* cells did not preferentially attach to citrus fruit. The water bath used in this experiment simulates packhouse conditions and the 10^6 inoculum concentration used represented a realistic concentration for spiking trials. In nature contamination with a single pathogen within a water ecosystem will in all likelihood never represent 10^6 cells/ml. Yet this study showed the potential of attachment given the correct set of conditions.

As proven by Pao and Davis (2001) attached *Salmonella* cells on the surface of fruit, not kept at uninterrupted cold storage conditions, will be transferred to the edible part of the fruit during slicing or peeling. The very low number of *Salmonella* detected from inoculated oranges during the citrus spiking experiment might be because normal municipal tap water that was treated with chlorine was used to fill the water tank, the chlorine is toxic to the bacteria and killed the bacteria that were not incorporated into the biofilm (Beuchat & Ruy, 1997). It is known that free-living *Salmonella* cells are less capable of surviving in chlorinated water compared to cells that has been incorporated into a protective biofilm (Joseph *et al.,* 2001; Rayner *et al,* 2004).

It should be noted that the fruit from which *Salmonella* was isolated was dipped into the tank soon after spiking the water with *Salmonella*. These oranges were kept at room temperature for a period of four days before processing. The findings in this study confirm

that *Salmonella* can survive on citrus fruit at room temperature (25 °C) as previously shown. Because no *Salmonella* could be detected from the oranges that were dipped four days later, this would indicate that the *Salmonella* was incorporated into the biofilm and did not survive in the dip tank for that period of time with the reported chlorine level.

Sixty min. after inoculation, cell attachment, replication and colonization on the fruit surface could be observed. The formation of what seems to be a slime matrix after 60 min, could also be seen, which represents biofilm formation on the surface of the orange fruit. Because this experiment shows that *Salmonella* can attach to the surface of fruit, it could potentially have serious implications for the industry in terms of fruit safety assurance. If the fruit becomes contaminated with *Salmonella* and the pathogen colonises the fruit surface, even if the fruit is exported or kept at cold storage conditions, the *Salmonella* could survive and potentially pose a risk to the consumer. This is particularly true if the citrus is used as un pasteurised juice where several examples already exist of outbreaks.

In all the temperature trials in 2004 (unpublished data) and 2005, *Salmonella* did not survive for longer than three weeks at 4 °C. These results together with the results of the simulated export chain highlighted the importance of effective cold chain management when transporting fruit throughout the supply chain to the consumer, as it can help to control *Salmonella* contamination on fruit. It is important to effectively maintain the cold chain to minimise stimulation of growth of microorganisms, which may be present on the fruit surface.

In the first 2005 simulated export trial, the *Salmonella* cells initially decreased in numbers when fruit was kept at room temperature. It continued to decrease when fruit was placed at 4 °C the following week. The numbers subsequently increased during the last week when the fruit was placed at room temperature. Variation in results between the different trials could be related to the physiological condition of the fruit (Obagwu & Korsten, 2003). In the 2004 trial the oranges were early- to mid-seasonal fruit while in the first trial in 2005 mid- to late-seasonal fruit was used. During the second trial in 2005, (mid - late-season), fruit was kept for an additional four weeks at 4 °C prior to using it in the trial. In the fourth

trial, oranges used represented very early-season fruit. Zagory (1999), concluded that viable populations of aerobic spoilage organisms might be a hurdle to the growth of human pathogens. Some of the oranges in the third and fourth trial showed signs of decay and fungal growth during the experiment which, in combination with physiological conditions of the fruit, could explain the similarity found in the counts of *Salmonella* cells isolated from the inoculated oranges in these experiments.

Salmonella enterica sv. Typhimurium was found incorporated into an existing biofilm in a packhouse with water maintained at 38 °C and only being replenished two to three times in the citrus season. These results show that if *Salmonella* contaminates water it could survive and be incorporated into the biofilms in commercial packhouses, given the current production practices. The occurrence of biofilms in water or food production system can have severe technical and health consequences (Lee & Kim, 2003). The formation of sessile communities and their inherent resistance to disinfectants and sanitizers are a significant cause of persistent deterioration of microbiological quality of water (Lee & Kim, 2003). *Salmonella* spp. are known to form biofilms in different environments under different conditions (Solano *et al.,* 2002). *Salmonella* can form biofilms on all kinds of surfaces such as plastic, metal and glass (Stepanović *et al.,* 2004). Studies showed that *S enterica* sv. Typhimurium normally forms better biofilms in nutrient-limited environments than in nutrient-rich environments (Stepanović *et al.,* 2004). This explains the rapid biofilm formation in both the dip tanks tested in the commercial (Chapter 3) and semi-commercial trials. This may represent a risk as the biofilms provide a continuous source of *Salmonella* contaminated water (Costerton, 1999).

It is recognised that the levels of *Salmonella* used in this study are far greater than may be found in packhouses. However, the numbers of bacteria that were used could be readily detected by the methods used in this study. Under natural conditions even very low numbers of *Salmonella* contamination could represent a significant health risk (Harris *et al.,* 2003).

Future studies should therefore be done to investigate whether *Salmonella* cells are able to multiply to high enough numbers to cause disease after export and cold storage conditions. Studies should further determine the minimum concentration of *Salmonella* able to survive the export chain and cold storage conditions.

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Chapter 6: Concluding Remarks

Citrus is an economically important crop produced globally and prominent in world trade (Spiegel-Roy & Goldsmith, 1996). Brazil, Spain, the USA and China are the four biggest producers and exporters of citrus. Africa produces about 7% of global production representing 67 362 564 tonnes of fresh fruit in world trade (PPECB, 2006). South Africa is a major exporter and is currently ranked third in terms of trade volumes, despite being only the world's fourteenth largest producer (Barry, 1996). Microorganisms most commonly associated with foodborne illness include Sa*lmonella* spp., *Campylobacter* spp., *Escherichia coli*, *Listeria* spp. *Staphylococcus aureus*, Hepatitis A virus and Jejuni spp. (www.who.int/foodsafety/micro/general/en.html, 2004). *Salmonella* has been implicated in diseases caused by the consumption of fresh-cut watermelons (Blostein 1993), mangoes (Penteado *et al.,* 2004), tomatoes (Cummings *et al.,* 2001; CDC 2002a; CDC 2005), unpasteurized orange juice (Bates 1999; CDC 1999), sliced cantaloupes (CDC 1991; CDC 2002b) and sprouts (Mahon *et al.,* 1997; CDC 2001; Van Duynhoven *et al.,* 2002). In some of these cases, water was found to be the source of contamination (Penteado *et al.,* 2004). *Salmonella* is, therefore, known to be transmitted via water to the fresh produce and represents a potential health risk for the consumer. *Salmonella enterica sv.* Typhimurium was identified as the causative agent in a disease outbreak that was associated with the consumption of fresh unpasteurized orange juice (Bates, 1999). In this case the source was xx. *Salmonella* can become sequestered in biofilms (Solano *et al.,* 2002), which provides increased protection against chemicals such as disinfectants (Costerton *et al.,* 1999). In order to determine the source of contamination in the citrus export chain, the quality of agricultural irrigation and packhouse water was determined, as was the survival potential of *Salmonella* in biofilms in packhouse dip tanks. The potential of *S. enterica sv.* Typhimurium to attach to citrus fruit surfaces, survive cold storage conditions and contaminate fruit moving through a spiked water dip tank in a simulated packline, was studied.

Biofilms were formed at an increased rate in warm water dip tanks of citrus packhouses. This is of some concern because biofilms can harbour a number of human pathogens. In a

subsequent shapter it was found that *S. enterica sv.* Typhimurium could be incorporated into the biofilm. If a pathogen such as *Salmonella* is incorporated into a biofilm in a packhouse dip tank, it is possible that the pathogen can increase and that flakes of biofilm can break off and be distributed, reattaching to fruit surfaces (Costerton *et al.,* 1999). Because biofilms provide increased protection, it is more likely that the pathogens will survive in such protected environments (Costerton *et al.,* 1999). In general most agricultural water is by nature polluted and reflect a general high indicator organism count. More than 50% of the water samples tested in this study had higher than the allowable limit of 10 MPN/100 ml, and more than 30% of the samples tested had higher than the allowable limits for *E. coli* of 1 MPN/100 ml (SABS, 2006). This indicate that water may pose a potential risk in terms of contamination of fresh produce. To what extent *S. enterica sv.* Typhimurium occur in general contaminated water remains to be determined.

Comparing water management practices on three farms (Appendix O), it was found that despite Farm 3 using clean borehole water and changing the dip tank water on a weekly basis, it had the highest bacterial and coliform counts. In this situation an average of 100 tonnes of fruit moved through the warm water dip tank on a daily basis. Compared to the farm that used contaminated water from a dam, the packhouse warm water dip tank had the lowest bacterial and coliform counts of all three farms. The warm water tanks at both Farm 1 (42 ºC) and 2 (45 ºC) were kept at higher temperatures than that of Farm 3 (38 ºC). A number of agricultural chemicals, Decomone, Imazalil sulphate, Kenopel, Ethrel, were also added to the water at Farm 1 and 2 contributing to the reduced bacterial and coliform counts.

No *Salmonella* spp. were isolated from any water samples tested from the three packhouses. Similarly, no *Salmonella* spp. were found on fruit samples, pickers' hands, trolleys, picking bags, washroom taps, sorters, packers, sorting bands, metal rollers, grading bands, packing bins and floors tested in this study. Although this study show the absence of *Salmonella* spp. in the South African citrus export chain, it remains an important issue to continually monitor as sample since might not truly reflect the general trend in the industry as a whole.. *Salmoenlla enterica* sv. Typhimurium is able to survive in

an interrupted cold chain for as long as four weeks in numbers higher than the minimum infectious dose. *Salmonella* could, however, not survive for longer than three weeks at continuous cold storage conditions. These findings emphasize the importance of maintaining the cold chain throughout the production and export chain. *Salmonella* was also incorporated into an already existing biofilm in a packhouse dip tank, and attached to the fruit surface after 5 s. This is important because biofilms provide increased protection and a protective environment for these organisms.

All these findings emphasize the fact that all role players participating in fruit production, packing, storage and postharvest handling of fresh fruit should be involved in ensuring fruit safety. Pre- and postharvest practices can affect safety of the product, and incorrect handling during marketing may nullify all previous efforts of safety assurance (Combrink *et al.,* 1994). Future studies should focus on determining the minimum concentration of *Salmonella* spp. able to survive the export chain and the development of a rapid system to indentify contamination points and the detection of waterborne and foodborne pathogens. These stydies should also include the establishment of appropriate microbiological standards for packhouse hygiene and agricultural water.

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APPENDIX A. Average $\log_{10}$ counts of Farm 1 on different sampling dates

		Date		Date		Date	
Sampling points	**n**	**15/8/2005**	**Standard Deviation**	**17/8/2005**	**Standard Deviation**	**19/8/2005**	***Standard Deviation***
Tank	3	5.6826A,a	486667 ± 89628.86	7.477A,b	30000000 ± 0	8.2708A,c	189666667 ± 4154877
Source	3	3.9332B,x	9333 ± 4932.883	3.665B,x	7000 ± 5291.503	5.0343B,y	108667 ± 11846.1
SEM		0.0924		0.236		0.0447	
F probability		<0.001		<0.001		<0.001	
CV%		3.3		7.3		1.16	

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX B. Average log_{10} counts of Farm 2 at different sampling points

Sampling point	n	Log_{10} means	SEM	Standard Deviation
Quattro tank	9	6.904A	0.509	107315778 ± 44824031.2
Chlorine tank	6	1.000C	0.623	333 ± 516.40
Warm Water tank	9	4.969B	0.509	100006333 ± 149996666.6
Imazalil spray	6	3.252BC	0.623	52167 ± 44174.65
Dam	9	7.595A	0.509	94033333 ± 118962178.9
Water channel	6	7.249A	0.623	100305000 ± 10932977636
Orchard 1	6	6.873AB	0.623	7683333 ± 1995419.76
Orchard 2	6	6.869AB	0.623	8416667 ± 4215447.78
Orchard 3	6	6.976AB	0.623	40588333 ± 46625100.54
Bathroom	6	8.226A	0.623	199166667 ± 112423307.2
Handwash stations	6	6.830AB	0.623	9998333 ± 8075394.73
F probability	<0.001			
CV%	25.0			

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX C. Average log_{10} counts of Farm 2 at different sampling groups

Sampling points	n	Log_{10} means	SEM	Standard Deviation
Packhouse	30	4.412A	0.37	62207133 ± 121107390.4
Source	15	7.457B	0.524	96542000 ± 111202518
Hygiene	12	7.528B	0.586	104582500 ± 124635468.5
Orchard	18	6.906B	0.478	18896111 ± 29916550.6
F probability	<0.001			
CV%	33.2			

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX D. Average log_{10} counts of Farm 3 at different sampling points

Sampling points	n	Log_{10} means	SEM	Standard Deviation
Warm water tank	9	7.525B	0.489	119607779 ± 13834013
Chemical tank	6	2.438A	0.599	37500 ± 41079.19
Chlorine tank	6	2.500A	0.599	333500 ± 516265.44
Orchard	6	7.361B	0.599	129750000 ± 147370282
Borehole	6	5.858B	0.599	1307833 ± 1199124.7
Dam water	6	7.118B	0.599	21900000 ± 30977573
Water channel	6	6.616B	0.599	13096667 ± 21794036
River	6	7.481B	0.599	41583333 ± 34480429
Bathroom	6	7.370B	0.599	11347333333 ± 146939763
F probability	<0.001			
CV%	24.0			

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX E. Average log_{10} counts of Farm 3 from different sampling groups

Sampling points	n	Log_{10} means	SEM	Standard Deviation
Packhouse	24	5.106A	0.464	77112333 ± 123121891
Source	24	6.768A	0.464	19471958 ± 28220560
Hygiene	6	7.370A	0.929	113473333 ± 146938763
Orchard	3	6.325A	1.314	2166667 ± 590956.85
F probability	<0.05			
CV%	37.3			

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX F. Average $\log_{10}$ counts of waxed and unwaxed orange fruit inoculated for a period of 3 and 5 min

		Time			
Treatment	**n**	**3 min**	**Standard Deviation**	**5 min**	**Standard Deviation**
Control (Waxed and Unwaxed)	3	0	0	0	0
Waxed	3	5.345A	378400 ± 441873.15	5.471A	310667 ± 119918
Unwaxed	6	5.021A	150217 ± 139191.24	5.456A	504333 ± 460649
SEM		0.4818			
F probability		0.531			
CV%		9.1			

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX G. Average log_{10} counts of *Salmonella enterica* sv. Typhimurium on inoculated oranges, over a period of four weeks during simulated export conditions. Fruit was kept at room temperature for one week, after which it war stored at 4 °C for a further two weeks. After two weeks the fruit was removed from the cold storage and kept at room temperature for another week

Time period	n	Log_{10} Means	Standard Deviation
Control	8	0	0
Week 0	8	5.706A	3761167 ± 7332666
Week 1	8	5.481AB	2494304 ± 5813174
Week 3	8	5.187B	681854 ± 1410354
Week 4	8	5.008B	2651407 ± 7166379
SEM	0.1800		
F probability	<0.05		
CV%	16.5		

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 5% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX H. Average log_{10} counts of *Salmonella enterica* sv. Typhimurium on inoculated oranges, over a period of four weeks during cold storage (4 °C) conditions

Time period	n	Log_{10} means	Standard Deviation
Control	8	0	0
Week 0	8	6.74A	10731500 ± 9712518
Week 1	8	5.04B	2242625 ± 3630565
Week 2	8	0.00C	0 ± 0
Week 3	8	0.00C	0 ± 0
SEM	0.412		
F probability	<0.001		
CV%	39.6		

SEM is the standard error of the means.

Mean per column followed by the same letter did not differ significantly at the 1% level.

Fisher's protected t-test least significant difference (LSD) was used.

CV is the coefficient of variance.

APPENDIX I. Pilot trial: Survival of *Salmonella* under cold storage conditions

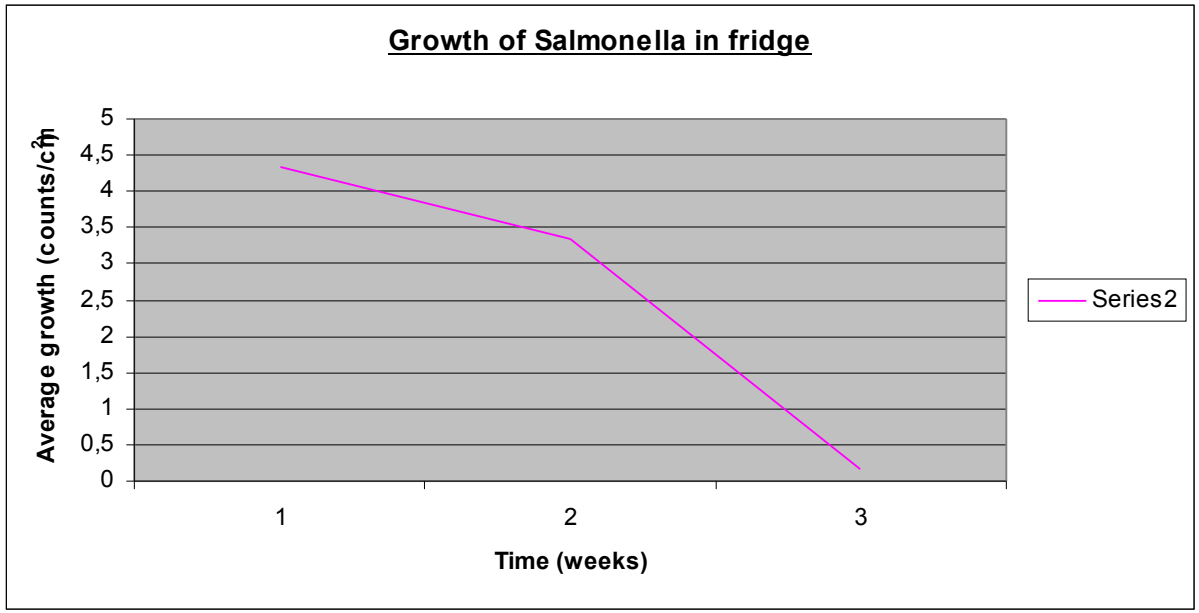

Figure 1. Monitoring the growth and survival of *Salmonella* on citrus kept under cold storage (4 °C) conditions over a period of three weeks.

APPENDIX J. Pilot trial: Survival of *Salmonella* under simulated export conditions

Figure 1. Monitoring the growth and survival of *Salmonella* under simulated export conditions. Fruit were kept at room temperature for one week, 4º C for two weeks and again at room temperature for one week.

APPENDIX K. Sampling points and pack house layout of Farm 1

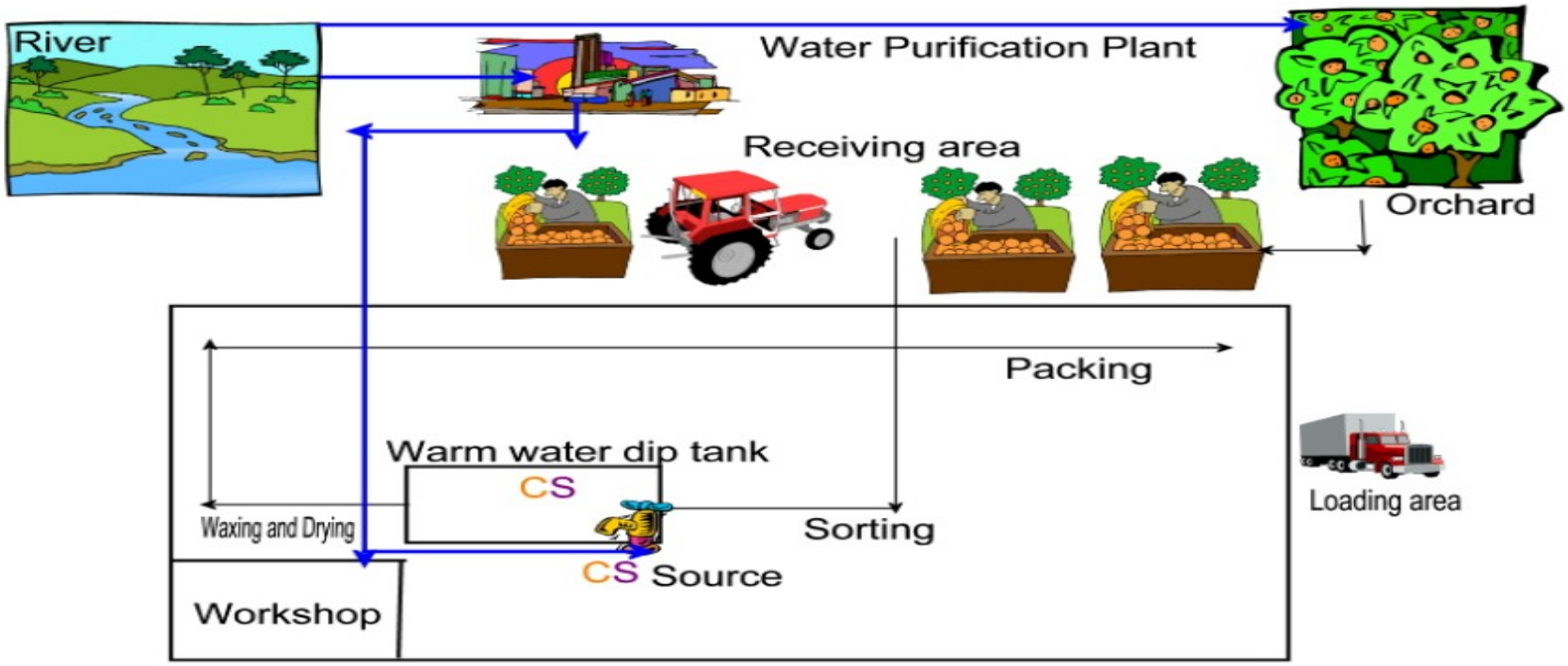

Figure 1. Sampling points and packhouse layout of Farm 1. Sampling points are indicated with the letter 'S'. Coliform counts higher than the allowable limit are indicated by the letter 'C'. If only a 'S' appears at a sampling point it means thatthe specific sampling point falls within the allowable limits for drinking water as per the national requirements (SANS 241:2005). The blue line indicates the source of water used to fill the dip tank. The black line indicates the flow of fruit through the citrus production chain.

APPENDIX L. Sampling points and packhouse layout of Farm 2

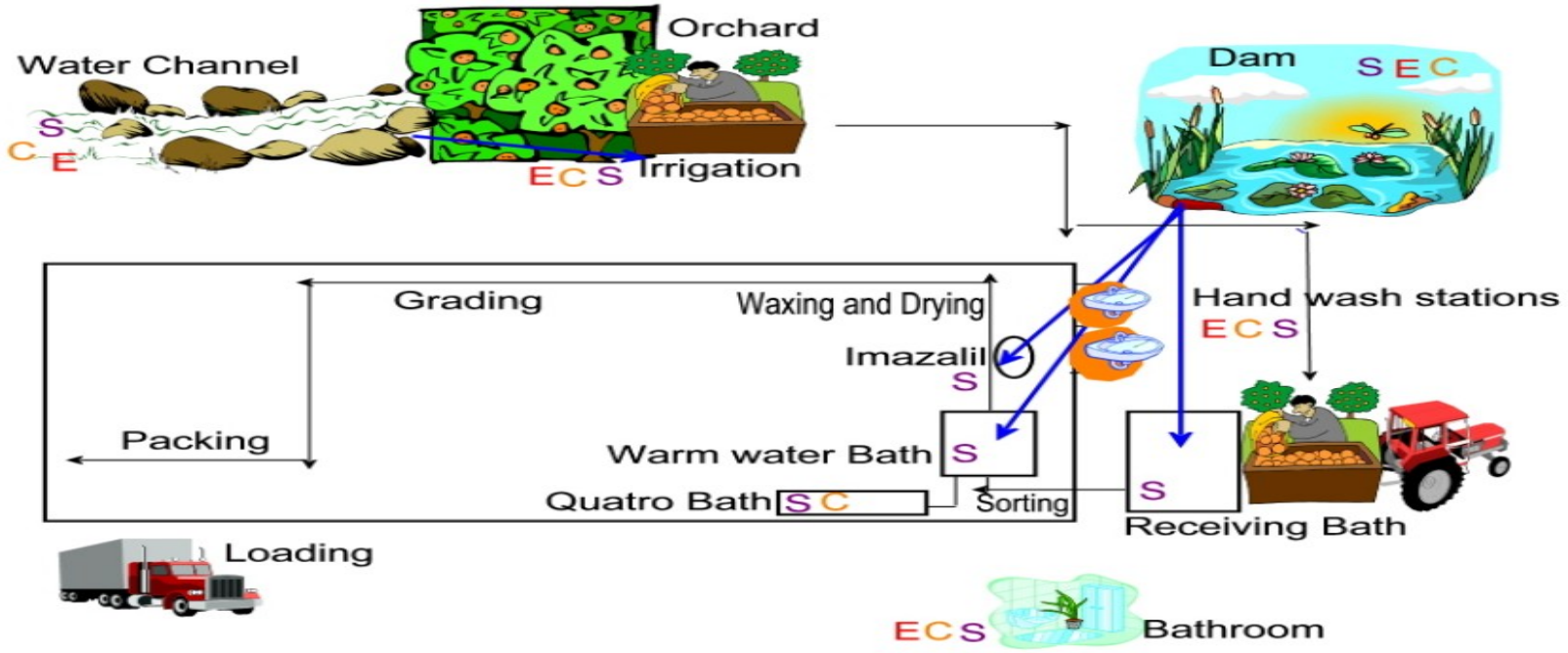

Figure 1. Sampling points and pack house layout of Farm 2. Sampling points are indicated with the letter 'S'. Coliform counts higher than the allowable limit are indicated by the letter 'C'. *E. coli* counts higher than the allowable limits are indicated by the letter 'E'. If only a 'S' appears at a sampling point it means thatthe specific sampling point falls within the allowable limits for drinking water as per the national requirements (SANS 241:2005). The blue line indicates the source of water used to fill the dip tanks. The black line indicates the flow of citrus fruit through the citrus production chain.

APPENDIX M. Sampling points and packhouse layout of Farm 3

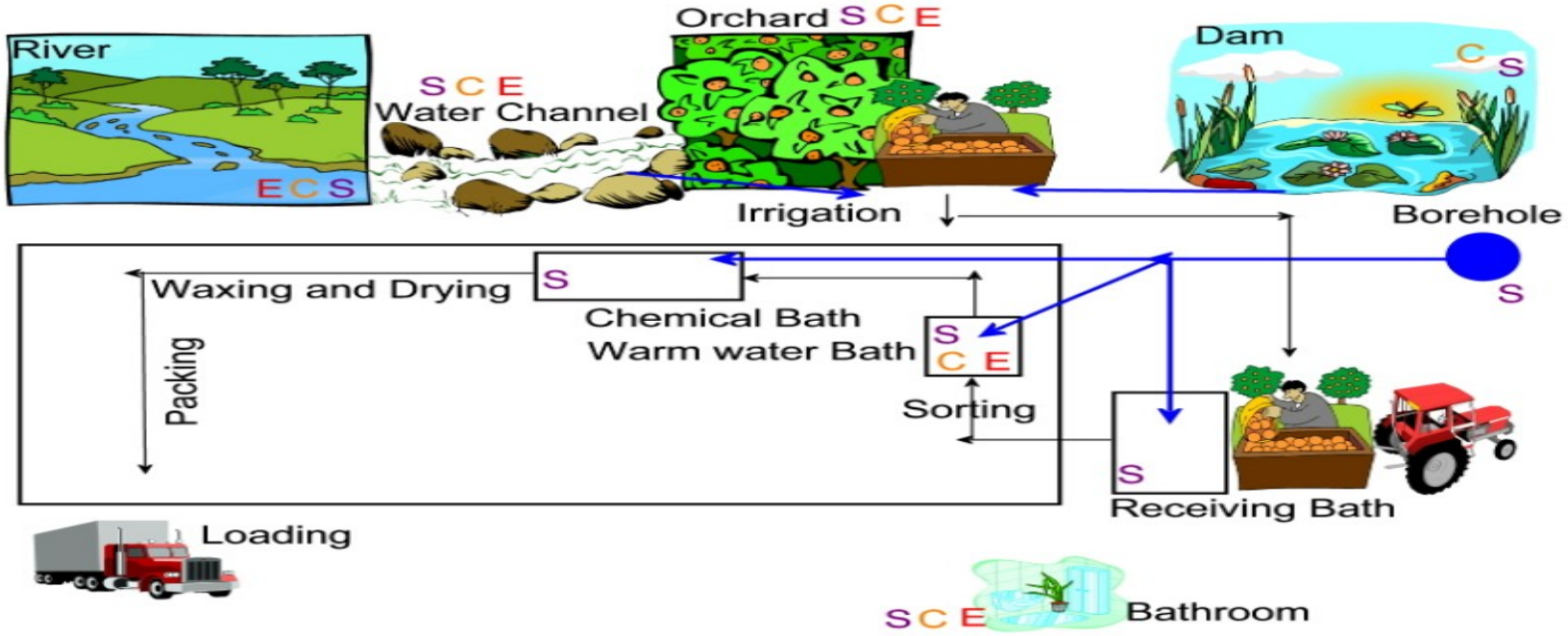

Figure 1. Sampling points and pack house layout of Farm 3. Sampling points are indicated with the letter 'S'. Coliform counts higher than the allowable limit are indicated by the letter 'C'. *E. coli* counts higher than the allowable limits are indicated by the letter 'E'. If only a 'S' appears at a sampling point it means thatthe specific sampling point falls within the allowable limits for drinking water as per the national requirement (SANS 241:2005). The blue line indicates the source of water used to fill the dip tanks. The black line indicates the flow of fruit through the citrus production chain.

APPENDIX N. Chemicals added to dip tanks studied

Table1. A list of the chemicals added to dip tanks of the three farms

Point of introduction	Farm 1	Farm 2	Farm 3
Receiving tank	N/A	Chlorine (Chlorine; HTH)	Chlorine (Chlorine; HTH)
Warm water tank	Imazalil sulphate (Imazalil sulphate) Universal Crop Protection Pty. (Ltd.); Meridian Agrochem Company (Pty) Kenopel (Guazatine) Makheshim AgriSA Pty. (Ltd)	Decomone (2,4-D) Bayer Ethrel (Ethephon) Bayer	Harvest wash activator (Chlorine dioxide) BTC Products and Services
Chemical tank	N/A	N/A	Imazalil sulphate (Imazalil sulphate) Universal Crop Protection Pty. (Ltd.); Meridian Agrochem Company (Pty) Ultracure (Guazatine) Natural Crop Protection (Pty.)

Table 1. Continued

			Megacide (Quazatine) ICA International Chemical Pty. (Ltd.) Decomone (2,4-D) Bayer

Sprays	N/A	Imazalil sulphate (Imazalil sulphate) Universal Crop Protection Pty. (Ltd.); Meridian Agrochem Company (Pty) Sporekill (Didesyl dimethyl ammonium chloride) ICA International Chemical Pty. (Ltd)	Harvest wash activators (Chlorine dioxide) BTC Products and Services

N/A – Not Applicable.

All chemicals are listed as used in the packhouses during the duration of this study.

All chemicals are replenished on a daily basis or per load basis.

APPENDIX O. A summary of warm water dip tank management at the three farms tested in this study

Table 1. A summary of warm water dip tank management practices at the three Farms tested in this study

Source	Contamination level of warm water dip tank	Dip tank temperature	Replenish rate	Average volume of tank	Average tonnes of fruit passing through tank per day

Farm 1	Purified River water	<1 MPN/100 ml *E.coli* <1 MPN/100 ml Coliforms	45 ºC	Seasonally	10 000 litre	150
Farm 2	Dam water	<1 MPN/100 ml *E. coli* <1 MPN/100 ml Coliforms	42 ºC	Seasonally	10 000 litre	300
Farm 3	Borehole	>2419.2 MPN/100 ml *E. coli* <1 MPN/100 ml Coliforms	38 ºC	Weekly	10 000 litre	100