Quantifying Microbial Risk Factors for the Consumption of Wastewater Irrigated Salad Crops

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CD	Drip under plastic sheet cover
CFU	Colony forming units
CS	Spray under plastic sheet cover
CSBL	Covered spray bed lettuces
d	Days
DALY	Disability adjusted life years
DGSE	Daily global solar exposure
FSANZ	Food Standards Australia New Zealand
h	Hours
HACCP	Hazard Analysis Critical Control Points
MPN	Most probable number
OD	Open drip
OS	Open spray
OSBL	Open spray bed lettuces
PFU	Plaque forming units
pppy	per person per year
QMRA	Quantitative Microbial Risk Assessment
s	Seconds
SD	Standard deviation
USFDA	the United States Food and Drug Administration
UV	Ultra violet
WHO	World Health Organization

SUMMARY

Wastewater reuse for irrigation in agriculture is increasingly used worldwide due to the freshwater shortage, the growth of population, and the growth of water demand for producing foods. The major public health concern is the risk from the consumption of the crops irrigated with wastewater, particularly salad crops which are eaten uncooked. This research assessed the microbial risk of wastewater irrigated lettuce from the 'farm to fork' continuum, to contribute to minimising the health risk from its consumption.

A field based experiment was conducted to determine the degree of *Escherichia coli* (*E. coli*) contamination on lettuces following partially treated domestic wastewater-spray and drip irrigation. A higher risk was found for spray irrigated-lettuces compared to the drip-irrigated lettuces. However, the microbial quality of irrigation wastewater, the time of harvest following the last irrigation, and climate conditions such as rainfall and sunlight, were also shown to impact the microbial quality of the lettuce at harvest.

A laboratory scale experiment investigated the volume of wastewater retained on the surface of three different lettuce cultivars, Iceberg, Cos, and Oak leaf, following submersion in wastewater of different microbial qualities as a surrogate method for estimation of contamination of spray-irrigated lettuce. The concentration of *E*. *coli* recovered from lettuce using the direct enumeration method, where *E*. *coli* were directly enumerated on the leaves after submersion or by the indirect method, where the *E*. *coli* concentration was estimated from the volume of wastewater retained by the lettuce and the *E.coli* concentration of the wastewater, were compared. The results demonstrated the different variety of lettuce has different wastewater retention capabilities ($p < 0.01$). No statistical differences ($p > 0.01$) were detected between *E*. *coli* counts obtained from different parts of lettuce, nor between the direct and indirect enumeration methods.

The survival of *E. coli* at postharvest and the decontamination process at home on wastewater-irrigated lettuce were also studied in this research. Wastewater-irrigated Cos lettuces were kept at different temperatures, 4°C and 20˚C. At 4˚C, there was no significant effect on the survival and growth of

E. coli on wastewater submersed lettuces over 48 h; while the populations of *E. coli* on lettuce decreased by 0.05 $\log_{10}$ *E. coli* MPN/ 100 g over 48 hours on the lettuce kept at 20˚C. Moreover, storage wastewater-irrigated Cos lettuces at 20˚C for more than 48 hours causes deterioration in visual quality. Ten different home washing methods were performed to remove *E. coli* on wastewater-irrigated Cos lettuce leaves. The results showed that these methods could remove *E. coli* by 1.3 – 3.3 $\log_{10}$ reduction, and the greatest $\log_{10}$ reduction was achieved from a method using 50 ppm sodium dichloroisocyanurate (NaDCC) based chlorine tablets. However, where chemical sanitisers are not available, pre-soaking for 3 min and running under tap water for 20s was the best alternative method to apply.

A quantitative microbial risk assessment (QMRA) was used to estimate the microbial risk from the consumption of wastewater irrigated Cos lettuce, and factors influencing the microbial risk was determined by Yates analysis. Sixteen exposure scenarios were simulated using a 2-level factorial experimental design based on 4 factors: the effect of rainfall on the concentration of *E. coli* in irrigating wastewater, withholding period, supply chain, and the effect of decontamination process prior consumption at home. The results from QMRA and Yates analysis determined that the decontamination process at home prior to consumption was the most important factor impacting the level of *E. coli* contamination at the point of consumption, hence, this step should be considered a Critical Control Point (CCP) from a Hazard Analysis Critical Control Point (HACCP) approach for a wastewater irrigated vegetable production chain from a ‘farm to fork’ perspective.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Prasert Makkaew

26th June 2017

PUBLICATIONS AND CONFERENCE PRESENTATIONS

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CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Overview of wastewater use in agriculture

The demand for freshwater resources is increasing due to population growth and urbanisation. It is projected to increase by 55% by 2050 (UN-Water, 2015), as a result, freshwater sources are becoming scarce. Water scarcity is emerging as a critical issue worldwide, not only in dry lands but also in other abundant freshwater regions (Bixio et al., 2006, Pereira et al., 2009, Steduto et al., 2012, Jiménez and Asano, 2008b). It is estimated that 60% of world's population will face water scarcity by 2025 (Sato et al., 2013). Nowadays, about 700 million people in more than 40 countries are facing water scarcity (Scheierling et al., 2011). In addition, by 2050, about 44% of the world's population is estimated to be living in such a condition (Drechsel et al., 2009). Consequently, wastewater reuse has become more attractive as an alternative source for water resources management.

There are variety of wastewater reuse applications, such as urban and industrial, recreational, aquaculture and agricultural reuse (Asano et al., 2007). The reuse in agriculture is by far the biggest user among all the applications (Scheierling et al., 2010a). Also, the agriculture itself is the largest user of freshwater, accounting for 70 % of total freshwater withdrawals globally (Jiménez and Asano, 2008b). Wastewater reuse in agriculture is not new, the Minoans did it on the Crete island, Greece, about 4000 years ago (Angelakis et al., 2005), and it has been practiced worldwide.

The global picture of wastewater irrigation is still unclear. Jiménez and Asano (2008b) estimated the volume of wastewater reuse for irrigation both treated and untreated in different countries around the world in 2008. It was found that China and Mexico had the largest area irrigated with wastewater, whereas China and USA were the biggest users by total volume used. Raschid-Sally and Jayakody (2009) estimated that about 200 million farmers irrigate with wastewater on an area of 20 million ha while Jiménez and Asano (Jimenez and Asano, 2008) estimated 4.5 million ha worldwide is irrigated with wastewater. However, a recent review about global wastewater generation,

treatment and use suggested based on those two estimations that wastewater irrigation is being used in about 1.5-6.6% of irrigated area of 301 million ha worldwide (Sato et al., 2013). The 20 countries with the largest volume of treated wastewater reuse for irrigation are shown in Table 1.1.

Reuse of wastewater generally occurs in developing countries of Asia and Africa, but wastewater recycling is also now being used in water shortage regions of the developed countries such as Middle East, South West of US and Australia (Marsalek et al., 2002). Wastewater reuse in agriculture can be beneficial for farmers because it is an available water source with nutrient content which is necessary for plants' growth, leading to the reduction of chemical fertiliser use, while increasing crop yields (Toze, 2006, Drechsel et al., 2009, Adrover et al., 2012). Although wastewater irrigation is beneficial for most countries, there are public health risks that we have to consider, principally from microbial contamination.

Table 1.1 Top twenty countries by volume of treated wastewater reused for agricultural irrigation (Jiménez and Asano, 2008a)

Country	Treated wastewater used for irrigation (m^3/ d)	Country	Treated wastewater used for irrigation (m^3/ d)
Mexico	4,492,800	Iran	421,918
Egypt	1,197,808	Chile	380,000
China	1,238,860	Jordan	224,658
Syria	1,182,000	UAE	200,000
Spain	931,507	Turkey	136,986
USA[1]	911,000	Argentina	129,600
Israel	767,123	Tunisia	117,802
Italy	741,262	Libya	110,000
Saudi Arabia	594,521	Qatar	80,000
Kuwait	431,520	Cyprus	68,000

[1]California and Florida

1.1.2 Public health concerns in wastewater irrigation

Wastewater irrigation in agriculture can be beneficial to the agriculture and water resources management. However, it can also pose public health risks due to the presence of pathogenic microorganisms such as bacteria, viruses and parasites.

1.1.2.1 Pathogenic microorganisms in wastewater

Wastewater may comprise a variety of excreted organisms that are harmful to human health, including bacteria (e.g., *Campylobacter*, *Salmonella* spp. and enteropathogenic *Escherichia coli*), viruses (e.g., norovirus and rotavirus), protozoan (e.g., *Cryptosporidium parvum* and *Giardia intestinalis*) and helminths (e.g., *Ascaris lumbricoides* and *Tricuris trichiura)* (Toze, 2006, WHO, 2006a). Pathogens in wastewater are agents of excreta-related diseases that can be spread by applying wastewater for agricultural irrigation. Possible pathogens that may be found in wastewater are detailed in Table 1.2.

Table 1.2 Excreted organism concentrations in wastewater (WHO, 2006b)

Organisms	Numbers in wastewater (per litre)
Bacteria	
Thermotolerant coliforms	10^8-10^{10}
Campylobacter jejuni	10-10^4
Salmonella spp.	1-10^5
Shigella spp.	10-10^4
Vibrio cholera	10^2-10^5
Helminths	
Ascaris lumbricoides	1-10^3
Ancylostoma duodinale/ Necator Americanus	1-10^3
Trichuris trichiura	1-10^2
Protozoa	
Crytosporidium parvum	1-10^4

Organisms	Numbers in wastewater (per litre)
Entamoeba histolytica	$1\text{-}10^2$
Giardia intestinalis	$10^2\text{-}10^5$
Viruses	
Enteric viruses	$10^5\text{-}10^6$
Rotavirus	$10^2\text{-}10^5$

The pathogenic microorganisms contained in wastewater stream in a community were excreted by infected and diseased population who live in that community. The disease transmission could occur when sewage, untreated wastewater or insufficient treated wastewater is applied for irrigation either by crops consumption or by aerosol exposure (Fig. 1.1). However, there are many factors influencing the disease transmission such as the ability of pathogens to survive or multiply in the environment and infect the host, latency period, host characteristics (e.g. immunity, health status, age, gender, and personnel hygiene) (Carr and Strauss, 2001, Feachem et al., 1983).

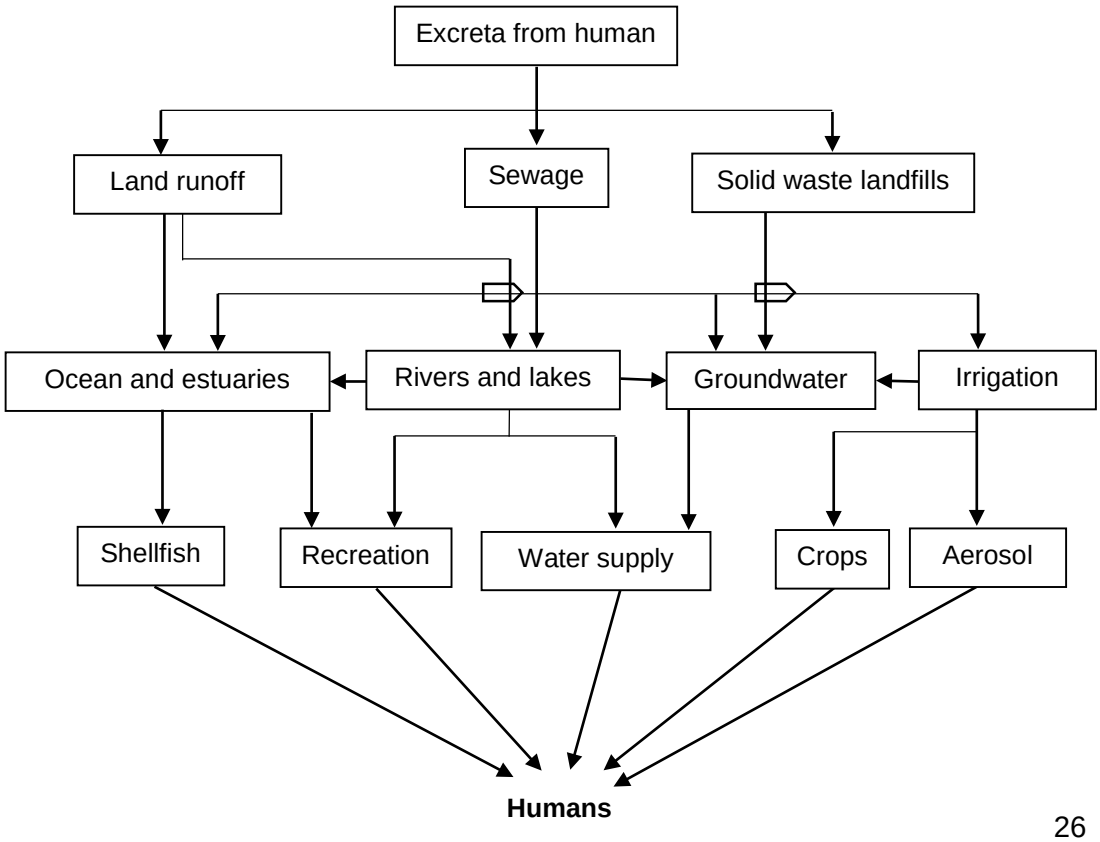

Figure 1.1 Routes of enteric microorganism transmission (Haas et al., 1999).

1.1.2.2 Health risk in a population

The health risk is not only specific to consumers who consume wastewater irrigated crops, but also workers and their families and communities in the vicinity of the wastewater irrigated zone could be affected by this practice. The major route of transmission, however, is the consumption of uncooked wastewater irrigated crops (Scheierling et al., 2010b, Shuval et al., 1984).

Shuval (1986) reviewed and reanalysed epidemiological data from wastewater irrigation in agriculture. It was concluded that there was a high risk of helminthic and bacterial infection among crop consumers and farm workers caused by using untreated wastewater in agricultural irrigation, but limited data about the health effect of people who lived near the wastewater-irrigated zone. Also, very little information was available on the transmission of viruses and protozoan at that time.

Blumenthal and Peasey (2002) conducted the first critical review of the epidemiological evidence of the health effect from using excreta and wastewater in agriculture. The summary of this study is presented in this chapter, together with some new evidence in order to illustrate the magnitude of the health risk related to wastewater reuse in agriculture from the past up to now.

Risk to consumers

Consumer risks relate specifically to consumption of crops irrigated with untreated wastewater and eaten uncooked (Shuval et al., 1984). Numerous research studies have revealed that wastewater applied to vegetables during irrigation resulted in an increased rate of helminths, bacterial and viral infections in those consuming the vegetables. In addition, there is some evidences of disease outbreaks associated with wastewater irrigated crops in some countries; for example, cholera (Fattal et al., 1985), typhoid (Shuval, 1993) and shigellosis (Porter et al., 1984), as well as helminthic infection (mainly from *Ascaris lumbricoides*) (Blumenthal et al., 2001, Gumbo et al., 2010, Habbari et al., 2000, Shuval et al., 1984, Shuval, 1993, Porter et al., 1984).

Risks to workers, their families and adjacent communities

Many studies demonstrate that enteric pathogen infections are high among people who live in areas where wastewater is used in agriculture in comparison to those who live in an area without wastewater reuse. Public health risks were categorised by various studies as bacterial (Melloul and Hassani, 1999, Shuval et al., 1984, Trang et al., 2007), viral (Margalith et al., 1990), protozoan (Ensink et al., 2006, Trang et al., 2007, El Kettani et al., 2008, Srikanth and Naik, 2004) and related to helminths (Blumenthal et al., 2001, Gumbo et al., 2010, Habbari et al., 2000, Shuval et al., 1984, Pham-Duc et al., 2013, El Kettani et al., 2008). On the other hand, children were found to be a susceptible group for infection (Cifuentes et al., 2000, Fattal et al., 1986, Hien et al., 2007). However, regarding the risk related to aerosol exposure from sprinkler irrigation, no difference was found between bacterial and viral infection risks in workers and people in an area exposed to wastewater aerosols from sprinkler irrigation; and to those who lived in an area unexposed to wastewater aerosols (Linnemann Jr et al., 1984, Fattal et al., 1985, Shuval et al., 1989).

1.1.3 Indicator microorganisms in wastewater

There is a variety of microorganisms contained in wastewater including pathogenic and non-pathogenic organisms as presented in Table 1.2. Pathogens can be spread through irrigation water when wastewater is applied for agriculture, it can cause waterborne disease that includes bacteria, viruses and parasites. Therefore, microbial quality of wastewater being used for irrigation should be monitored. However, identification and isolation of each organism individually is impractical as analysis methods are generally complex, labour extensive, expensive, time consuming with detection limit problems at the normally low concentration of pathogens (WHO, 2011, Harwood et al., 2005). Instead, indicator microorganisms are used as surrogates for the presence of pathogens in wastewater. These are usually analysed to monitor water quality and treatment process performance. To be qualified as a suitable surrogate (WHO, 2011, Tchobanoglous et al., 2003), indicator organisms should be :

- found when faecal contamination is present;
- presented whenever pathogens existed in great numbers;

- more (or at least equal) persistent than pathogens in the environment;
- unable to reproduce in the environment;
- inexpensive and relatively easy to detect and quantify; and
- non-pathogenic and generally found in the faecal material of human and other warm-blooded animals.

In practice, there is no indicator organism that meets all the requirements above. Some pathogens can survive longer than indicator organisms, so absence of the indicators does not always mean no pathogens exist (WHO, 2011). However, widely used indicator organisms (mainly bacteria) are total coliforms, *E. coli* and enterococci and clostridia.

Coliforms have been used as indicator organism of faecal contamination for a long time. Coliform bacteria are non-spore forming Gram negative, oxidase negative, facultative anaerobic rod shape bacteria that can produce acid and gas by fermenting lactose (with β-glucuronidase) at 36 ± 2^{o}C within 24-48 hours (Ashbolt et al., 2001). Coliforms belong to *Enterobacteriaceae* family, which comprise of many genera and species. Some common coliforms are shown in Table 1.3. Although coliforms are globally recognised as indicator organism of faecal contamination, they also naturally found in non-faecal pollution impacted soil and water environments. Therefore, *E. coli* is considered as an appropriate indicator microorganism as it exists in a large numbers in human faeces and highly related to faecal pollution. (Stevens et al., 2003, Tripathi and Prasad, 2016). However, it is very poor at predicting the presence of viruses as there are some significant differences in their properties. Unlike bacteria, viruses are more resistant than other microorganisms and very difficult to detect (Toze, 2006). Hence, other organisms such as bacteriophage has been proposed as a surrogates for pathogenic viruses (Duran et al., 2002, Lucena et al., 2004). Bacteriophage MS2 is one of the most recognised surrogates for pathogenic viruses which is a non-enveloped f-RNA coliphage that infects male specific *E. coli* via the f pilli (Jolis, 2002).(Mesquita and Emelko, 2012).

Table 1.3 Most common coliforms divided by genera and species (Stevens et al., 2003).

Family	Genera	Species
Enterobacteriaceae	*Escherichia*	*Escherichia coli*
	Klebsiella	*Klebsiella pneumoniae*
	Enterobacter	*Enterobacter amnigenus*
	Citrobacter	*Citrobacter freundii*

1.1.4 Crop contamination from using wastewater for irrigation

1.1.4.1 Importance of irrigation method

Pathogens in wastewater may contaminate cultivated crops by differing amounts depending on the irrigation practice chosen. A variety of methods are used such as spray, furrow, drip irrigation and subsurface drip irrigation. A number of studies have investigated the microbiological quality of vegetables according to different irrigation methods (Alum et al., 2011, Armon et al., 2002, Bastos and Mara, 1995, Fonseca et al., 2011, Song et al., 2006).

Bastos and Mara (1995) found that there was no difference between bacterial quality of radishes and lettuces in terms of *Escherichia coli* (*E. coli*) and *Salmonella* spp. whether they were drip or furrow irrigated using waste stabilisation pond effluent (1.7-5 x 10^3 MPN/ 100 mL *E. coli*) and tricking filter effluent diluted with potable water (2.5-2.7 x 10^3 MPN/ 100 mL *E. coli*) under dry weather conditions. The concentration of *E.coli* ranged from non-detectable to 10^4 MPN/ 100 g in drip-irrigated lettuces, 10^2 and 10^3 MPN/ 100 g in radishes irrigated with drip and furrow irrigation system, respectively, and *Salmonella* spp. were not detected in all samples. However, the number of *E. coli* increased and *Salmonellae* were detected in dripped-lettuces when rainfall occurred.

Regarding drip irrigation method, this can be divided into 2 types; surface drip irrigation and subsurface drip irrigation. Alum et al. (2011) compared the viral contamination (using bacteriophage MS2, P22, Poliovirus type 1, Enteric virus 40 and Hepatitis A virus) between tomatoes and cucumbers irrigated with viral

spiked secondary treated wastewater by both surface drip and subsurface drip irrigation. MS2, P22 and Enteric virus 40 were spiked into the irrigation water for growing tomato, while cucumber irrigated water was spiked by MS2, P22 and Hepatitis A virus. They found that surface drip irrigation resulted in viral contamination of both the above and below the ground surfaces of tomatoes and cucumbers. It was also found that roots were contaminated more than the leaves and fruits. However, no viruses were detected in any of above ground crops surface where wastewater was applied by subsurface irrigation.

In addition, surface and subsurface drip irrigation were compared with sprinkler irrigation by Armon et al. (2002). They compared the contamination of *Cryptosporidium* and *Giardia* in field crops (mainly vineyard and zucchini) using different water qualities from different stages of wastewater treatment plant (raw wastewater, outlet from settling pond, facultative pond effluent, filtered effluent). The study demonstrated that crops irrigated by sprinkler irrigation had the highest potential for contamination by *Cryptosporidium* and *Giardia* compared to drip and subsurface drip irrigation. Moreover, Fonseca et al. (2011) found that Romaine and Iceberg lettuces grown with10^8-10^9 cells/ mL *E. coli* K-12 spiked water were positive for *E. coli* K-12, and could survive on the crops' surfaces up to 7 days when using sprinkler irrigation, whereas only one sample was found to be positive using other irrigation methods (Subsurface drip irrigation and furrow).

Other studies have compared furrow and subsurface drip irrigation. Song et al. (2006) determined the contamination of *E. coli*, bacteriophage PRD1 and *Clostridium perfringens* (*C. perfringens*) in lettuces, bell peppers and cantaloupes. Mixed microorganisms; 2.58 x 10^{13} CFU PRD-1, 1.72 X 10^{11} PFU *E. coli* and 5.93 x 10^8 PFU *C. perfringens* were spiked in to irrigated water and applied to each irrigation system. The results of this study indicated that crops grown in plots with furrow irrigation were subject to greater contamination than those in subsurface irrigated plots. PRD1 was more often detected on the surface of lettuces and cantaloupes compared to *E. coli*, while these microorganisms were not recovered from any bell pepper samples, and *C. perfringens* was under the detection limit in all crop samples. In contrast, Choi et al. (2004) showed that bacteriophage PRD1 and MS2 were detected in greater numbers in subsurface plot lettuces than in the furrow plot when using

PRD1 and MS2 spiked water for irrigation. Therefore, there is clearly no consensus on this issue since there are more factors that can influence the crops contamination when furrow and subsurface drip system are being applied such as water movement and the soil surface wetting pattern (Song et al., 2006).

1.1.4.2 Variation of plant morphology on microbial quality of vegetables

Different parts of plants have different potential to be contaminated by microorganisms. Research has shown that lettuces and parsley where the edible part developed close to the ground surface were more contaminated by *Salmonella* spp. than tomatoes and pimentos where the edible part grows well above the soil surface when they were grown using raw wastewater (Ait Melloul et al., 2001). Similarly, Song et al. (2006) found that bacteriophage PRD1, *E. coli* and *C. perfringens* were recovered from the surface of cantaloupes and lettuces, but never detected on bell peppers when theses crops were irrigated with mixed microorganisms spiked water by either drip or furrow irrigation system. This could be described by their direct contact with irrigated wastewater when crops which developed near the surface of the soil, are more exposed to contaminated irrigated water and/ or soil when drip and furrow irrigation systems are being applied, this would result in high microbial contamination on the crops surface.

Furthermore, the different types of crops also suffer contamination from different groups of parasites possibly due to the texture of the crops. Amahmid et al. (1999) grew some vegetables using raw wastewater from stabilisation pond for irrigation. The results indicated that coriander and mint were found to be more contaminated with *Giardia* and helminth eggs than carrots and radish because of their dense foliage. These foliage allows more surface to be contaminated, also it could prevent microorganisms being exposed to external environmental factors (Shuval et al., 1986, Armon et al., 1994).

1.1.4.3 Impact of degree of water contamination on the microbial quality of vegetables

The degree of contamination of water is an important factor; vegetables irrigated with a high concentration of bacteria would result in a high level of the pathogen contamination at harvest. This was confirmed by research of

Solomon et al. (2003), where butter head lettuces were spray irrigated with 10^4 CFU/mL *E. coli* O157: H7 inoculated water were more contaminated at harvest time (up to 5 log CFU/ g) compared with those irrigated with 10^2 CFU/mL *E. coli*O157: H7 inoculated water, and the level of contamination also increased when repeated irrigation occurred. Similarly, Erickson et al. (2010b) found that spray-irrigated spinach had a higher level of contamination when using water containing 10^6 CFU/ mL *E. coli* O157:H7 compared to 10^4 CFU/ mL *E. coli* O157:H7 water, and there was no *E. coli* O157:H7 detected in any sample when 10^2 CFU/ mL *E. coli* O157:H7 water was applied.

In addition, Al-Lahham et al. (2003) studied the effects of the irrigation between different ratios of potable water to treated wastewater on microbial quality of tomatoes. Four different ratios of potable water to treated wastewater (contamination with up to 42 CFU/ 100 mL total coliforms) were applied to furrowed irrigate (1:0, 1:1, 1:3 and 0:1) tomatoes. The results of this study revealed that coliform contamination on tomato fruit surfaces increased with increasing proportion of wastewater applied.

Nikaido et al. (2010) compared the microbiological quality of lettuce and rocket salad irrigated with 2 types of irrigation waters, treated wastewater without chlorination and treated wastewater with chlorination. Although there was no difference in the number of parasites (*Hymenolepsis nana*, *Enterobius vermicularis*, *Entamoeba coli* and nematode larvae) in the different types of irrigation water, the crops which irrigated with treated wastewater without chlorination were more contaminated with faecal coliforms.

1.1.4.4 The persistence of microorganisms on crop surfaces and in soil used for growing vegetables

Microorganisms can persist in soil and on crop surfaces for a period of time after irrigation. Many factors affect the survival of pathogens on crop surfaces and in soil such as humidity, soil content, temperature, pH, sunlight and plant type (WHO, 2006b). There are a number of studies, which investigated the persistence of pathogenic microorganisms on crops surface caused by direct contact with contaminated irrigating water. However, there were many different factors in these studies design such as setting (field based VS laboratory based), plant species, pathogen strains, the concentration of pathogens in

irrigation water, irrigation system. A summary of each study is shown in Table 1.3 and Table 1.4 which is separated in to a field based setting and a laboratory based setting, respectively. The damage by either physical (e.g. bruising, punching) or degradation by plant pathogens could enhance the persistence of the pathogens, also promoting their proliferation particularly at ambient temperature.

Table 1.4 The persistence of pathogens on crops and in soil following the application of contaminated irrigated water (Field based)

Microorganisms	Concentration in water (log CFU/ mL)	Crop	Irrigation method	Survival after contamination (day)		References
				On crop surface	In soil	
E. coli K-12	8 - 9	Lettuce	Spray	7	17	Fonseca et al. (2011)
			Subsurface drip	N/A	7	
			Furrow	N/A	7	
E. coli O157:H7	8	Lettuce	Spray	27	N/A	Erickson et al. (2010b)
E. coli O157:H7	5	Lettuce	Spray	56	119	Islam et al. (2004a)
		Parsley	Spray	154	140	
E. coli O157:H7	5	Carrot	Spray	154	168	Islam et al. (2005)
		Onion	Spray	63	140	
E. coli O157:H7	4	Lettuce	Surface	15	N/A	Mootian et al. (2009)
Salmonella spp.	N/A	Lettuce	Spray	3	N/A	Ait Melloul et al. (2001)
S. enterica	5	Parsley	Spray	28	N/A	Kisluk and Yaron (2012)
Salmonella Typhimurium (*S*. Typhimurium)	5	Carrot	Spray	172	217	Islam et al. (2004b)
		Radish	Spray	70	217	
S. Typhimurium	5	Lettuce	Spray	63	161	Islam et al. (2004c)
		Parsley	Spray	231	231	

Table 1.5 The persistence of pathogens in crops and soil through the application of contaminated irrigated water (Laboratory based)

Microorganisms	Concentration in water (log CFU/ mL)	Crop	Irrigation method	Survival after contamination on crop surface (day)	References
E. coli O157:H7	7	Lettuce	Surface Spray	20* 20**	Solomon et al. (2002)
E. coli O157:H7	4	Lettuce	Spray	30	Solomon et al. (2003)
E. coli O157:H7	5	Lettuce	Spray	6	Wood et al. (2010)
E. coli O157:H7	7	Lettuce	Spray	28	Oliveira et al. (2012)
Listeria innocua	7	Lettuce	Spray	28	Oliveira et al. (2011)

**6/32 samples positive, **29/32 samples positive*

Note: Table 1.4 – 1.5 were modified from Uyttendaele et al. (2015)

From Table 1.4-1.5, most of the studies have been done using bacteria, there are very few regarding the persistence of viruses on crops and in soil. Choi et al. (2004) compared the persistence of two viral indicators, bacteriophage PRD1 and MS2 on lettuces and in soil. Tertiary treated wastewater seeded with bacteriophages PRD1 and MS2 was irrigated on lettuces using subsurface drip and furrow irrigation methods. The results indicated that numbers of bacteriophage PRD1 and MS2 detected from the lettuces in the subsurface irrigation plots were greater than those in the furrow-irrigated plots. Moreover, bacteriophage PRD1 was more persistent in dry conditions and more tolerant to higher temperature than bacteriophage MS2.

1.1.4.5 The internalisation of microorganisms into plants

It has clearly been demonstrated that pathogens can be transported from contaminated irrigation water and soil onto vegetables, but it also has a potential to be internalised into the edible parts of the crops. Exposure to *E. coli* O157:H7 and *Salmonella* spp.,; foodborne pathogens, by this route have been linked to several outbreaks in the USA (Deering et al., 2012, Erickson, 2012).

Critical reviews about internalisation of plant by pathogens have been published lately by Erickson (2012) and Deering et al. (2012). Studies including a variety of crops, source of contamination, detection techniques have been collected and analysed. It could be concluded that human pathogens could enter into the internal plant tissues either through natural openings of the plant surface (e.g. stomata, lateral root emergence sites including physical damage site), or by introduced into the plants' internal tissue along with the water used for soaking seeds, irrigating, or washing crops. Internalisation could happen during pre- and postharvest. At pre-harvest, internalisation could occur via either plant roots or leaf stomata, for example under the laboratory conditions when crops were exposed to high concentration of pathogens in the media; > 6 log/ g soil or 6 log/ mL water. However, they could survive inside the plant tissues for a short period of time. In addition, controlling effective dose of sanitising solution in waters during harvesting and minimal processing could minimise the internalisation of bacteria via damaged surface of crops at postharvest. The studied papers were summarised in Table 1.6, however, due to the large numbers of crops studied, only leafy greens were focused on this

section as it linked to the crop used in this current research.

There are few studies about internalisation of viruses in plants. Carducci et al. (2011) found that Enteric virus (Coxsackie B virus) can penetrate through the roots and transport to the leaves of lettuce when plants were exposed via a hydroponic system, Urbanucci et al. (2009) also reported that lettuce leaves were positive for Canine calicivirus when grown under hydroponic conditions. Viral internalisation in plants was not done only under hydroponic system, murine norovirus could internalise to lettuce leaves when they were submersed to 5 log PFU/ mL murine norovirus solution (Wei et al., 2010). Not only bacteria and viruses could internalise to the plants tissue, but also protozoa, Macarisin et al. (2010) noted evidence of internalised *Cryptosporidium parvum* oocysts in spinach leaves when they were sprayed with 10^3 oocysts/ 100 mL *Cryptosporidium parvum* inoculated water. This area clearly requires more research but is beyond the scope of the current proposed research.

Table 1.6 Internalisation of some pathogens in leafy greens

Pathogens	Crop	Contamination source	Concentration of pathogens in/ spiked to the source	Internalised pathogen detected (+/-)	References
E. coli	Spinach	Seed (soaked in solution)	7 log CFU/ mL	+	Warriner et al. (2003)
	Spinach	Soil	2 log CFU/ g	-	Warriner et al. (2003)
	Spinach	Water (hydroponic)	2-3 log CFU/ mL	+	Warriner et al. (2003)
	Lettuce	Wash water	8 log CFU/ mL	+	Gomes et al. (2009)
	Lettuce	Soil	7 log CFU/ g	+	Habteselassie et al. (2010)
	Lettuce	Irrigation water	6 log CFU/ mL	+	Habteselassie et al. (2010)
E. coli O157:H7	Lettuce	Irrigation water	7 log CFU/ mL	+	Seo and Frank (1999)
	Lettuce	Irrigation water (submersion)	7, 8 and 9 log CFU/ mL	+	Takeuchi and Frank (2000)
	Thale cress	Water (hydroponic)	4 and 6 log CFU/ mL	+	Cooley et al. (2003)
	Thale cress	Soil	8 log CFU/ g	+	Cooley et al. (2003)
	Spinach	Soil (irrigated with spiked water)	6 log CFU/ mL	+ (only in roots)	Hora et al. (2005)
	Cress	Seed (soaked in solution)	2 log CFU/ mL	+	Jablasone et al. (2005)
	Lettuce	Seed (soaked in solution)	2 log CFU/ mL	+	Jablasone et al. (2005)
	Spinach	Seed (soaked in solution)	2 log CFU/ mL	+	Jablasone et al. (2005)
	Lettuce	Soil (added with spiked water)	9 log CFU/ mL	+	Franz et al. (2007)
	Lettuce	Water (Hydroponic)	7 log CFU/ g	+	Nthenge et al. (2007)

Pathogens	Crop	Contamination source	Concentration of pathogens in/ spiked to the source	Internalised pathogen detected (+/-)	References
	Lettuce	Inoculated leaf	4-6 log CFU/ cm^2	+	Li et al. (2008)
	Spinach	Inoculated leaf	6 log CFU/ mL	+	Mitra et al. (2009)
	Spinach	Soil (injected with spiked water)	3 and 7 CFU/ mL	+	Mitra et al. (2009)
	Lettuce	Soil	1-4 log CFU/ g	+	Mootian et al. (2009)
	Lettuce	Irrigation water	1-4 log CFU/ mL	+	Mootian et al. (2009)
	Spinach	Soil (injected with spiked water)	7 log CFU/ mL	+	Pu et al. (2009)
	Spinach	Soil	4 and 8 log CFU/ g	+ (only in roots)	Sharma et al. (2009)
	Spinach	Water (hydroponic)	5 and 8 log CFU/ mL	+	Sharma et al. (2009)
	Lettuce	Soil	4 and 6 log CFU/ g	-	Zhang et al. (2009a)
	Lettuce	Inoculated leaf	6-7 log CFU/ plant	-	Zhang et al. (2009b)
	Lettuce	Soil	3 and 6 log CFU/ g	-	Zhang et al. (2009b)
	Spinach	Irrigation water	2, 4 and 6 log CFU/ mL	+	Erickson et al. (2010b)
	Lettuce	Irrigation water	2, 4 and 6 log CFU/ mL	+	Erickson et al. (2010b)
	Spinach	Irrigation water	2, 4 and 6 log CFU/ mL	-	Erickson et al. (2010a)
	Spinach	Irrigation water	3 and 5 log CFU/ g	-	Erickson et al. (2010a)
	Lettuce	Compost	3 and 5 log CFU/ g	-	Erickson et al. (2010a)
	Parsley	Compost	3 and 5 log CFU/ g	-	Erickson et al. (2010a)

Pathogens	Crop	Contamination source	Concentration of pathogens in/ spiked to the source	Internalised pathogen detected (+/-)	References
		Compost			
Salmonella spp.	Parsley	Wash water	6 log CFU/ mL	+	Duffy et al. (2005)
S. Typhimurium	Lettuce	Seed (soaked in solution)	2 log CFU/ mL	+	Jablasone et al. (2005)
	Spinach	Seed (soaked in solution)	2 log CFU/ mL	+	Jablasone et al. (2005)
	Lettuce	Soil (added with spiked water)	9 log CFU/ mL	+	Franz et al. (2007)
	Lettuce	Water (hydroponic)	7 log CFU/ mL	+	Franz et al. (2007)
	Lettuce	Soil	7 log CFU/ g	-	Klerks et al. (2007)
	Thale cress	Water (hydroponic)	9 log CFU/ mL	+ (only in roots)	Schikora et al. (2008)
	Parsley	Irrigation water	7.6 log CFU/ mL	+	Lapidot and Yaron (2009)
	Lettuce	Irrigation water (submersion)	8 log CFU/ mL	+	Kroupitski et al. (2011)
	Lettuce	Irrigation water (submersion)	8 log CFU/ mL	+	Golberg et al. (2011)
	Arugula	Irrigation water (submersion)	8 log CFU/ mL	+	Golberg et al. (2011)
	Parsley	Irrigation water (submersion)	8 log CFU/ mL	+	Golberg et al. (2011)
	Basil	Irrigation water (submersion)	8 log CFU/ mL	+	Golberg et al. (2011)
Salmonella Enteritidis	Lettuce	Soil	7 log CFU/ g	-	Klerks et al. (2007)
Salmonella Dublin	Lettuce	Soil	7 log CFU/ g	+	Klerks et al. (2007)
Salmonella Newport	Lettuce	Soil	6 log CFU/ g	+	Bernstein et al. (2007)

Pathogens	Crop	Contamination source	Concentration of pathogens in/ spiked to the source	Internalised pathogen detected (+/-)	References
	Thale cress	Water (hydroponic)	4 and 6 log CFU/ mL	+	Cooley et al. (2003)
	Thale cress	Soil	8 log CFU/ g	+	Cooley et al. (2003)
Listeria monocytogenes	Cress	Seed (soaked in solution)	2 log CFU/ mL	-	Jablasone et al. (2005)
	Lettuce	Seed (soaked in solution)	2 log CFU/ mL	-	Jablasone et al. (2005)
	Spinach	Seed (soaked in solution)	2 log CFU/ mL	-	Jablasone et al. (2005)
	Thale cress	Irrigation water (submersion)	8 log CFU/ mL	+	Mililllo et al. (2008)

Note: This table was adapted from Deering et al. (2012) and Erickson (2012).

1.1.5 Microbial safety of wastewater irrigated crops related to postharvest handling

Microbial contamination of fresh produce could occur through many steps from the farm-to-plate continuum including planting, harvesting, processing, transporting, retailing and home handling (Ailes et al., 2008, Olaimat and Holley, 2012, Matthews, 2013). At wastewater irrigated farms, microbial quality of irrigation water is one of the major sources of crops contamination on farm, but there are many other factors influencing microbial safety of the crops including postharvest handling. A number of studies have demonstrated that an increased microbial load on crops at postharvest persist at the point of sale and/ or consumption at home (Johnston et al., 2005, Ailes et al., 2008, Ensink et al., 2007, Ilic et al., 2008).

There are many sources of postharvest contamination such as harvesting tools, transport containers and vehicles, processing equipment and cross contamination by human handling (Beuchat and Ryu, 1997). In addition, there are various steps which potentially could be other sources of contamination during processing such as cooling, cutting, washing/ sanitising and packaging/ storing (Jung et al., 2014). However, the process steps could be diverse and vary due to crop types, product types and region. Fig. 1.2 shows the general supply chain of lettuce/ leafy greens in developed countries. Fresh cut leafy greens (generally also known as minimal processed vegetables) tend to be more complex in terms of processing, while food agricultural commodity is simpler with fewer steps.

In this section, more focus is on transportation and storage and home handling steps since these relate more to this current research on lettuce commodity, not a minimal process lettuce. It is clearly known that time and temperature play an important role in controlling microbial safety of food (FSANZ, 2002). In the production chain of fresh produce, pathogens could die-off when the crops were kept under low controlled temperature, but the number will increase if the temperature cannot be controlled and the time between harvest and consumption is extended (Drechsel et al., 2009). The United States Food and Drug Administration (USFDA) recommended that leafy greens should be kept under 5^{o}C throughout the commercial supply chain in order to minimise the risk of pathogens proliferation during distribution (USFDA, 2010). Handling at

consumption point either in the restaurant or home is also important. Decontamination process at home is a crucial step as it is the last intervention that could remove the microbial risk before consumption; however, the data available for the efficacy of home washing methods are limited, as the majority of the research about the effectiveness of the decontamination process on fresh produce is primarily focused on the performance of sanitisers in food industries. The detail of the importance of transport/ storage and household washing process to control microbial safety of vegetable is considered further below.

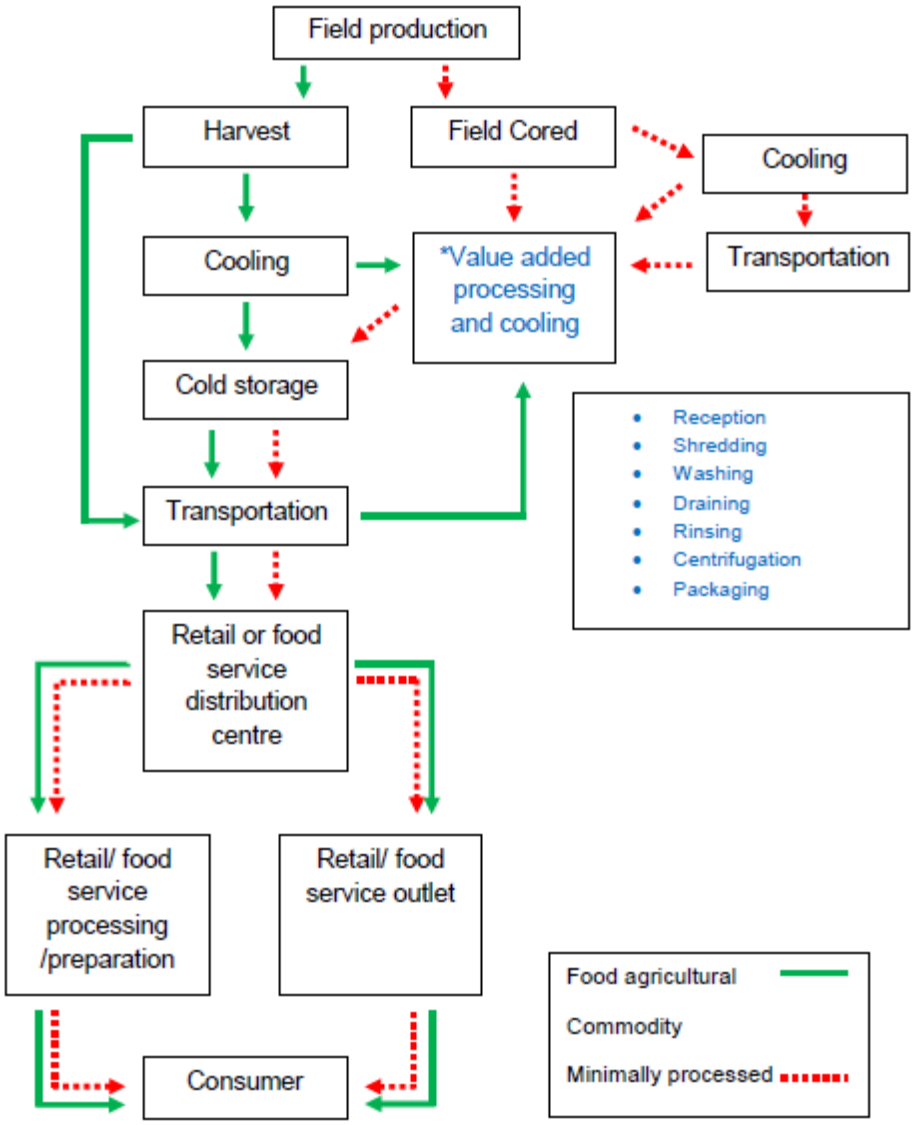

Figure 1.2 General supply chain of lettuce/ leafy greens (Gorny et al. (2006)

1.1.5.1 Transportation and storage

Transportation is one of the main factors influencing microbial safety of fresh produce as it is a link between crop producers and consumers. The most important factor that cause the increase of microbial load on fresh produce during transportation and storage is temperature. Poor temperature management and control in the cold chain have been identified as the cause of foodborne diseases (Brackett, 1999, McCabe-Sellers and Beattie, 2004, Rosset et al., 2004, Todd et al., 2010). To minimise foodborne illness arising from transportation and storage, fresh fruit and vegetable should be strictly controlled throughout the supply chain by being kept under 5^{o}C for reducing spoilage and pathogens proliferation process (USFDA, 2010, Rediers et al., 2009, Ukuku and Sapers, 2007). However, some pathogenic bacteria such as *L. monocytogenes* is still able to grow or survive longer under refrigerated temperature in a variety of fresh produce (Beuchat and Brackett, 1990, Brackett, 1999, Thomas and O'Beirne, 2000, Oliveira et al., 2010, Ells and Truelstrup Hansen, 2010, Ongeng et al., 2007), as are some viruses, for instance bacteriophage MS2 (Dawson et al., 2005, Carratalà et al., 2013), adenoviruses(Carratalà et al., 2013), hepatitis A (Croci et al., 2002), poliovirus (Kurdziel et al., 2001) and rotavirus SA-11 (Badawy et al., 1985).

Temperature control during distribution chain is not only beneficial for controlling the pathogenic microorganisms on vegetables, but also the physical produce qualities. High temperature accelerates the physiological activities of fresh produce such as transpiration and respiration, resulting in product's weight loss, decrease in the quality of internal flesh, tissue softening and discolouration (Vigneault et al., 2009, Brosnan and Sun, 2001, de Castro et al., 2005). In addition, the growth rate of spoilage microorganisms such as *Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp., *Xanthomonas* spp. as well as some yeasts and moulds could be inhibited while vegetable are being stored at low temperature so that the produce remains fresh (Brackett, 1994, Francis et al., 1999).

Although the effective temperature control is the key factor for microbial safety of fresh produce during the distribution chain, fresh fruits and vegetables in many developing countries are generally transported from farms to markets and/ or retailers by open and non-refrigerated vehicles (Ilic et al., 2010, Ahmad

and Siddiqui, 2015). In China and Thailand, fresh produce are transported by open-air vehicles, and crushed ice is sometimes put between the products to reduce the temperature in case of long distance transportation (Feng, 2001, Vadhanavikit et al., 2006), while bullock/ buffalo carts are commonly used in rural area of northern India (Ilic et al., 2010). Fresh produce tend to be more susceptible to loss in quality when transported by non-refrigerated vehicles; also, the ambient temperature itself spoils the produce (Ahmad and Siddiqui, 2015). In addition, rising temperature during distribution chain can enhance the proliferation of pathogenic bacteria on vegetables which could pose a risk to public health (Brackett, 1999, McCabe-Sellers and Beattie, 2004, Rosset et al., 2004, Todd et al., 2010, Rediers et al., 2009).

Although the data on microbial quality of wastewater irrigated salad crops through the distribution chain is scarce, there are number of studies which demonstrated the effect of rising temperature or temperature abuse on the microbial safety of vegetables. Most found that the number of pathogens increased when fresh produce was stored at 5^{o}C, studied pathogens including *E. coli* O157: H7 (Puerta-Gomez et al., 2013, Ding et al., 2012, Tian et al., 2012, Khalil and Frank, 2010, Carey et al., 2009, Luo et al., 2009, Doering et al., 2009, Kim and Harrison, 2008, Koseki and Isobe, 2005, Choi et al., 2011, Luo et al., 2010), *S*. Typhimurium (Puerta-Gomez et al., 2013, Tian et al., 2012), *S. hadar* (Piagentini et al., 1997) and *Staphylococcus aureus* (*S. aureus*) (Tian et al., 2012). The microbial quality of wastewater irrigated salad crops during transport and storage needs to be explored further since there is a lack of available data on this issue.

1.1.5.2 Home washing methods

Although postharvest setting plays an important role in microbial safety of fresh produce, transportation, markets and retail could be controlled by government, while it could hardly be expected to control the consumers' behaviour at a domestic level (Fischer et al., 2005). Consumers food handling and preparation, including fresh fruit and vegetables, at home is one of the important causes of foodborne diseases internationally (Milton and Mullan, 2010, Taché and Carpentier, 2014). Studies revealed that some population groups handle fresh produce unsafely at home such as consumers over 45 year-old, non-college degrees and lower-income people (Williamson et al.,

1992, Li-Cohen and Bruhn, 2002, Altekruse et al., 1996). Anderson et al. (2004) revealed that about 6 % of subjects participated a research in Utah University, the USA tend not to clean vegetables when preparing salad, moreover some consumers hardly ever or never wash the produce before consumption (Li-Cohen and Bruhn, 2002).

A washing step is crucial to minimise the level of microbial contamination on fresh produce as it has been known that raw fruits and vegetables are vehicles of human disease for many decades (Beuchat, 1998).It is thus necessary to find the effective decontamination process to reduce the threat caused by pathogenic microorganisms on the surface of fresh produce before consumption, particularly salad crops which are consumed raw.

Research on the decontamination of fresh produce which has been performed is primarily focused on the efficacy of sanitisers in food industry (Vijayakumar and Wolf-Hall, 2002b). While there is a limited data available comparing effective washing method at home or in the restaurants (Nastou et al., 2012). A variety of antimicrobial agents have been used in the food industry, such as hypochlorite, chlorine dioxide, hydrogen peroxide, peroxyacetic acid, lactic acid, irradiation, ozone, electrolysed water, etc. (Kondo et al., 2006, Rodgers et al., 2004, Vijayakumar and Wolf-Hall, 2002a, Zhang et al., 2009c, Lin et al., 2002, Niemira, 2008, Hadjok et al., 2008, McKellar et al., 2004, Luo, 2007). Despite the limitation of data on the effectiveness of home washing methods to reduce the microbial load, a number of studies have shown that some industrial sanitisers are also being used at household level as discussed below.

Chlorine

Chlorine is the major sanitizing agent used for washing fresh produce. The common form which is the active compound being used in food industry, and in household bleach is sodium hypochlorite (NaClO). In aqueous solution, hypochlorite is transformed to hypochlorous acid (HOCl) and sodium chloride (NaCl), then HOCl may further dissociate to hypochlorite ion (ClO^-) and hydrogen ion (H^+) (Jongen, 2005, Block, 2001).

$$NaClO + H_2O \longrightarrow HOCl + NaOH$$

$$HOCl \longrightarrow H^+ + ClO^-$$

Chlorine chemistry is strongly related to pH, at the pH 6.0-7.5 HOCl dominates which is the most antimicrobial effective form of chlorine. It is reported that the percentages of HOCl and OCl^- are 78% and 22% at the pH 7, which is the appropriate pH value used in the fresh-cut vegetable industry. However, it could be shifted to OCl^- under alkaline conditions (pH > 8) (Dawson, 2002, Jongen, 2005, Warriner and Namvar, 2014b) (Fig. 1.3).

HOCl destroys microorganisms by several mechanisms, it is commonly considered to produce highly oxidative, hydrolysis and amination reactions which may affect a variety of subcellular compounds. Microorganisms could be destroyed by HOCl combining with proteins to form N-chloro compounds (Baker, 1925), or oxidise sulfhydryl groups of proteins (Venkobachar et al., 1977, Knox et al., 1948, Green and Stumpf, 1946). Other mechanisms including α-amino acids oxidation (Patton et al., 1972), cell membrane damage by oxidising cytochromes, iron-sulphur proteins and nucleotides (Venkobachar et al., 1977, Haas and Engelbrecht, 1980), metabolism and protein synthesis disruption (LeChevallier and Au, 2004) and genetic defects caused by purine and pyrimidine bases modification (Hoyano et al., 1973, Haas and Engelbrecht, 1980).

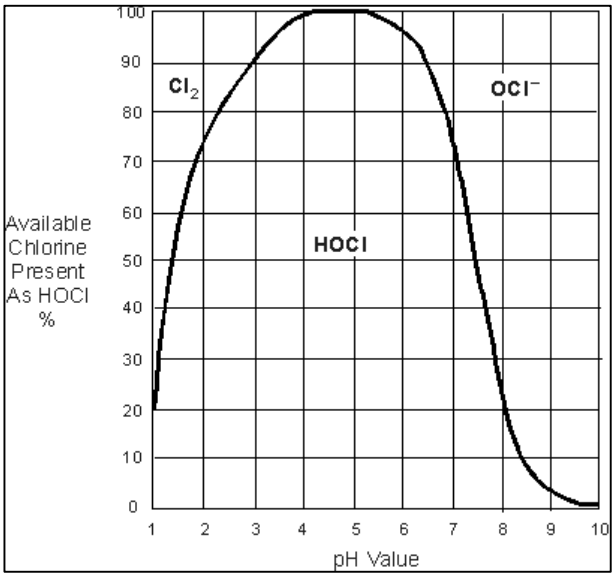

Figure 1.3 Chlorine forms in different pH (available from https://aquaox.wordpress.com/2010/01/27/chlorine-efficacy/)

The efficacy of chlorine based washing method depends on several factors namely; the quantity of hypochlorous acid formed, the pH of the water, temperature and amount of organic matter in the solution, washing time (Jongen, 2005, Block, 2001). Moreover, types of produce also influence the efficacy of the crops' surface disinfection, as well as the characteristics of crops' surface (cracks, crevice, hydrophylic tendency and texture) and the location of the tissue (outer leaves and inner leaves) (Kondo et al., 2006, Gil et al., 2009a).

Chlorine based washing is widely used in surface decontamination of fresh produce, the concentration range from 50-200 ppm with 1-2 minute-contact time is recommended (Beuchat, 1998). Relevant studies regarding the efficacy of chlorine to reduce the microbial load of pathogens is summarised in Table 1.7.

Table 1.7 The performance of Chlorine for reducing microbial load of vegetables

Target microorganisms	Types of vegetable	Chlorine concentration (ppm)	Contact time (minute)	Log reduction	Reference
Total Aerobic Count	Lettuce	200	5	1.3	Escudero et al. (1999)
E. coli	Lettuce	50	0.5	1.9 – 2.8	Behrsing et al. (2000)
	Broccoli	50	0.5	1.7 – 2.5	Behrsing et al. (2000)
	Broccoli	100	2	2.4	Behrsing et al. (2000)
	Lettuce	100	5	0.5 – 1	Francis and O'Beirne (2002)
	Lettuce	100	2	2.5	Ölmez (2010)
E. coli O157:H7	Shredded carrots	200	2	0.87	Gonzalez et al. (2004)
	Lettuce	200	10	1.2	Kondo et al. (2006)
	Lettuce	300	3	0.5	Niemira (2007)
	Lettuce	600	3	0.5	Niemira (2007)
	Lettuce	20000	1	2.43	Palma-Salgado et al. (2014)
S. aureus	Lettuce	200	10	1.4	Kondo et al. (2006)
Salmonella spp.	Lettuce	100	1	0.9	Betts et al. (2005)
	Lettuce	100	5	1.8	Betts et al. (2005)

Target microorganisms	Types of vegetable	Chlorine concentration (ppm)	Contact time (minute)	Log reduction	Reference
S. Typhimurium	Lettuce	200	10	1	Kondo et al. (2006)
L. monocytogenese	Lettuce	200	10	1.7	Zhang and Farber (1996)
	Lettuce	100	1	1.3	Betts et al. (2005)
	Lettuce	100	5	1.8	Betts et al. (2005)
	Lettuce	100	1	0.7	Hellström et al. (2006)
L. inocua	Lettuce	100	5	1 -1.5	Francis and O'Beirne (2002)
Yesinia enterocolitica	Lettuce	100	10	2	Escudero et al. (1999)
	Lettuce	300	10	3	Escudero et al. (1999)

Organic acids

Since the growth of pathogens and spoilage microorganisms may be limited under acidic conditions, organic acids are also frequently used to decontaminate the surface of produce. Organic acids are naturally found in fruits and vegetables or as a product of a fermentation process (Beuchat, 1998). Their antimicrobial properties occur by hydrogen ion dissociation, reducing the pathogens' intracellular pH, and disruping the cell's ability to maintain pH homeostasis. As a result, membrane permeability and substrate transport are dysfunctional (Betts et al., 2005).

Organic acids such as acetic, citric, sorbic, benzoic, tartaric, succinic and malic are the major acids found in fruits and vegetables, among these, acetic, malic, citric and lactic are more affective due to their stability at high organic loading (Beuchat, 1998, Warriner and Namvar, 2014b). However, these organic acids have low antimicrobial properties compared to strong acids, so they require high concentration and longer contact time to reduce the microbial load on the crop surfaces (Olaimat and Holley, 2012). Nevertheless, mostly are defined as Generally recognised as Safe (GRAS) for food treatment (Beuchat, 1998, Warriner and Namvar, 2014b).

Park et al. (2011) investigated the efficacy of different organic acids in reduction of microbial load (*E. coli* O157: H7, *S.* Typhimurium and *L. monocytogenes*) of sliced apples and lettuce. 1-2 % propionic, acetic, lactic, malic and citric acids were used to decontaminate produce with 10 minute contact time. It was reported that these acids supported 2 log reduction; also, malic and citric acids were relatively effective compared to others.

Treatment of salad crops by dipping them in some organic acids acid has also been studied. Acetic, citric and lactic acid applied for 1 minute can reduce *E. coli* O157: H7 on shredded iceberg lettuce by 0.2, 0.8 and 1.1 log reductions, respectively. Acetic, citric and lactic acid could reduce *L. monocytogenes* on shredded iceberg lettuce 0.6, 1 and 0.93 log reductions, respectively (Yuk et al., 2006). However, Zhang and Farber (1996) found that lactic and acetic acids could reduce *L*. monocytogenes on shredded iceberg lettuce only by about 0.5 and 0.2 log reductions, respectively. They suggested that the performance of organic acids to reduce pathogens can vary due to the different

acid tolerance among various strains.

Despite the high cost, peroxyacetic acid which is produced from the reaction between hydrogen peroxide and acetic acids is more useful in fresh produce decontamination due to its high stability at high organic matter loading (Vandekinderen et al., 2009, Ölmez and Kretzschmar, 2009). The reduction of *E. coli* 0157: H7, *L. monocytogenes* and *S.* Typhimurium exposed to 20-80 ppm peroxyacetic acid, ranged from 0.4-3.5 log reductions. Generally, pathogens are not peroxyacetic acid resistant; however, the reduction varies among different produce types; for instance, the microbial load log reduction on cabbage was higher than on leek when peroxyacetic was used to decontaminate (Grace Ho et al., 2011, Vandekinderen et al., 2009).

It is known that organic acids are naturally present in fruits and vegetables. Vinegar and lemon juice are generally used at home because they are inexpensive and accessible. However, the aroma and flavour of the vegetables could be changed after they are treated. (Beuchat, 1998).

Other washing methods at home

In response to increasing consumers concern about the adverse side effects of chemical sanitisers, plant extracts could be an alternative choice for fresh produce decontamination. However, the performance to reduce the microbial load depends on types of produce and target microorganisms (Tirpanalan et al., 2011).

Kim et al. (2011) studied the feasibility of 12 plants extracts to reduce microbial load on lettuce with S. Typhimurium, *E. coli* O175: H7 and L. *monocytogenes* as target pathogens. The study found that clove extract (*Syzygium aromoticum*) showed the highest effectiveness; 2 and 3 log reductions of *S.* Typhimurium and *E. coli* O175: H7 could be achieved when 5% and 10% clove extracts treatments were used. The effectiveness of thyme essential oil against *E. coli* O175: H7 on shredded lettuce was also investigated (Singh et al., 2002). The study showed that the oil could reduce *E. coli* O175: H7 on shredded lettuce by 1.91 and 2.33 log reductions when 1and 10 mL/ L were applied.

Plant extracts are typical ingredients in commercial produce wash solutions. These products were developed due to the increasing consumer demand, seeking alternative, less toxic washing technologies which are user friendly. They not only have antimicrobial properties, but also an ability to remove soil and fruit waxes from the surface of produce (Tang, 2010). Although, there are numbers of these solutions sold in the market, research determining the effectiveness of these commercial products is very limited. Fishburn et al. (2012) used a commercial washing solution called Veggie Wash (Beaumont Products, Inc., Kennesaw, GA) to remove *S. enterica*, *E. coli* O157: H7 and *L. monocytogenes* from some fresh produce' surfaces. The results showed that this solution could reduce these pathogens about 5 and 0.5 to ~1 log reductions in spinach and lettuce, respectively.

All methods mentioned above are related to decontamination using chemical agents either natural or synthetic. However, the most common method which has been using to wash fresh produce is running under tap water which is recommended by U.S. Food and Drug Administration (Fishburn et al., 2012, USFDA, 2011).

Nthenge et al. (2007) applied tap water to reduce inoculated (7.66 $\pm$ 0.01 log CFU/ g *L*. *inocua*) lettuce. Four treatments were applied namely; (1) rinse 15 seconds under tap water,(2) soak 2 minutes in tap water and rinse 15 seconds under running tap water, (3) soak 2 minutes in tap water and rinse 15 seconds under running tap water twice and (4) soak 30 minutes in tap water and rinse 15 seconds under running tap water twice. Each treatment could reduce the microbial load 1.41, 1.79, 1.86 and 1.84 log, respectively. Fishburn et al. (2012) also investigated the efficacy of running tap water on reducing the microbial load on lettuce. Target microorganisms were *E. coli* 0157: H7, *L. monocytogenes* and *S. enterica*, the mean log reduction values were 1.69, 1.41 and 1.58, respectively.

Nastou et al. (2012) determined log reductions of *L*. *monocytogenes* on lettuce surfaces, stored at different temperature (5^{o}C, 15^{o}C and 30^{o}C), prior immersion in tap water for 5 minutes. The mean decrease ranged 0.06 – 0.83 log reductions. Also, this study revealed that the storage temperature before immersion influenced the effectiveness of the decontamination process using

water immersion, as the reduction of the microbial load was smaller when lettuce had been stored at higher temperature.

1.1.6 International guidelines and regulations related to the microbial quality of irrigation wastewater

Wastewater reuse in agriculture is increasing, which also contains pathogenic microorganisms that could harm or potentially harm human health (Becerra-Castro et al., 2015, Rizzo et al., 2013, Varela and Manaia, 2013). Therefore, many countries have developed local guidelines and/ or regulations to control irrigation wastewater quality in order to protect human health.

The state of California, USA established the first regulation of wastewater reuse in agriculture in 1978. Subsequently, the United States Environmental Protection Agency (US EPA) set up new regulations and criteria towards wastewater reuse for agricultural irrigation in 2012. WHO also delivered guidelines about wastewater reuse in agriculture which promotes multi barrier approach to reduce the adverse effect on human health. These regulations and guidelines are being used as references by many countries around the world such as Portugal, France, Spain, Australia, Oman and Israel (Navarro et al., 2015, Becerra-Castro et al., 2015).

Microbial irrigation water quality criteria are generally based on indicator organisms. Generally, coliforms, faecal coliform and *E. coli* have been used as the indicators. However, nematodes eggs have also been proposed (Blumenthal et al., 2000). Some microbial irrigation water quality standards and/ or guidelines applied to crops to be eaten raw are summarised in Table 1.8 which was modified from Becerra-Castro et al. (2015)

Table 1.8 Microbial guidelines on agricultural wastewater irrigation (unrestricted) in some countries

Country/ State (Year)	Indicator organisms			
	Total coliforms (CFU/ 100 mL)	**Faecal coliforms (CFU/ 100 mL)**	***E. coli* (CFU/ 100 mL)**	**Nematode eggs (no./ L)**
California (1978)	-	2.2×10^2	-	-
Mexico (1987)	-	240*	-	≤ 1
Tunisia (1989)	-	-	-	≤ 1
Oman (1993)	-	2.2×10^2	-	< 1
Israel (1999)	-	10	-	-
Australia (2000)	10	-	-	-
Saudi Arabia (2000)	2.2*	-	-	1
Kuwait (2001)	-	20*	-	< 1
Jordan (2002)	-	-	10^2*	< 1
Italy (2003)	-	-	10^2	-
Portugal (2006)	-	10^2	-	-
WHO (2006)	-	-	10^3	≤ 1
China (2007)	-	2×10^4	-	-
Spain (2007)	-	-	10^2	≤ 1(/ 10 L)
France (2010)	-	-	2.5×10^2	
US EPA (2012)	-	Absent	-	-
				-

* MPN/ 100 mL

1.1.7 Quantitative Microbial risk assessment (QMRA)

In general, risk assessment is the process of estimating the potential impact of a chemical, physical, microbiological or psychological hazard on a specified human population or ecological system under a specific set of conditions and for a certain time frame (EnHealth Council, 2012). Obviously, the principle of risk assessment can be applied to assess the microbiological risks in food and water, its aim is to illustrate the magnitude and likelihood of disease or infection associated with human exposure to the microbial hazard (bacteria, viral, parasites, fungal, or their metabolites), and identify factors that affect or potentially affect the risk throughout the supply chain. The information from risk assessment is used by risk managers, particularly policy makers and regulators in order to provide interventions and risk management options to reduce the risk. Risk assessment can be categorised into qualitative and quantitative. The level of risk in qualitative risk assessment can be described by putting risk into ‘low’, ‘medium’ and ‘high’, while risk in quantitative risk assessment is expressed in numerical terms such as probability of illness or infection (EnHealth Council, 2012, Craig, 2013).

Quantitative Microbial Risk Assessment (QMRA) is used to assess and to estimate the consequences from a planned or actual exposure to infectious microorganisms (Haas et al., 1999). Generally, it follows four steps, namely: Hazard identification, dose-response assessment, exposure assessment, and risk characterisation.

Hazard identification

Hazard identification is the determination of the microbial agent and the magnitude of human illness caused by specific microorganisms.

Dose-response assessment

Dose-response assessment describes a quantitative relationship between microbial dose and the likelihood of adverse health effects.

Exposure assessment

Exposure assessment determines the affected population, exposure pathway, microbial dose and frequency and length of time of exposure.

Risk characterisation

Risk characterisation is the estimation of probability of an adverse effect, uncertainty and variability of hazard by combining the information from exposure and dose-response assessment.

Various studies regarding quantitative microbial risk assessment (QMRA) for wastewater irrigation in agriculture have been published (Forslund et al., 2010, Hamilton et al., 2006, Mara et al., 2007, Petterson et al., 2001, Sales-Ortells et al., 2015, Pavione et al., 2013, Forslund et al., 2012, Shuval et al., 1997, Bastos et al., 2008, Seidu et al., 2008, Barker, 2014). Most of them have explored the health risk caused by the consumption of wastewater irrigated salad crops. An outline of the general QMRA for wastewater irrigated salad crops can be described in Fig. 1.4.

The model from Fig. 1.4 describes the general QMRA approach which has been used to estimate health risk associated with wastewater irrigated vegetables consumption. The probability of illness or infection is the output of the model which is computed by using the dose and the number of consumption days as inputs. With regard to the dose, it is computed by using pathogen concentration in wastewater and amount of water retained on vegetable surfaces, thus the fraction of pathogen transferred to produce, fraction of pathogen surviving until harvest, pathogen die-off after harvest and the quantity consumed daily. After QMRA associated with wastewater reuse was adopted by World Health Organisation (WHO) in 1989 (WHO, 1989), it has been applied bacteria, viruses and protozoa (WHO, 2006b).

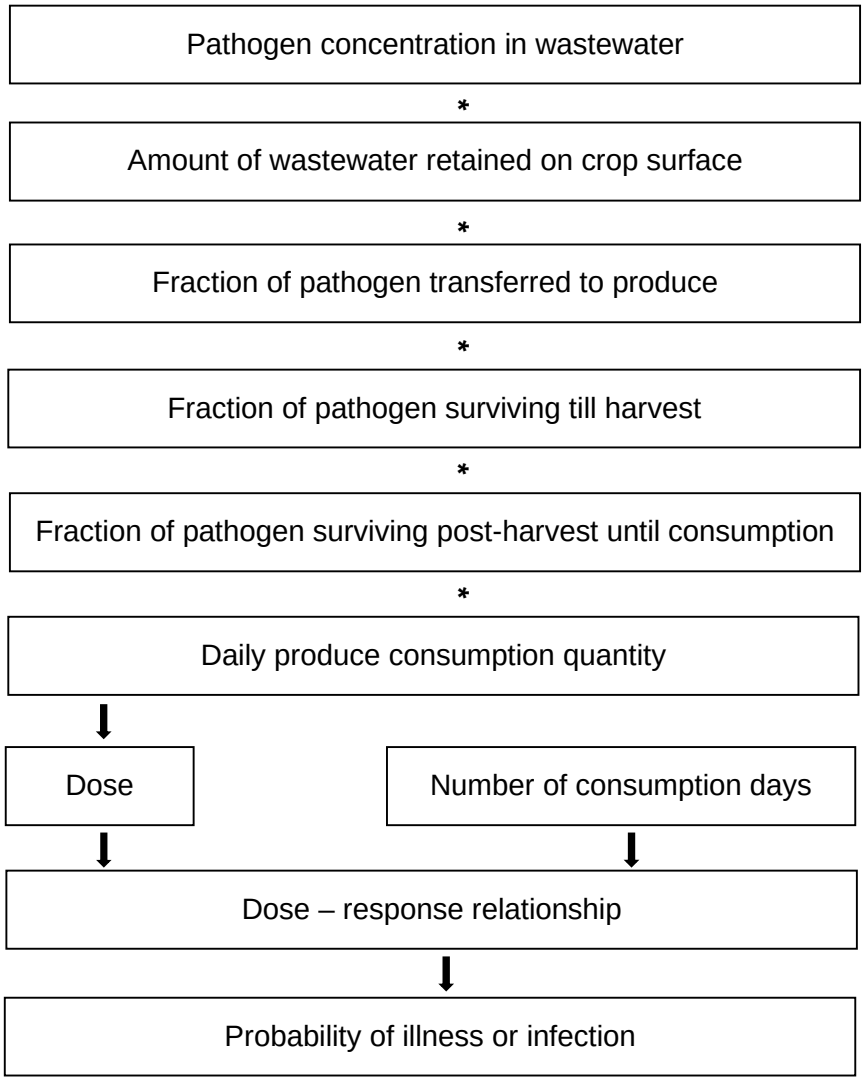

Figure 1.4 An outline of the general QMRA for agricultural wastewater irrigation due to consumption of salad crops (Modified from Pachepsky et al., 2011)

The first study on QMRA associated with salad crops irrigated with wastewater was performed by Shuval et al. (1997). The study was based on the assumption that any microorganisms contained in the residual wastewater remaining on the crop surfaces would cling to them even after the wastewater itself evaporated, and also that people consumed 100 grams of lettuce per person on alternate days. The results showed that the risk of hepatitis A infection from eating lettuces irrigated with raw wastewater was 10^{-2}-10^{-4} per person per year. However, the hepatitis A and rotavirus infection was 10^{-6}-10^{-8} and 10^{-5}-10^{-6}, respectively when eating lettuces irrigated with treated wastewater.

There are debates about the approach used in QMRA applied to the consumption of salad crops following wastewater irrigation. In a number of

QMRA studies, including the current WHO guidelines for the safe use of wastewater, excreta, and wastewater (Volume II Wastewater use in agriculture, (WHO, 2006b), the degree of microbial contamination of the crops were estimated using exposure assessment based on the assumption of Shuval et al. (1997) that any microorganisms contained in the residual wastewater remaining on the vegetables surfaces would cling to the crops, even after the wastewater itself evaporated (Hamilton et al., 2006, Mara et al., 2007, Petterson et al., 2001, Forslund et al., 2010, WHO, 2006a), whereas there were others which determined the level of microbial contamination directly from the crops' surfaces (Bastos et al., 2008, Aiello et al., 2012, Forslund et al., 2012, Pavione et al., 2013). Also, in some studies, the survival/ activation of microoorganisms at postharvest, and the decontamination process prior the consumption at home were not considered (Bastos et al., 2008, Barker-Reid et al., 2010, Mara and Sleigh, 2010a, Forslund et al., 2012, Pavione et al., 2013, Shuval et al., 1997).

Moreover, previous QMRA studies on microbial risk from wastewater irrigation applied to salad crops mostly refer to conventional wastewater treatment as the ultimate measure to reduce health risk from consumption of wastewater irrigated crops. However, wastewater treatment is not well employed in developing countries with more than 90% of untreated wastewater being discharged in to natural water bodies (Corcoran, 2010). Therefore, from food safety management perspective, other measures to reduce the risk would be more effective to apply in developing countries where the wastewater treatment level is very low. This includes at the farm level, post-harvest management and handling at home. As illustrated in Fig. 1.2, microbiological risk could be either increased or decreased throughout the supply chain. Hence, the QMRA applied to wastewater irrigated salad crops consumption in this thesis involved a variety of pathways in line with the principle of the Hazard Analysis and Critical Control Points (HACCP) from farm to fork, to identify the health risk mitigation and safety measures necessary before the salad crops are consumed.

1.2 RESEARCH OBJECTIVES

The main objective of this research was to estimate microbial risk from consumption of salad crop irrigated with partially treated wastewater in with exposure scenarios. This includes the specific objectives to;

- determine the degree of microbial contamination on lettuce following irrigation by partially treated wastewater using microorganism indicators *E. coli*, as well as factors which may affect crop contamination; irrigation practice, microbial wastewater quality and harvesting time.
- examine the volume of water retained on different types of lettuce and number of recovered *E. coli* after irrigation with different microbial wastewater qualities.
- explore the influence of the storage time and temperature on microbial quality of wastewater irrigated lettuce.
- investigate the performance of home washing method for removing microbial load on wastewater irrigated lettuce leaves.
- employ exposure scenarios to determine practices and interventions which reduce potential adverse health effects relating to the consumption of partially treated wastewater irrigated salad crops.

CHAPTER 2

GENERAL METHODOLOGY

2 GENERAL METHODOLOGY

2.1 INTRODUCTION

This chapter describes the general methodology applied in this thesis including the experimental site, the analysis wastewater and lettuce, and the statistical analyses used. Specific methodologies for each individual research objective are described in the respective chapters.

2.2 MATERIALS AND METHODS

2.2.1 Experimental site

A site to study the contamination of lettuce irrigated with wastewater (detail in Chapter 3), was constructed at Mt Barker Wastewater Treatment Plant, the District Council of Mt Barker, South Australia (Plate. 2.1). In brief, Mt Barker wastewater treatment plant treats septic tank effluent from Mount Barker, Littlehampton and Nairne in a series of waste stabilisation ponds. The wastewater was stored in the ponds for more than 60 days, then discharged to the Mount Barker Creek. The plant currently treats 3.2 ML d^{-1} (Mt Barker District Council, 2016). The details of the construction of the experimental site and wastewater sampling are described specifically in Chapter 3-6.

Plate 2.1 Mt Barker Wastewater Treatment Plant, South Australia

2.2.2 Wastewater analysis

2.2.2.1 Quantification of E. coli

E. coli in wastewater was quantified by the Colilert “Quanti-tray” method (IDEXXX Laboratories, Maine, USA) which is an MPN (Most Probable Number) based method, and comparable to other standard methods to analyse wastewater samples (Eckner, 1998).

Colilert®, uses Defined Substrate Technology (DST) to simultaneously detect total coliforms and *E. coli*. Two nutrient-indicators, ONPG (ortho-nitrophenyl-ß-D galactopyranoside) and MUG (4-methylumbelliferyl-ß-D-glucuronide), are the major sources of carbon in Colilert and can be metabolised by enzymes *β*-galactosidase and *β*-glucuronidase by coliforms and *E. coli*, respectively. As coliforms grow in Colilert, they use *β*-galactosidase to metabolise ONPG and change it from colourless to yellow (Plate 2.2), *E. coli* uses *β*-glucuronidase to metabolise MUG and create fluorescence (Plate 2.3). Since most non-coliforms do not have these enzymes, they are unable to grow and interfere (IDEXX, 2016).

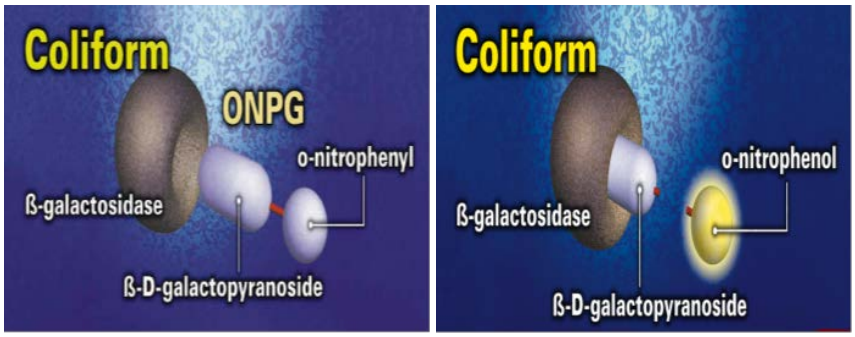

Plate 2.2 Enzymatic metabolism of OPNG to the yellow o-nitrophenol by *β*-galactosidase (pictures provided by IDEXX, Main, USA)

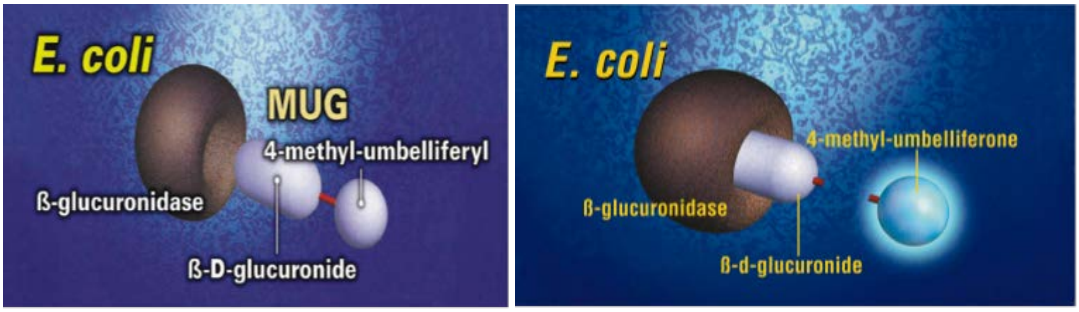

Plate 2.3 Enzymatic metabolism of MUG to the fluorescent 4-methyl-umbelliferone by *β*-d-glucuronide (pictures provided by IDEXX, Main, USA)

For the wastewater analysis, where necessary, 10 fold serial dilutions of samples in sterile 0.1 % buffered peptone water (BPW; Oxoid) was conducted. The concentration of *E. coli* (MPN/ 100 mL) was determined and described by the manufacturer (IDEXX, 2016).

2.2.2.2 Quantification of bacteriophage MS2

A modified, double layer agar method using *E. coli* Famp (ATCC 700891) as a bacterial host, described by Noble et al. (2004) and Debartolomeis and Cabelli (1991) was used to quantify F-specific RNA bacteriophage MS2 in wastewater. *E. coli* Famp (ATCC 700891) was grown at 35°C for 18 h in 10 mL of 1.5% tryptone soya agar (TSA; Oxoid Ltd.) with 1 mL, 0.2 µm filter sterilized, ampicillin/ streptomycin antibiotic stock (0.15 g ampicillin sodium salt/ 0.15 g streptomycin sulphate (Sigma) in 100 mL reverse osmosis treated water (RO).

Wastewater samples were analysed in duplicate. 5 mL of wastewater sample was added into 5 mL of molten 1.5 % TSA mixture, inverted gently to mix and poured over previously made 1.5% TSA base plates, swirled and allowed to set. The plates were then inverted and incubated at 35°C for 18 – 24 h. Plaques were counted and expressed as plaque forming units (PFU)/ 100 mL.

2.2.2.3 Physicochemical analysis of wastewater

pH

The wastewater pH was measured by using a handheld Jenway 370 pH meter (Crown Scientific Pty. Ltd, New South Wales).

Biochemical Oxygen Demand (BOD_5)

The five-day BOD (BOD_5) was determined at 20°C using OxiTop® BOD measuring system, as per the manufacturers instructions, which employs the same principle as in American Public Health Association standard method (APHA, 1992). This system consisted of OxiTop® OC 100 controller, OxiTop®-C measuring heads, inductive stirring system in a temperature controlled cabinet, and dark brown sample bottles to exclude light.

Ammonium Nitrogen (NH_4-N)

Ammonium nitrogen (NH_4-N) was determined using the Automated Phenate Method described in American Public Health Association standard method for ammonium-N concentration analysis (APHA, 1992) as performed in the FIAStar 5000 analyser (Foss, Sweden).

Orthophosphate (PO_4-P)

Orthophosphate (PO_4-P) was determined using the Stannous Chloride Method described in American Public Health Association standard method for ammonium-N concentration analysis (APHA, 1992) as performed in the FIAStar 5000 analyser (Foss, Sweden).

2.2.3 Lettuce samples analysis

Each 25 g lettuce sample was added to stomacher bags containing 225 mL 0.1% sterile buffered peptone water (BPW; Oxoid) and homogenized using a stomacher (Model 2X; IDEXX) for 1 minute. 100 mL of suspension from the homogenate was collected into 120 mL sterile tube and enumerated for *E. coli* by Quanti-Tray Colilert®-18 MPN method as described in 2.2.2.1 and expressed as the most probable number (MPN) of *E. coli* / 100 g of lettuce (MPN/ 100 g).

2.2.4 Statistical analysis

Statistical analysis was performed using SPSS (PASW Statistics 18) and graphs were produced by Microsoft Excel 2010 (Microsoft Cooperation, USA). Statistical significance was accepted at 95% confidence ($p < 0.05$) level. Results were generally expressed as the mean ± standard deviation (SD) of three determinations, unless otherwise stated.

CHAPTER 3

MICROBIAL CONTAMINATION OF LETTUCE IRRIGATED WITH PARTIALLY TREATED DOMESTIC WASTEWATER

The main content of this chapter has been published as:

Makkaew P., Miller M., Fallowfield H. J. & Cromar N. J. (2016) Microbial risk in wastewater irrigated lettuce: Comparing *Escherichia coli* contamination from an experimental site with a laboratory approach. *Water Science and Technology*, 74(3), 749-755.

3 MICROBIAL CONTAMINATION OF LETTUCE WITH PARTIALLY TREATED DOMESTIC WASTEWATER

3.1 INTRODUCTION

Water scarcity is becoming a serious problem worldwide resulting in the increased use of wastewater in agriculture (Scott et al., 2004). However, this practice can also pose a public health risk due to the presence of pathogens such as bacteria, viruses and protozoa (Blumenthal and Peasey, 2002, WHO, 2006b). These pathogens could contaminate agricultural products and could be transmitted to humans by consumption; therefore, public health concerns associated with the consumption of raw vegetables require management. The pathogens of concern can be a source of foodborne disease such as salmonellosis, shigellosis, cholera, and typhoid when eaten uncooked (Shuval et al., 1984, Fattal et al., 1986, Shuval, 1993, Porter et al., 1984). Thus, consuming uncooked vegetables such as leafy greens is the critical public health risk associated with wastewater irrigation in agriculture (Beuchat, 2002, Scheierling et al., 2010b).

Fresh produce could be contaminated by pathogenic microorganisms during any steps from the farm-to-fork perspective; production field, harvest, processing, storage, transportation/ retailing, and home handling (WHO, 2008, USFDA, 2001). Furthermore, the irrigation water quality, types of crops, irrigation methods, and environmental conditions are the factors influencing the degree of contamination at the production sites (Uyttendaele et al., 2015).To manage the public health risk concerns, a guideline for the safe use of wastewater in agriculture has been established by the World Health Organization (WHO, 2006). The guidelines propose multiple measures to protect human health together with the $\log_{10}$ reduction in pathogen numbers required for wastewater treatment to meet a health-based target $\leq 10^{-6}$ disability adjusted life years (DALY) per person per year.

Numbers of studies have been performed which demonstrate that using wastewater to irrigate vegetables in farms results in the contamination of vegetables with microorganisms at the production fields (Nikaido et al., 2010, Manas et al., 2009, Ensink et al., 2007, Rai and Tripathi, 2007, Ait Melloul et

al., 2001, Amahmid et al., 1999, Bastos and Mara, 1995, Rosas et al., 1984). However, most of those studies measured the concentration or contamination of pathogens at the end of growing period. In the study reported here, the time course of microbial contamination of wastewater irrigated vegetables was explored.

In this study, partially treated domestic wastewater was used to irrigate lettuce. The objective of this study was to assess the contamination of lettuce grown using different irrigation systems by applying a widely used indicator microorganisms of faecal contamination, *E. coli*.

3.2 MATERIALS AND METHODS

3.2.1 Lettuce plots and irrigation systems

A field experiment was conducted at Mount Barker Wastewater Treatment Plant, District Council of Mt Barker, South Australia during January – March 2014. The fenced field site (14.6 m (L) x 5.4 m (W)) was divided into 4 beds; open bed with spray irrigation (OS), covered bed (covered with polyethylene sheet) with spray irrigation (CS), open drip irrigation (OD) and covered drip irrigation (CD) (Fig. 3.1 and Plate 3.2). Each bed (2.4 m x 2.4 m) was filled with a commercial, agricultural sandy-loam. Combo mixed lettuce seedlings (commercial name), which comprised of Oak leaf, Mignonette and Salad bowl lettuce (*Lactuca sativa* L.) were bought from a local nursery near the site, and grown in 6 rows with approximately 30 cm spacing between plants (Plate 3.2). Forty two plants were grown in each bed. All beds were irrigated by wastewater pumped directly from the last pond of the waste stabilisation pond series (Plate 3.1). The wastewater irrigation practice was adapted from the guideline of Department of Primary Industries, Queensland Horticulture Institute, Australia (Lovatt and Heisswolf, 1997). The practice required the seedlings received ‘light’ irrigation (10-15 mm) every other day for the first 2 weeks after transplantation, then 15 - 20 mm every 2 days for the remaining growing period.

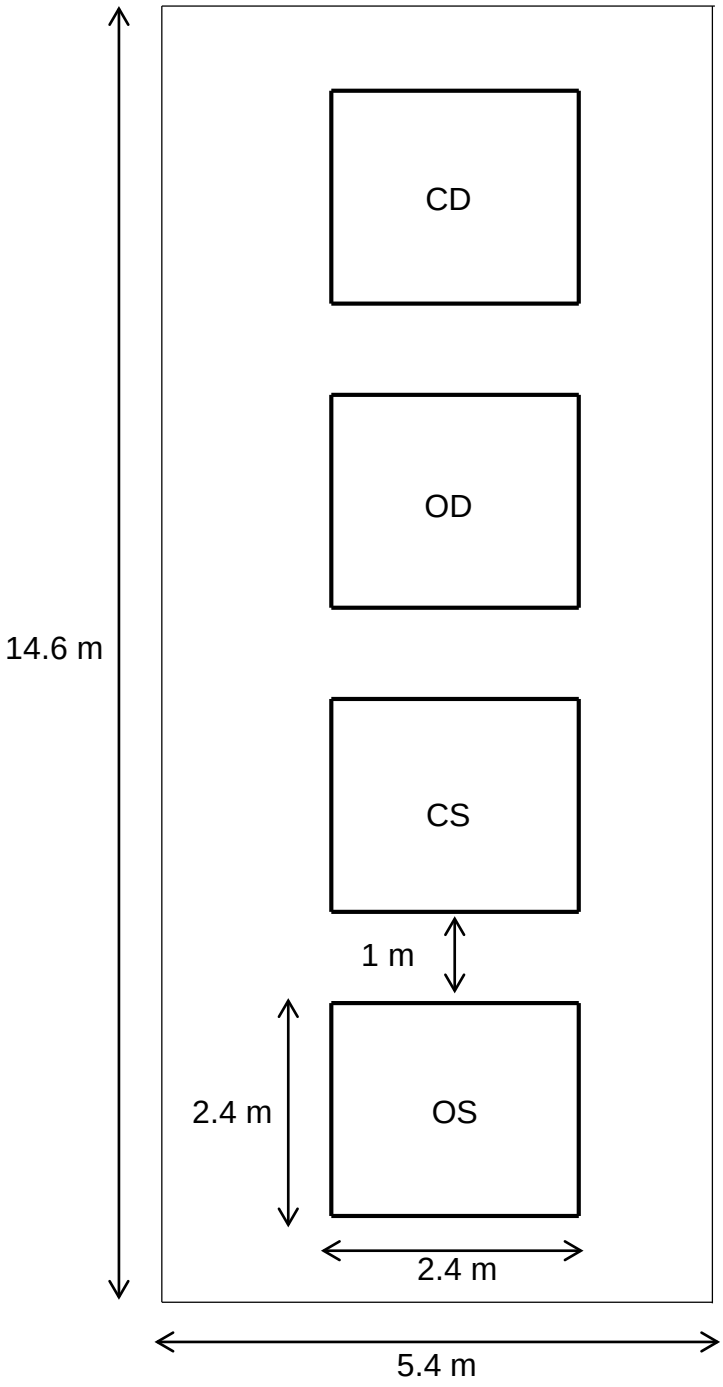

Figure 3.1 Diagram of lettuce plots

Plate 3.1 Wastewater pumped from the last pond of waste stabilisation pond series

Plate 3.2 Lettuce plots at Mt Barker, South Australia

3.2.2 Sample collection

Three samples of lettuce were collected randomly from each irrigation bed within 2 hours of the cessation of irrigation. Sampling commenced on week 4 as the lettuce plants were only then large enough to enable analysis (Plate 3.3). Subsequent, sampling was conducted on week 5 and week 6 since lettuce sold commercially are normally harvested at age 6 - 8 weeks. Within the weeks, lettuce samples from drip irrigated beds were collected on the day of irrigation (D_0), whilst spray bed lettuces were collected on D_0, D_1 and D_2 (0, 1 and 2 days after irrigation respectively). This enabled determination of the change in concentration, post irrigation, of *E. coli* on lettuce spray-irrigated with wastewater. The samples were cut from the stem and 3 - 4 outer leaves discarded using aseptic technique. Irrigated wastewaters were also sampled for analysis at the same time as crop collection. In addition, three samples of Oak leaf lettuce were bought from a local market and analysed for *E. coli* weekly (week 4 – 6). All samples were transported in cold packs and were analysed within 1 hour of sampling.

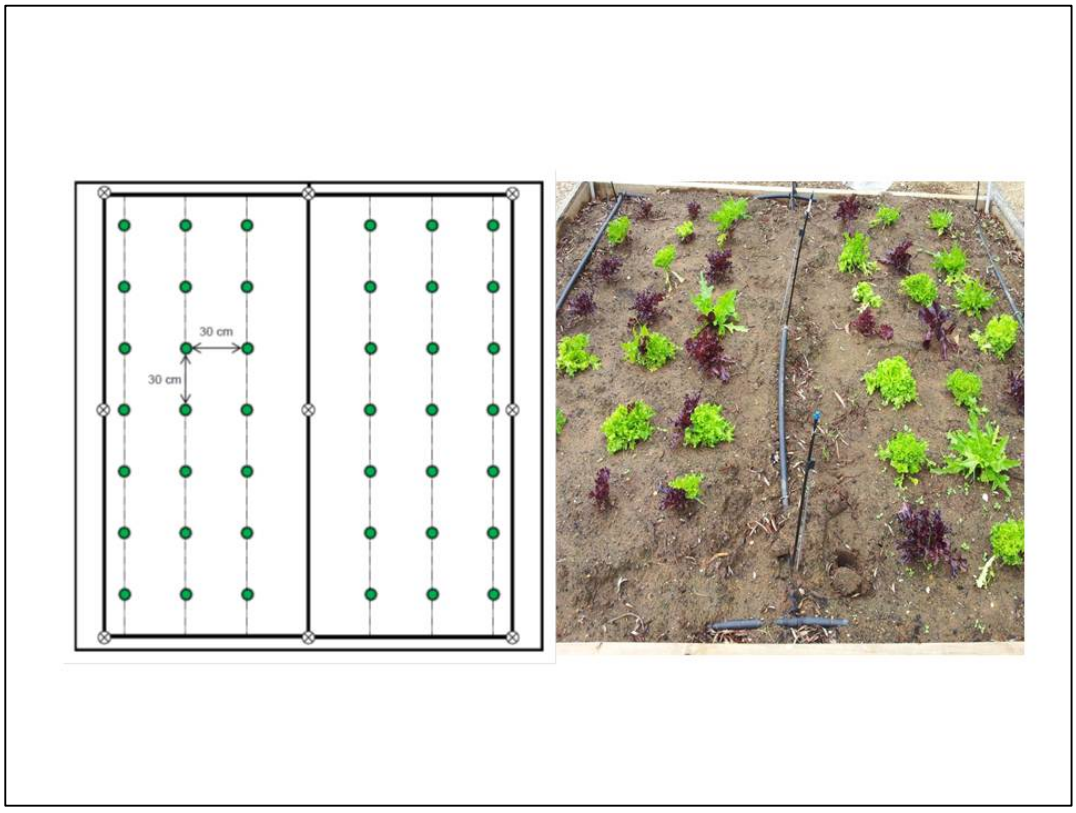

Figure 3.2 A spray irrigated bed

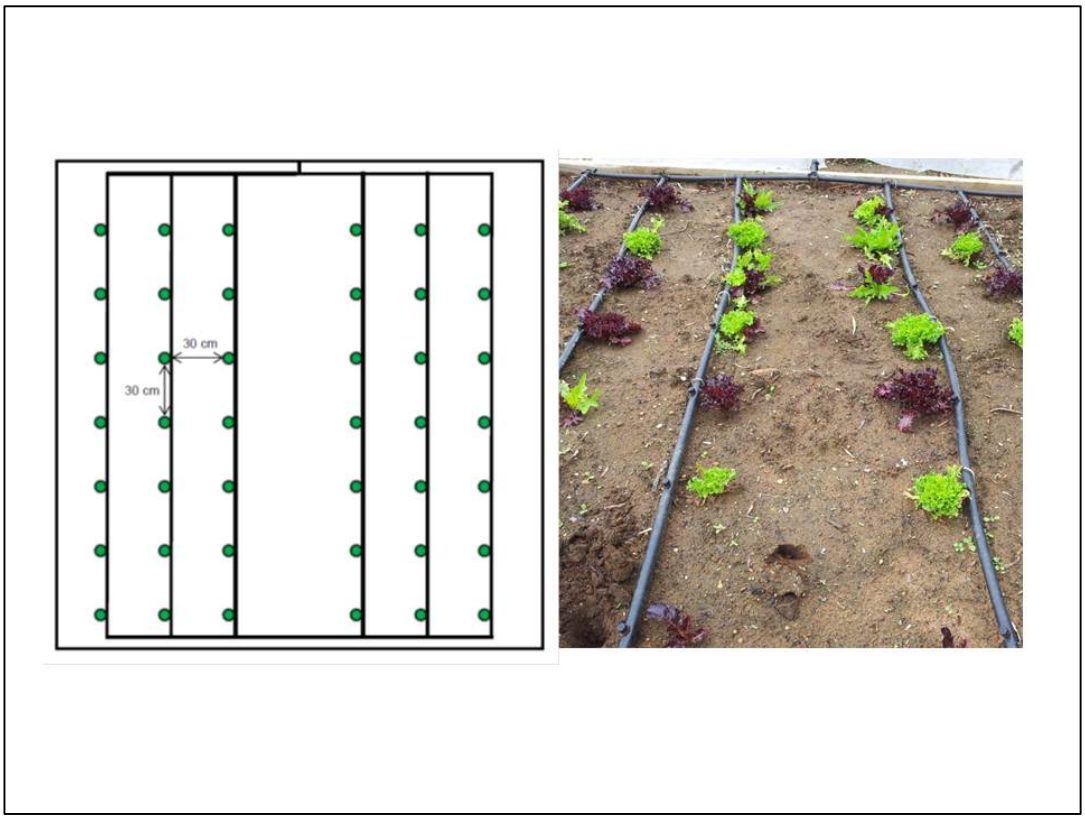

Figure 3.3 A drip irrigated bed

3.2.3 Meteorological data

Temperature (Temp), rainfall and daily global solar exposure (DGSE) for Mt Barker during growing period were obtained from the Bureau of Meteorology, Australian Government (www.bom.gov.au/sa/).

3.2.4 Microbial assay and physicochemical analysis

The microbial analysis of wastewater and lettuce samples were analysed as described previously in Chapter 2. Lettuce sample handling in the laboratory is show in Plate 3.4. The irrigation wastewater was also analysed for; pH, BOD_5, NH_4-N, PO_4-P, MS2 and *E. coli*, the methods were described previously in Chapter 2.

3.2.5 Statistical analysis

The difference in *E. coli* concentration within weeks between lettuces grown in the open spray bed and the covered spray bed was analysed using the Independent – Samples T Test. The difference in *E. coli* concentration of spray bed lettuces between different weeks and different sampling times were analysed using One – Way Analysis of Variance (ANOVA). The difference in

E. coli concentration on lettuces between different irrigation methods was also analysed using ANOVA. All ANOVA analysis was performed together with Bonferroni *post-hoc* test. The *E. coli* data used in the analysis were from D_0, except for the analysis of the difference in *E. coli* concentration of spray bed lettuces between different sampling times (D_0, D_1 and D_2). The correlation of *E. coli* concentration between irrigated wastewater and lettuces was analysed using Pearson Correlation Test. All statistical analyses were performed using SPSS (PASW Statistics 18) with the confidence level of 95 %.

Plate 3.3 Partially treated wastewater grown lettuce at week 4

Plate 3.4 Lettuce handling in the laboratory

3.3 RESULTS

The characteristics of the irrigation wastewater are shown in Table 3.1. During the growing period, *E. coli* in irrigation wastewater from the field experimental site ranged from 141 - 962.6 MPN/ 100 mL (489.8 ± 391.6 MPN/ 100 mL, mean ± standard deviation). All lettuce samples from both spray beds were positive for *E. coli*, while no *E. coli* was detected on drip irrigated lettuces. The samples from lettuce sold in the local supermarket were also free from *E. coli* during the sampling time. The microbial quality of the irrigation wastewater (*E. coli* MPN/ 100mL), and spray bed lettuces (*E. coli* MPN/ 100 g) sampled at different weeks of the irrigation period on the day of irrigation (D_0) and one day after irrigation (D_1), and two days after irrigation (D_2) are presented in Table 3.2. In addition, the metereological data during the sampling time is shown in Table 3.3.

Table 3.1 The characteristics of irrigation wastewater before growing period

Parameter	**Unit**	**Mean value**
pH	-	7.2
BOD_5	mg/ L	53.5
NH_4-N	mg/ L	26
PO_4-P	mg/ L	5.5
MS2	PFU/ 100 mL	10
E. coli	MPN/ 100 mL	210

Table 3.2 *E. coli* concentration of the irrigation wastewater (*E.coli* MPN/ 100 mL mean ± standard deviation), and of spray bed lettuces (*E. coli* MPN/ 100 g mean ± standard deviation) sampled within different weeks of the irrigation period on the day of irrigation (D_0) and one day after irrigation(D_1) and two days after irrigation (D_2).

Sampling week		***E. coli* concentration**					
	Irrigation wastewater (MPN/ 100 mL)	**OSBL (MPN/ 100 g)**			**CSBL (MPN/ 100 g)**		
		D_0	**D_1**	**D_2**	**D_0**	**D_1**	**D_2**
W_4	210.0 ± 99.8	94 ± 36.3	37.7 ± 5.8	ND	93.7 ± 26.6	70.7 ± 23.5	ND
W_5	177.8 ± 27.8	74.3 ± 11.5	244.3 ± 190.2	93.7 ± 17.2	83.7 ± 12.5	141.7 ± 18.3	63.3 ± 11.5
W_6	962.6 ± 72.4	235.7 ± 100.9	727.3 ± 670.9	ND	249.9 ± 68.3	770.7 ± 730.5	103.3 ± 62.5

ND: not detected (< 10 MPN/ 100 g); OSBL: Open Spray Bed Lettuces; CSBL: Covered Spray Bed Lettuces

Table 3.3 Meteorological data during the sampling times

Sampling week	Sampling day	DGSE[1] (mJ/m^2)	Rainfall (mm)	Temperature[2] (^{o}C)
W_4	D	24.8	0	20.8
	D_0	13.9	0	17.5
	D_1	24.4	0	12.5
	D_2	24.6	0	12.8
W_5	D	9.7	0	20.3
	D_0	8.9	0.2	22.5
	D_1	22.0	3.4	14.5
	D_2	21.4	0	12.5
W_6	D	14.6	9.2	11.8
	D_0	11.9	7.4	16.2
	D_1	14.0	2.0	16.9
	D_2	21.9	0.2	14.8
Monthly mean		17.2	*29.6	15.5

[1]DGSE: daily global solar exposure

[*]Monthly mean rainfall is not provided, the data shown in this table is the total amount within a month

[2]Temperature was the value at 9.00 am.

D: The day before irrigation; D_0 = Irrigation day; D_1: 1 day after irrigation; D_2: 2 days after irrigation

There was no statistical difference in *E. coli* concentration (mean ± standard deviation) between OSBL (53.87 ± 37.36) and CSBL (56.93 ± 35.51) within each week or over the entire experimental period ($p > 0.05$). However, there were statistical differences in *E. coli* concentration between weeks in both OSBL (W_5& W_6) ($p < 0.05$) and CSBL (W_4& W_6 and W_5& W_6) ($F2, 6 = 14.11$, $p < 0.05$). The highest contamination was found at W_6 for both OSBL and CSBL, the lettuce contamination was significantly correlated with the microbial quality of irrigated wastewater ($r^2 = 0.99$, $p < 0.05$).

The difference in *E. coli* concentration on lettuce at different sampling times was also explored. In week 4 there was a statistically significant difference in *E. coli* concentration of lettuce sampled on the day of irrigation (D_0) and one (D_1) and two (D_2) days after irrigation for both OSBL ($p < 0.05$) and CSBL ($p < 0.05$). The numbers of *E. coli* decreased one day after irrigation, no *E. coli* was detected on the lettuce 2 days after irrigation. In week 5& 6, the concentration of *E. coli* increased from D_0 to D_1, and decreasing afterwards until D_2.

3.4 DISCUSSION

This study examined the degree of *E. coli* contamination on lettuces following spray and drip irrigation in a field experiment, using partially treated domestic wastewater. The results from the spray irrigation method implied a higher risk than drip irrigation, since there was no *E. coli* detected in any of the drip-irrigated lettuce samples in this study. Irrigation method plays an important role in microbial contamination of crops on site. Drip irrigation can reduce the microbial risks by minimising the exposure of the edible part of crops to irrigated wastewater, when compared to spray or springer irrigation method. Armon et al. (2002) compared the contamination of *Cryptosporidium* and *Giardia* in field crops (vineyard and other vegetables such as cabbage, zucchini, tomato, cauliflower etc.) using different water qualities from different stages of wastewater treatment plant (raw wastewater, outlet from settling pond, facultative pond effluent, filtered effluent). The study demonstrated that crops irrigated by sprinkler irrigation had the highest potential for contamination by *Cryptosporidium* and *Giardia* in comparison to drip and subsurface drip irrigation. Moreover, Fonseca et al. (2011) found that Romaine and Iceberg lettuces grown with water spiked with 10^8 - 10^9 *E. coli* K-12 / mL

were positive for *E. coli* K-12, and could survive on the crops' surfaces up to 7 days when using sprinkler irrigation, whereas only one sample was found to be positive using other irrigation methods (subsurface drip irrigation and furrow).

There was no statistical difference in *E. coli* between OSBL (53.87 ± 37.36) and CSBL within each week or over the entire experimental period. However, there were statistical differences in *E. coli* concentration between weeks in both OSBL (W_5& W_6) ($p < 0.05$) and CSBL (W_4& W_6 and W_5& W_6) ($p < 0.05$). The highest contamination was found at W_6 for both OSBL and CSBL, the lettuce contamination was significantly correlated with the microbial quality of irrigated wastewater ($r^2 = 0.99$, $p < 0.05$). The concentration of *E. coli* in irrigated wastewater at W_6 was 962.6 ± 72.4 MPN/ 100 mL which was the highest concentration during the growing period, while the concentrations at W_4 and W_5 were 210.0 ± 99.8 and 177.8 ± 27.8 MPN/ 100 mL, respectively. It can be seen that the degree of microbial contamination of the irrigating wastewater was another important factor influencing the level of pathogen contamination of produce at harvest. These data are comparable with research by Solomon et al. (2003), where lettuces irrigated with water inoculated with 10^4 CFU *E. coli* O157: H7/ mL were more contaminated at harvest time when compared with those irrigated with water containing 10^2 CFU *E. coli* O157: H7/ mL.

The time elapsed after irrigation also influenced the degree of *E. coli* contamination of the lettuce. In week 4 there was a statistically significant difference in *E. coli* concentration of lettuce sampled on the day of irrigation (D_0) and one (D_1) and two (D_2) days after irrigation for both OSBL (< 0.05) and CSBL ($p < 0.05$). The numbers of *E. coli* decreased one day after irrigation, no *E. coli* was detected on the lettuce 2 days after irrigation. This may be explained by the daily solar exposure data during W_4, which was very high, greater than monthly mean (17.2 mJ/ m^2), one day after irrigation (24.4 - 24.6 mJ/ m^2). Although the effect of sunlight on the survival of microorganisms on fresh produce surface has not been well described, sunlight is an important factor which inactivates microorganisms in contaminated water and wastewater (Bolton et al., 2010). Bichai et al. (2012) planted lettuce using solar

disinfected wastewater to irrigate. Two types of low cost solar disinfection reactors were made; (1) 20-L reactors made from borosilicate glass equipped with compound parabolic concentrators (CPC) to optimise solar radiation, and (2) 1.5-L PET bottles. These methods could reduce *E. coli* in wastewater from $< 10^3 – 10^4$ CFU/ mL to < 2 CFU/ mL, resulting in 26 out of 28 samples were absent of *E. coli* on grown lettuce.

Therefore, the elapse time after irrigation is important because of its influence on the microbial quality of lettuce at harvest. From this study, in week 4, in which there was no potential interference from a rainfall event (discussed below), *E. coli* was not detected on the lettuce 2 days after irrigation for both OSBL and CSBL. It seems that, in hot and dry conditions, harvesting lettuce 2 days after the cessation of irrigation with wastewater may be a management option to reduce the crop's contamination. However, this option may be impracticable in wet conditions – following a rainfall event. Vaz da Costa et al. (1996) cited in WHO (2006b) suggested that crops grown with wastewater should be harvested one or 2 weeks after the last irrigation to provide the optimum time for pathogens to die-off. Keraita et al. (2007) showed that thermotolerant coliforms reduced 0.65 log units per day on wastewater grown lettuce harvested 6 days after the last irrigation in dry season in Ghana. From those studies, the irrigation cessation periods were longer than the result from this study (2 days), however, the microbial load on irrigating wastewater was slightly different. Wastewater used to irrigate lettuce of Keraita et al. (2007) study was higher (4.44 – 8.58 $\log_{10}$ thermotolerant coliforms MPN/ 100 mL) than this study (2.32 $\log_{10}$ *E. coli* MPN/ 100 mL). It is likely to be difficult to apply 6 – 14 day cessation to leafy vegetables that need watering to keep their freshness for market value. Additionally, prolonged withholding period can adversely impact the yield of the production. Keraita et al. (2007) found that lettuces lost 0.14 kg/ m^2 fresh weight on average when harvested 6 days after the last irrigation. Fonseca (2006) observed that the whole lettuce fresh weight reduced by 10% when the last irrigation was terminated 16 days before harvest. However, the withholding period after the last irrigation is site-specific, it depends on the climate of the growing area. There might be difference between the tropical climate countries and cold climate countries. Therefore, watering with clean water after the cessation could be helpful, or

complementary risk reduction measures would be required.

It is not only sunlight that could influence the microbial contamination and pathogens persistence on fresh produce at pre-harvest. Rainfall events may also be potential factors. Rainfall events occurred during the growing period in week 5 and 6, at W_5 (0.2 and 3.4 mm on D_0 and D_1, respectively) and W_6 (7.4, 2.0 and 0.2 mm on D_0, D_1 and D_2, respectively, also 9.2 mm on the day prior to irrigation day (D)). The *E. coli* was remained on lettuce on D_2 (2 days after irrigation) for both OSBL (93.7 ± 17.2 MPN/ 100 g) and CSBL (63.3 ± 11.5 MPN/ 100 g) in week 5, and CSBL in week 6 (103.3 ± 62.5 MPN/ 100 g). It has been reported that resuspension of sediments during or after rainfall events may result in the increasing numbers of *E. coli* in stream water (Hunter et al., 1992).There is no evidence regarding this effect in wastewater pond systems. However, in this study, the concentration of *E. coli* in irrigation wastewater at W_6 was the highest of the growing season (962 ± 72.4 *E. coli* MPN/ 100 mL) and could be a consequence of rainfall, and possibly less solar radiation exposure. The high concentration of *E. coli* in irrigation wastewater resulted to the recovery of high numbers of *E. coli* in lettuces. In addition, Monaghan and Hutchison (2012) noted that rainfall created soil splash which could transfer microorganisms from soil to vegetables. Soil splash created by heavy rainfall on D_1 could have also affected the *E. coli* concentrations on the lettuce which were higher in OSBL than on D_0 in W_5 and W_6. However, at W_6, no *E. coli* was detected on open dripped bed lettuces, although there was heavy rain both on D and D_0. The geometry of the irrigation systems may explain this observation. The open, unvegetated surface area of irrigated soil in the dripped beds was much smaller than sprayed beds. The wetted zone, following irrigation of drip beds, extended only around the drippers, furthermore, the canopy of the mature lettuce protected this area from the direct impact of rain, which may minimise contamination of the lettuce by soil splash. In contrast, the entire area of the spray beds was contaminated with irrigated wastewater, providing greater opportunity for lettuce in spray irrigated beds to be contaminated by soil splash. Further studies, determining the relative concentration of *E. coli* in soil samples taken from transects across irrigation beds, are required to confirm the potential effect of irrigation geometry on lettuce contamination by soil splash. It was observed from the meteorological data in Table 3.3, that

DGSE was relatively high even on the day of maximum rainfall (D at Week 6, DGSE was 14.6 mJ/ m^2 and rainfall was 9.2 mm). UVA and UVB are recognised as the main disinfection wavelengths from sunlight (Bolton et al., 2010). While they were not measured directly in this study the measurement of DGSE was considered a surrogate measurement for likely UV exposure. Cloud cover will obviously moderate surface exposure to UV and it might be expected that DGSE and cloud cover are inversely related. However, low DGSE was not always associated with rainfall; there were low values of DGSE recorded in W_4 and W_5 on days when there was no rain – presumably due to cloud cover moderating DGSE but not resulting in precipitation. It is important to note that the rainfall and DGSE data are daily total values; no hourly data was available. The high rainfall could have occurred at night in W_6 resulting in high rainfall and maintenance of a high DGSE, alternatively, as occurs frequently in this location the rainfall event (and cloud cover) could have been of high intensity but short duration, which again would have had a minor effect on the DGSE value. These factors may explain apparent contradiction for the results obtained for W_6 whereby the rainfall was the maximum recorded for the study while the DGSE was also high, although slightly less than the monthly mean. For further research, climatic conditions should be monitored onsite in order to more understand about the environmental factors influencing the microbial quality of grown lettuce.

Strong wind during rainfall in W_6 also blew off the plastic sheet cover of CSBL, which also resulted in the contamination of the produce in CSBL. At week 6 (W_6) of the growing season, there was no *E. coli* detected in OSBL on D_2, while there was some still detected in CSBL (103.3 ± 62.5 *E. coli* MPN/ 100 mL). The polyethylene film use to cover the CBSL has the ability to absorb ultraviolet (UV) and visible light (Kamweru et al., 2014) which could minimise the exposure of *E. coli* on the lettuce leaf surfaces to UV and visible light, resulting in the numbers of *E. coli* still retained on CSBL. This was supported by the observation that, on D_0 at W_4 - W_6, *E. coli* was only detected on a third of the OSBL, whereas *E. coli* was detected on two - thirds of the CSBL sampled, suggesting that the UV disinfection in open spray bed was greater than the covered bed covered by polyethylene film. It may have been desirable to include in the study control plots irrigated with potable water, however, potable

water was unavailable at the site. It can, however, be concluded from the absence of *E. coli* on lettuces from the drip-irrigated plots that the *E. coli* contamination was derived from the wastewater. Two mechanisms for the contamination are considered, firstly, directly from the wastewater following spray irrigation and secondly, indirectly by *E. coli* present on the spray irrigated soil surface subsequently contaminating lettuce via ‘soil splash’ during rainfall events.

In summary, spray irrigation method implied a higher risk than drip irrigation, since there was no *E. coli* detected in any of the drip-irrigated lettuce samples in this study. Therefore, in order to minimise public health risk, drip irrigation should be the recommended irrigation method when wastewater is applied to crops. However, when drip irrigation is not applied and spray irrigation is used, the microbial quality of irrigating water, the time of harvest following the last irrigation and climate conditions such as rainfall and sunlight all have the potential to influence the degree of contamination of the harvested lettuce.

CHAPTER 4

THE INFLUENCE OF THE MICROBIAL QUALITY OF WASTEWATER, LETTUCE CULTIVARS AND ENUMERATION TECHNIQUE IN ESTIMATING THE HEALTH RISK FROM THE CONSUMPTION OF WASTEWATER IRRIGATED LETTUCE

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4 THE INFLUENCE OF THE MICROBIAL QUALITY OF WASTEWATER, LETTUCE CULTIVARS AND ENUMERATION TECHNIQUE IN ESTIMATING THE HEALTH RISK FROM THE CONSUMPTION OF WASTEWATER IRRIGATED LETTUCE

4.1 INTRODUCTION

In an effort to minimise the adverse health effects from human exposure to pathogens associated with wastewater reuse in agriculture, the World Health Organization (WHO) published the third edition of the guidelines for the safe use of wastewater, excreta and greywater in 2006 (WHO, 2006b). The guidelines offer multiple approaches to risk management to meet the health-based target for the burden of waterborne disease, $\leq 10^{-6}$ Disability Adjusted Life Years (DALY), associated with working in wastewater-irrigated farms, or consuming wastewater irrigated crops. In this guideline a Quantitative Microbial Risk Assessment (QMRA) approach (Haas et al., 1999) was used to estimate the health risk from wastewater irrigation. In brief, QMRA translates the exposure of consumers to pathogens under a specific set of conditions (exposure scenarios) to the probabilities of infection by applying four steps, namely: hazard identification, dose-response assessment, exposure assessment and risk characterisation. Although QMRA could be an effective tool for health risk estimation, the challenges of using this tool include the lack of data or the poor quality of data available for inclusion in the estimation of the risk. Many studies have applied QMRA to the consumption of wastewater irrigated salad crops, and lettuce, consumed worldwide, has been widely used to estimate this health risk.

In most of the QMRA studies, including the current WHO guidelines, the level of microbial contamination of the crops was estimated using exposure assessments derived from the water retained on the crops' surface and assuming that any microorganisms contained in the residual wastewater would be retained on the vegetable surfaces, even after the wastewater evaporated (Shuval et al., 1997, Petterson et al., 2001, Hamilton et al., 2006, Mara et al., 2007). Based on this assumption, it is important to identify the water retention in various morphological varieties of lettuce since it has only been determined

for one type of lettuce (long leaf lettuce) by Shuval *et al*. (1997) who determined water retention from the difference in weight following submersion of lettuce in a bucket of water. There is a variety of lettuce cultivars with different morphology, grown across the world, Iceberg, Cos and Oak leaf lettuce are varieties which are most commonly consumed (Mou, 2012, Van Treuren and van Hintum, 2009). At present, there is limited information about water retention in various varieties of lettuce, which could be useful for estimating risk using QMRA. Furthermore, there are few studies that attempt to determine directly the numbers of microorganism retained on the plants' surfaces to estimate the risk, rather than using the volume of wastewater, of known concentration of microorganisms of concern, retained on the crops' surface to estimate numbers (Bastos et al., 2008, Forslund et al., 2010, Aiello et al., 2012). Nevertheless, Mok and Hamilton (2014) argued that this direct method was not flexible for modelling multiple scenarios compared to the water retention method as it will only allow modelling on a particular set of conditions. However, enumeration directly from the surface of crops is the standard method for the microbiological examination of fresh fruits and vegetables used by food standard regulatory agencies (Thatcher, 1974, USFDA, 2003, FSANZ, 2001).

There is no clear evidence if the numbers estimated from the water retention on the surface of plant following submersion is comparable to the number of microorganisms quantified directly from plants' surface. The aims of the investigation outlined in this chapter were to:

- Determine wastewater retention volumes for three different varieties of lettuce (Iceberg, Cos and Oak leaf).
- Compare the *E. coli* concentration on composite samples and samples of outer and inner leaves from the three different varieties of lettuce after submersion with wastewater.
- Determine the effect of microbial wastewater quality on the contamination of *E. coli* on lettuce leaves, and to compare the direct enumeration of *E. coli* on lettuce leaves with the indirect method, which estimates contamination using the *E. coli* concentration and the volume of wastewater retained on the leaves.

4.2 MATERIALS AND METHODS

4.2.1 Sample selection

4.2.1.1 Lettuce variety

Three varieties of lettuce, Iceberg, Cos and Oak leaf lettuce (Plate 4.1), which are widely consumed, were selected for this study as they have different leaf structures, which could potentially affect water retention. Lettuce samples were bought from local supermarkets in Adelaide, South Australia. Each lettuce was contained individually in clear polythene freezer bags, and was transported, chilled in a freezer box, to Environmental Health Laboratories, Flinders University for analysis. Oak leaf lettuces normally came with the roots attached, which were removed aseptically with a sterile knife (wiped with 70% ethanol and flamed).

(a) **(b)** **(c)**

Plate 4.1 The three varieties of lettuce used in this study (a) Iceberg (http://www.samsclub.com/sams/taylor-farms-iceberg-lettuce-2-heads/133615.ip) (b) Cos (http://www.samsclub.com/sams/romaine-hearts-6-ct/prod1941521.ip) (c) Oak leaf (http://montecitourbanfarms.com/shop/salanova-green-oakleaf-lettuce/#prettyPhoto).

4.2.1.2 Wastewater samples

In order to determine the effect of microbial wastewater quality on the contamination of lettuce, 4 different target concentrations of *E. coli* in wastewater were selected; 10, 10^2, 10^3 and 10^4 *E. coli* MPN/ 100 mL. The wastewater samples (40 L) were collected at different points from the wastewater stabilisation ponds (WSP), Mt Barker wastewater treatment plant, South Australia. The concentration of *E. coli* in the various wastewaters used in the experiments and the collection points are shown in Table 4.1, and Plates 4.2 – 4.4.

Table 4.1 The concentration of *E. coli* (MPN/ 100 mL) in wastewater samples from various sources.

Target concentration	Actual concentration	Wastewater source (Mt Barker*)
10	6.3	The outlet of wastewater DAF**plant.
10^2	75.9	Final pond in the WSP series
10^3	1,299.7	The dilution of the first facultative pond: with the outlet water from the DAF** (10: 1)
10^4	27,550	Wastewater from the first facultative pond

*Mt Barker Community Wastewater Treatment Plant, South Australia.

** DAF: Dissolved Air Flotation.

Plate 4.2 Dissolved Air Flotation

Plate 4.3 The final pond in series

Plate 4.4 The first facultative pond in the series

4.2.2 Wastewater retention

To determine the influence of lettuce cultivars on water retention and *E. coli* contamination, lettuces were contaminated with wastewater in the laboratory using the bucket submersion technique (Hawley, 2012) which was adapted from (Shuval et al., 1997). A 10 L plastic bucket, placed on a larger aluminum tray to contain spillage, was used for lettuce submersion. The whole lettuces were submerged, individually, upside down into the bucket for 20s. Then, each submersed lettuce was held above the bucket and gently flicked right to left, left to right, and up and down, eight times each way. This sequence was performed 4 times, the lettuce was then held above the water for 20s after the last submersion to drain surplus water. Six samples of each type of lettuce were contaminated using this procedure for each of four experiments using wastewaters with the different concentrations of *E. coli* (Table 4.1).

This bucket submersion method was applied to 72 lettuces (3 varieties of lettuce, 6 samples per lettuce variety with 4 different of *E. coli* concentration in wastewater). Each lettuce was weighed individually in an aluminum foil lined plastic bowl before and after submersion. The volume of water retained was calculated using Eq. 4.1.

$$W_r = W_a - W_b \qquad \textbf{Equation 4.1}$$

Where,

W_r was the volume of water retained (mL/g lettuce), W_a was the weight (g) of lettuce after submersion and W_b was the initial weight (g) of lettuce before submersion. Then, the volume of water retained was calculated (Eq. 4.2) and expressed as water retained per 100 g of lettuce based on the current guidelines (WHO, 2006b, Shuval et al., 1997). The volume of water retained was calculated by:

$$W_{r100} = (W_r / W_b) * 100 \text{ g Lettuce} \qquad \textbf{Equation 4.2}$$

Plate 4.5 Submersion equipment

Plate 4.6 Cos lettuce weighting

4.2.3 Enumeration of *E. coli*

4.2.3.1 Indirect method

The *E. coli* content of the respective wastewater in which the lettuces were submerged was determined using the Colilert®-18 MPN method (IDDEX Laboratories). The number of *E. coli* on lettuce leaves was calculated from the *E. coli* concentration of the wastewater and the volume retained by the lettuce using Eq. 4.3.

[(*E. coli* concentration/ 100 mL wastewater) * (Volume of water retained /100 g of lettuce)]/100 **Equation 4.3**

4.2.3.2 Direct method

Following wastewater submersion (described above), the lettuce was aseptically separated into two components; 3-4 outer leaves and 3-4 inner leaves in order to determine the *E. coli* concentration retained on lettuce leaves from different leaf locations. The outer and inner leaf samples were cut aseptically into 25 g (Plate 4.7 – 4.8). A second wastewater submersed lettuce, of the same type, was cut into quarters and then aseptically dissected into 25 g to include all parts of the lettuce leaves; this was designated the composite leaves (Plate 4.9). This experiment was conducted using three lettuce varieties each at 4 different *E. coli* concentration in wastewater. The respective dissected leaf parts were analysed in triplicate. Each 25 g lettuce sample was added to a stomacher bag containing 225 mL, 0.1% sterile buffered peptone water and homogenised using a stomacher (Model 2X (IDEXX)) for 1 minute. Afterwards, 100 mL of suspension from the homogenate was collected into a 120 mL sterile tube and dispensed into Quanti-Tray for enumerating *E. coli* using the Colilert®-18 MPN method. The results were expressed as the most probable number (MPN) of *E. coli* /100 g of lettuce.

4.2.4 Statistical analysis

The difference in wastewater retained, and recovered *E. coli* between different varieties and parts of lettuce were analysed by using Two – Way ANOVA together with Bonferroni *post-hoc* test. The difference in *E. coli* concentration between the direct and indirect method of enumeration was analysed using the Paired-T test. The relationship between the *E. coli* concentration of the wastewater and lettuces following submersion was analysed using linear

regression. All statistical analyses were performed using SPSS (PASW Statistics 18) with a confidence level of 95%.

Plate 4.7 Outer leaves of Cos lettuce

Plate 4.8 Inner leaves of Cos lettuce

Plate 4.9 Composite samples of Cos lettuce

4.3 RESULTS

4.3.1 Wastewater retention in three varieties of lettuce

The mean volume of wastewater retained by the three lettuce varieties using the indirect method of determination is shown in Fig. 4.1 There was a statistically significant difference in the volume of wastewater retained by the three different varieties of lettuce ($p < 0.01$). It can be seen that Oak leaf lettuce retained the highest volume (42.9 ± 4.9 mL/ 100 g), following by Cos (22.6 ± 4.8 mL/ 100 g) and Iceberg (15 ± 4.6 mL/ 100 g).

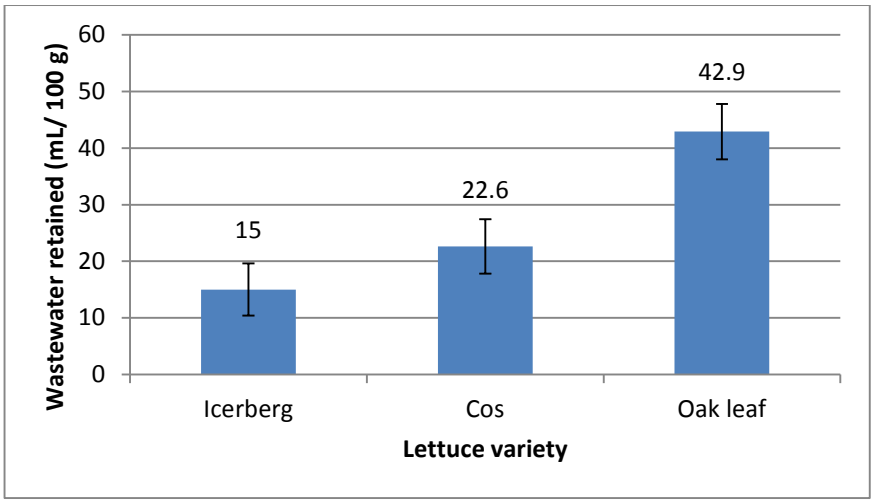

Figure 4.1 Wastewater retained (mL/ 100 g) on the leaves of Iceberg, Cos and Oak leaf lettuce (n = 24 for each lettuce cultivar).

4.3.2 *E. coli* retained in different parts of the three varieties of lettuce

E. coli retained by parts of three different varieties of lettuce after submersion in wastewaters of differing *E. coli* concentration, enumerated using the direct method, is shown in Fig. 4.2 – 4.4. No *E. coli* was detected (< 10 MPN/ 100 g) in any lettuce samples following submersion in wastewater containing 6.3 *E. coli* MPN/ 100 mL. There were, however, statistically significant differences ($p < 0.01$) in *E. coli* concentration retained between Iceberg and both the Cos and Oak leaf varieties, with Iceberg retaining significantly less *E. coli* following submersion in wastewaters containing 75.9, 1,299.7 and 27,550 *E. coli* MPN/ 100 mL. In addition, there was no statistically significant difference between outer and inner leaves and the composite samples ($p > 0.01$) following submersion in the wastewaters with *E. coli* concentrations of 75.9 and 1,299.7 *E. coli* MPN/ 100 mL (Figs 4.2 and 4.3). However, the location of the lettuce leaf becomes an important factor following submersion in the wastewater with an *E. coli* concentration of 27,550 *E. coli* MPN/ 100 mL, when there were statistically significant differences in *E. coli* concentration between outer and inner leaves and the composite samples ($p < 0.01$) with outer leaves retaining significantly more *E. coli* following submersion than inner and composite ones (Fig. 4.4).

4.3.3 The comparison of *E. coli* concentration enumerated by direct and indirect method

The direct method (quantified from the lettuce sampled after wastewater

submersion) and indirect method (estimate the concentration based on the retained wastewater on the lettuce leaf surfaces as described in Eq. 4.3) for determining the retention of *E. coli* were compared. The mean *E. coli* counts of the three composite samples from each lettuce type was used to represent the concentration of *E. coli* enumerated by the direct method. The composite samples were used in this comparison as they were considered to better represent the number of *E. coli* enumerated using the standard method for the microbiological examination of fresh produce employed by food regulatory agencies (Thatcher, 1974, USFDA, 2003, FSANZ, 2001). The data are shown in Table 4.2. There were no statistically significant differences in *E. coli* concentration between the direct and indirect method of enumeration ($p > 0.01$) for all three varieties of lettuce.

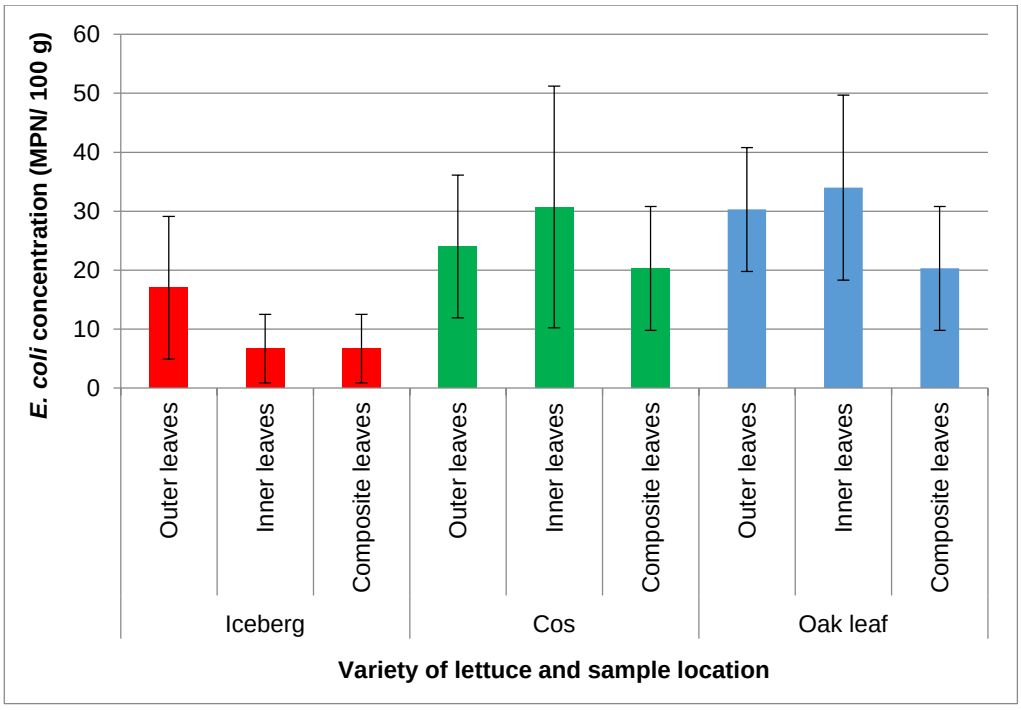

Figure 4.2 *E. coli* enumerated on outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 75.9 *E. coli* MPN/ 100 mL.

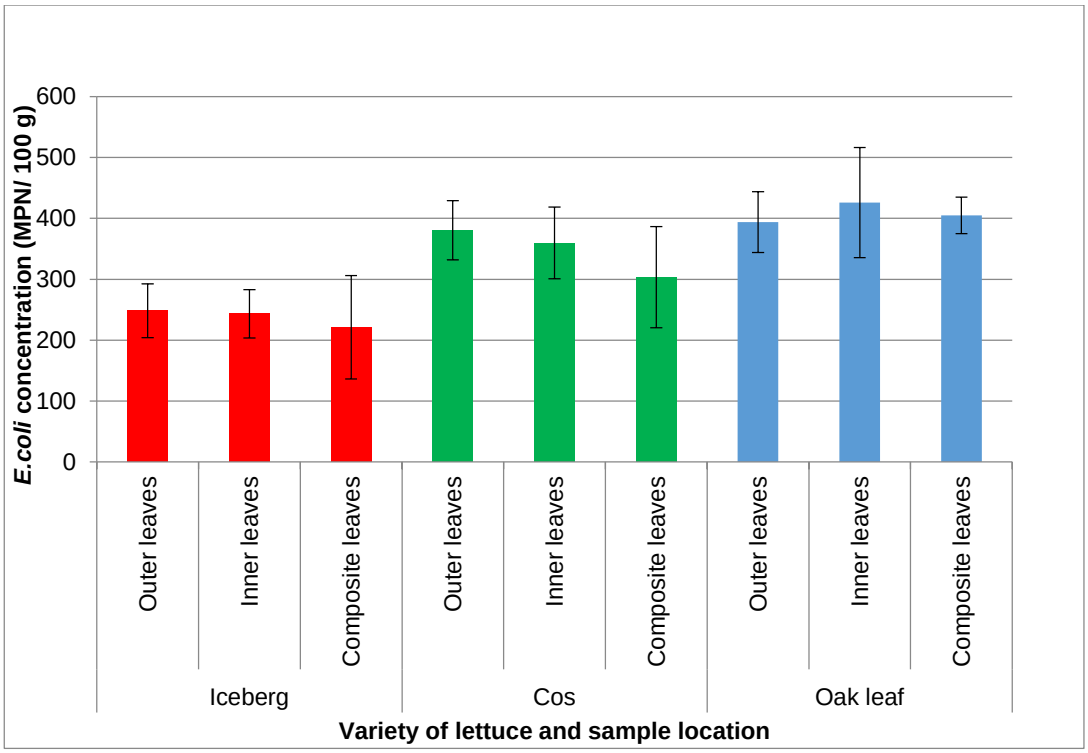

Figure 4.3 *E. coli* enumerated on outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 1,299.7 *E.coli* MPN/ 100 mL.

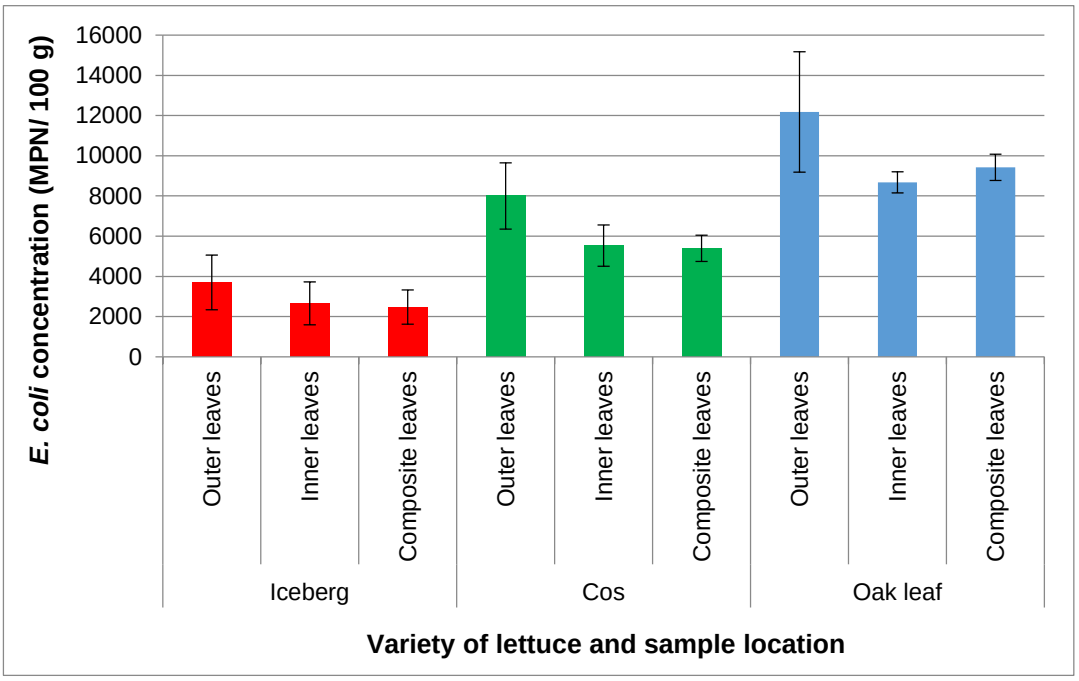

Figure 4.4 *E. coli* enumerated on outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 27,550 *E. coli* MPN/ 100 mL.

Table 4.2 The comparison of *E. coli* concentration quantified by the direct method, enumerated from a 25g composite sample of lettuce, and the indirect method, estimated from the *E. coli* concentration of the wastewater and the volume retained by the lettuce.

Lettuce varieties	**Microbial wastewater quality (*E. coli* MPN/ 100 mL)**	***E. coli* concentration (MPN/ 100 g) (Mean ± S.D.)**	
		Direct method	**Indirect method**
Iceberg	75.9 (n = 3)	6.7 ± 5 .8	10.2 ± 3.3
	1,299.7 (n = 3)	221.3 ± 84.9	237.4 ± 57.4
	27,550 (n = 3)	2,470.7 ± 848.8	3,278.5 ± 600.4
Cos	75.9 (n = 3)	20.3 ± 10.5	17.1 ± 2.9
	1,299.7 (n = 3)	303.3 ± 83.1	262.5 ± 15.8
	27,550 (n = 3)	5,395.7 ± 652.9	5,234.5 ± 1,360.6
Oak leaf	75.9 (n = 3)	20.3 ± 10.5	32.0 ± 1.6
	1,299.7 (n = 3)	405.0 ± 29.8	510.8 ± 20.4
	27,550 (n = 3)	9,424.0 ± 658.2	11,470.0 ± 937.2

4.3.4 The relationship between the microbial quality of lettuce and that of irrigation wastewater

The *E. coli* counts enumerated from composite leaves of the respective lettuces were used to determine the relationship between the *E. coli* concentrations of the wastewater in which they were submersed. The *E. coli* concentration on lettuces was significantly ($p < 0.01$ for Cos and Oak leaf, and $p < 0.05$ for Iceberg) related to *E. coli* concentration of the wastewater (Fig. 4.5).

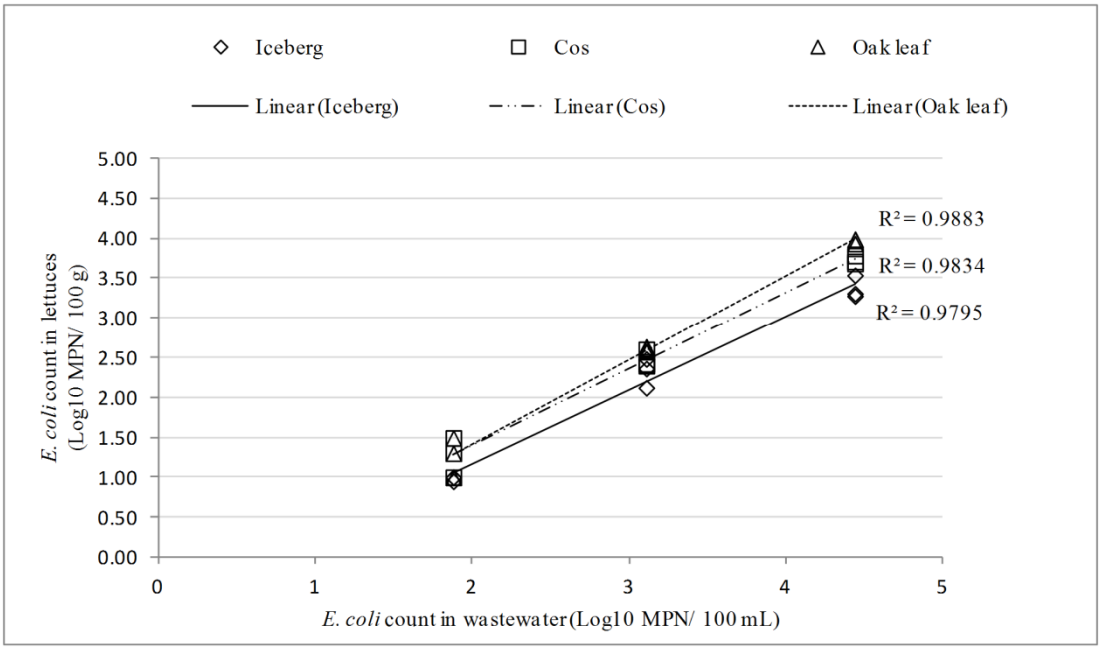

Figure 4.5 The linear regression of *E. coli* count (log_{10} *E. coli* MPN/ 100 g) recovered from composite leaf samples of Iceberg, Cos, and Oak leaf lettuce and the *E. coli* count in the wastewater in which they were submersed (log_{10} *E. coli* MPN/ 100 mL).

4.4 DISCUSSION

Wastewater irrigation in agriculture is an emerging public health risk as it is becoming more widely used worldwide, but there are still knowledge gaps with regards to assessing the health risk from consuming crops irrigated with wastewater. Shuval *et al*. (1997) were the first to estimate the health risk based on the captured volume of water on the surface of lettuce leaves, which was subsequently used by a number of other studies (Petterson et al., 2001, Mara et al., 2007, Hamilton et al., 2006). The study reported here determined the volume of wastewater retained on three different varieties of lettuce (Iceberg, Cos and Oak leaf) since there was limited data available regarding water retention by different lettuce varieties. The results of this study showed that the different lettuce varieties retain differing amounts of wastewater; Oak leaf retained significantly more wastewater per 100 g of lettuce than either Cos or Iceberg. The differences are likely due to differences in leaf morphology between different varieties of lettuce and consequently, the relative surface area exposed to submersion in wastewater. Iceberg has tightly compacted, overlapping leaves, while Oak leaf has a loose head with broad and elongated

rosette leaves (Mou, 2008, Křístková et al., 2008, Hawley, 2012). These leaf structures affect the wastewater retention on the lettuce leaf surfaces. The compact leaves of the Iceberg variety could prevent the water getting inside the heart of the lettuce; whereas the more open leaf varieties like Oak leaf retains more of the water. In addition, crop leaves also have some properties, which could potentially influence the wastewater retention on the leaf surfaces. Hunter *et al*. (2010) found that Iceberg lettuce leaves had slightly more wax content than other lettuce varieties, causing the water to roll off the surface immediately instead of being adsorbed into the leaves (Neinhuis and Barthlott, 1997).

In this study, the retained wastewater volume of the three lettuce varieties (15, 22.6 and 42.9 mL/ 100 g for Iceberg, Cos and Oak leaf lettuce, respectively) was higher than the 10.8 mL/100 g , which was the mean volume captured by lettuce reported by Shuval *et al*. (1997) and which has been widely used in subsequent QMRAs (Petterson et al., 2001, Mara et al., 2007, Hamilton et al., 2006)). Possible explanations for the difference in the retained wastewater volume include; the varieties of lettuce used in this experiment were different from the long leaf lettuces used by Shuval *et al*. (1997). The sample size also differed, only 12 long leaf lettuces were used to determine the captured water in the study by Shuval *et al*. (1997), while the sample size of the study reported here was 24 plants per lettuce variety. The water applied could also be another factor influencing the amount of retained water on the crop surfaces. Lettuces were submersed into wastewater in this study, whereas there was no description of the water type used in the experiment conducted by Shuval *et al*. (1997). Hawley (2012) compared the amount of water retention on lettuce following submersion in either potable water or domestic wastewater from a waste stabilisation pond. She noted that the volume of retained water was greater when lettuces were submersed in wastewater. Suspended solids are the major component of organic contaminants in domestic wastewater (Mckinney, 2004). These could adhere to the lettuce leaf surfaces, resulting in the greater weight gain, interpreted as the volume of water retained, compared with when potable water was used in the experiments.

Mok and Hamilton (2014) investigated the captured water volume of some

Asian vegetables as well as Oak leaf lettuce. The captured volume of Oak leaf lettuce in their study, 1 mL/ 100 g was far less than from this study, 42.9 mL/ 100 g. This difference could possibly be explained by the difference in experimental procedures used to examine the water retained on the crops' surfaces. Mok and Hamilton (2014) collected a hundred Oak leaf lettuce from a field irrigated with freshwater by overhead sprinklers, to determine the captured volume by weight differential before and after spinning and drying with a paper towel, while a laboratory submersion technique, as described above, was used in the study reported here. The bucket submersion technique exposed a greater surface area of the crops to the wastewater in comparison to irrigation by overhead sprinklers where the water falls as droplets on to the surface of the crop. In addition, the weighing protocol was also different between these two studies. In this laboratory study the whole lettuce was submersed into wastewater contained within a bucket, excess water was removed using a well defined 'shaking' protocol and the captured volume was calculated by difference in weight before and after submersion. In contrast, in the study reported by Mok and Hamilton (2014) significant manipulation of the samples occurred before the water retention value was determined. The Oak lettuces were cut from the field, transported to a laboratory, weighed, cut into small pieces before being spun, and weighed. The water retained on crops' surfaces was potentially lost during transportation, leaf dissection and spinning, resulting in a much reduced value for water retention than that reported here (42.9 mL/100g) or the 10.8 mL/100g reported by Shuval *et al*. (1997). In addition, the volume of wastewater captured by the lettuce reported by Mok and Hamilton (2014) was determined by the weight lost before and after spinning, whereas Shuval *et al*. (1997) and this study determined wastewater retention from the difference in the weight of the lettuce pre- and post-submersion. The sample manipulations conducted by Mok and Hamilton (2014), could, arguably, be considered to more accurately reflect the microbial contamination where transport and handling is more intense and consequently reduces both the wastewater retained and the associated pathogens. The submersion technique (indirect method) reported here might, however, be the more valid approach where worse-case scenario, rapid risk assessment for use in exposure models in QMRA studies is required

The morphology of crops' leaves also affects the microbial contamination, in this study *E. coli* were detected in larger numbers in Oak leaf compared to Cos and Iceberg. Dense foliage crops have also been observed to be more contaminated by parasites. Amahmid *et al*. (1999) reported greater numbers of *Giardia* cysts and *Ascaris* eggs detected in coriander and mint compared to carrot and radish when irrigated with raw wastewater because the larger surface of the herbs could capture more irrigated wastewater.

Microbial contamination might plausibly be influenced by the relative exposure of the leaves to the irrigating wastewater and, further, by the location from which the sample was obtained for analysis. There was no statistically significant difference ($p > 0.01$) amongst outer and inner leaves and composite samples of leaves following submersion in wastewaters with *E. coli* concentrations of 75.9 and 1,299.7 *E. coli* MPN/ 100 mL. However, when submersed in wastewater with an *E. coli* concentration of 27,550 *E. coli* MPN/ 100 mL, the number of *E. coli* was higher on the outer leaves than on both inner and composite leaf samples of all three varieties of lettuce studied. A similar result was observed by Oliveira *et al*. (2012) under a field condition where Cos lettuces were exposed to water contaminated with 10^7 *E. coli* O157:H7 CFU/ mL, manually applied by surface or hand spray irrigation. They reported more contamination on the outer leaves than the inner ones, presumable since the outer leaves were more exposed to the contaminated spray and were potentially in direct contact with contaminated soil and water. Therefore, a possible risk mitigation approach for food regulators may be to recommend that consumers discard the outer leaves of lettuce before washing in order to reduce the risk posed by contaminating pathogenic microorganisms.

Uniquely, the study reported here compared both direct (sampling the leaves) and indirect (water retention) methods for determining or estimating microbial contamination of lettuce following submersion in wastewater contaminated with differing concentrations of *E. coli*. An important finding was that there was no statistical difference ($p > 0.01$), irrespective of the lettuce variety, in the *E. coli* concentration recovered from the lettuce using the direct method of determination and that estimated indirectly from the weight of water retained

following submersion. The indirect estimation approach was used in many studies (Shuval et al., 1997, Petterson et al., 2001, Hamilton et al., 2006, Mara et al., 2007), few have used the direct method to determine the numbers of microorganism on the crops' surfaces to estimate the risk associated with consumption (Bastos et al., 2008, Aiello et al., 2012, Forslund et al., 2012, Pavione et al., 2013). The findings reported here suggest that the results from studies using either the direct or the indirect methods are broadly comparable. Consequently, both the direct and indirect methods are valid for estimating the health risk from consumption of wastewater irrigated salad crops.

This study shows that *E. coli* counts on lettuces were significantly ($p < 0.01$) correlated with the *E. coli* concentration in the wastewater in which they were submersed. High concentration of *E. coli* in irrigating wastewater results in high contamination of wastewater irrigated crops. Furthermore, the relationship between the *E. coli* concentration in the wastewater and in composite lettuce samples following submersion was shown to be linear, the *E. coli* concentration in lettuce could be predicted from the *E. coli* concentration in wastewater (Eq. 4.4 – 4.6).

Oak leaf

$\log_{10}$ (*E. coli* lettuce) = 1.06 $\log_{10}$ (*E. coli* wastewater) – 0.71 (n = 9, R^2 = 0.9998)

Equation 4.4

Cos

$\log_{10}$ (*E. coli* lettuce) = 0.93 $\log_{10}$ (*E. coli* wastewater) – 0.53 (n = 9, R^2 = 0.9997)

Equation 4.5

Iceberg

$\log_{10}$ (*E. coli* lettuce) = 0.93 $\log_{10}$ (*E. coli* wastewater) – 0.70 (n = 9, R^2 = 0.9889*)*

Equation 4.6

Where;

E. coli wastewater = *E. coli* MPN/ 100 mL

E. coli lettuce = *E. coli* MPN/ 100 g

Bastos et al. (2008) also derived similar equations, using results from field studies, relating wastewater quality to contamination of salad crops. Two equations were derived to estimate the *E. coli* concentration of high-growing crops (kale and green pepper) and low-growing crops (lettuce, spinach, and arugula; Eq. 4.7) at harvest.

Low-growing crops

$\log_{10}$ (*E. coli* crops) = 0.83$\log_{10}$ (*E. coli* wastewater) – 0.73

Equation 4.7

Where;

E. coli wastewater = *E. coli* MPN/ 100 mL

E. coli crops = *E. coli* MPN/ g

Although the equation of Bastos *et al*. (2008) was different to those reported here there was no statistical difference (Independent – Samples T Test, $p > 0.01$; Eq. 4.7& 4.4, 4.7& 4.5, and 4.7& 4.6) in the predicted microbial quality of crops (Table 3) modelled using irrigation wastewater qualities of 10, 10^2, 10^3 and 10^4 *E. coli* MPN/ 100 mL. The predicted *E. coli* of lettuce from Eq. 4.4 – 4.6 was converted from *E. coli* MPN/ 100 g to *E. coli* MPN/ g in order to be consistent with Eq. 4.7 of Bastos *et al*. (2008).

Table 4.3 The predicted *E.coli* concentration on lettuce irrigated with wastewater containing 10^2, 10^3 and 10^4 *E. coli* MPN/ 100 mL calculated using Eq. 4.4 – 4.6 (this study) and Eq. 4.7 (Bastos et al., 2008).

Wastewater qualities (*E. coli* MPN/ 100 mL)	**Predicted bacterial quality of lettuce (*E. coli* MPN/ g)**			
	Eq. 4.4	**Eq. 4.5**	**Eq. 4.6**	**Eq. 4.7**
10	0.02	0.03	0.02	1.26
10^2	0.26	0.21	0.14	8.51
10^3	2.95	1.82	1.23	57.54
10^4	33.88	15.48	10.47	398.05

However, Eq. 4.7 (Bastos *et al*. 2008) was derived from low- growing salad crops data, which including spinach, arugula, and lettuce (no cultivar defined in their study), while equations reported here were derived from the data obtained from three different varieties of lettuce. The equations derived from this study, as well as the equation derived from Bastos *et al*. (2008), could be used for the *preliminary* assessment of microbial risk in salad crops when the *E. coli* concentration of irrigation wastewater is known.

In summary, the laboratory based experiment using the bucket submersion technique as a surrogate for field spray irrigation, showed that the different cultivars of lettuce had different wastewater retention capabilities; the volume of wastewater retained by Oak leaf was greater than that retained by either Cos or Iceberg lettuce. There was no statistical difference in the *E. coli* count obtained from outer, inner and composite samples of leaves following submersion in wastewaters with *E. coli* concentrations of 10^2 and 10^3 *E. coli* MPN/ 100 mL. However, the *E. coli* count was higher on the outer leaves than on either inner or composite leaf samples of lettuce following submersion in wastewater with an *E. coli* concentration of 10^4 *E. coli* MPN/ 100 mL. Equations were derived which described the statistically significant linear relationship between the *E.coli* concentration of the wastewater and the

subsequent *E.coli* count obtained from composite leaf samples following submersion. Uniquely, this study was the first to confirm that using the direct enumeration technique, where *E*. *coli* was enumerated on the leaves after submersion in wastewater was comparable with indirect technique, where the *E*. *coli* concentration was estimated from the volume of wastewater retained by the lettuce and the *E. coli* concentration of the wastewater. This finding will be useful for conducting QMRA associated with the consumption of wastewater irrigated salad crops.

CHAPTER 5

THE SURVIVAL OF *E. COLI* IN WASTEWAER IRRIGATED LETTUCE DURING STORAGE AT DIFFERENT TEMPERATURES

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5 THE SURVIVAL OF *E. COLI* IN WASTEWATER IRRIGATED LETTUCE DURING STORAGE AT DIFFERENT TEMPERATURES

5.1 INTRODUCTION

Although the microbiological quality of irrigation water is the main factor affecting the contamination of crops grown on wastewater irrigated farms, there are many risk factors, which influence the degree of microbial contamination of crops when considering the journey from 'farm to fork'. On farm, existing microorganisms in irrigating wastewater can contaminate vegetables; however, the degree of contamination also depends on the irrigation system, microbial wastewater quality, types of crops, time elapsed after the last irrigation and environmental conditions as described in previous chapters. Postharvest is also important as it could be another source of bacterial contamination in the food chain, therefore, the WHO guidelines for safe use wastewater irrigation address the 'multiple barrier' approach to reduce the health risk, particularly in cases where wastewater treatment is not well performed (WHO, 2006b).

A control temperature for transportation and storage is very important in food safety (Piagentini et al., 1997). Temperature plays an important role in controlling bacterial growth during transportation and storage as it has been shown that poor temperature control during the cold chain could cause foodborne diseases (Brackett, 1999, McCabe-Sellers and Beattie, 2004, Rosset et al., 2004, Todd et al., 2010). USFDA (2010) recommended fresh fruits and vegetables should be kept under 5^{o}C throughout the supply chain to reduce the proliferation of spoilage and pathogenic microorganisms, which could prevent foodborne illness arising from poor conditions during transportation and storage.

Bacterial growth in fresh produce caused by the temperature abuse during transportation and storage is relatively well documented. Tian et al. (2012) investigated the survival and growth of *S.* Typhimurium, *S. aureus*, *L. monocytogenes* and *E. coli* O157: H7 on fresh vegetables stored at 4 and 15°C. They found that the populations of these pathogens increased when the

fresh vegetables were stored at 15°C for 7 days. Similarly, Luo et al. (2009) found that *E. coli* O157:H7 on packaged baby spinach increased when kept at 8 and 12°C, whereas decreased on those stored at 1 and 5°C for 3 days. Not only bacteria but also viruses have been studied regarding the survival and growth on fresh produce stored under different temperatures. Carratalà et al. (2013) determined the persistence of adenoviruses on strawberries and lettuce maintained at 4 and 30°C over 24 hours. The results showed that no virus decay was observed on produce stored at 4°C both in dark and under sunlight experiments, but inactivation of about 3 - 4 log reduction occurred over 24 hours at 30°C. Similarly, Dawson et al. (2005), reported that MS2 on fresh produce stored at 4 and 8°C slightly decreased over 7 days, while the decrease was greater at 22°C.

However, the majority of reported research has focused mainly on minimally processed leafy greens, not on fresh commodities which was used in this study; also, it has been done by spiking particular pathogens on the produce. It did not really represent the microbial quality of wastewater irrigated salad crops during transportation and storage on the fresh commodity. Due to the lack of available data on the survival of microbes in wastewater irrigated vegetables during transportation and storage, more research on this issue is required as it could improve the assessment of risk associated with the consumption wastewater irrigated salad crops.

Many developing countries commonly use non-refrigerated, open vehicles to transport fresh produce, even though it is well documented that temperature control is a key factor for microbial food safety during the distribution system. The objective of the study reported in this chapter was to investigate the effect of temperature on the survival of *E. coli* on wastewater irrigated lettuce stored at different temperatures (4 and 20°C). The study was conducted to contribute to a better understanding of the behaviour of *E. coli* on wastewater irrigated crops transported at different temperatures. The results of this study will be incorporated in to the QMRA associated with the consumption of wastewater irrigated salad crops.

5.2 MATERIALS AND METHODS

5.2.1 Wastewater and lettuce samples collection

Wastewater samples were collected from the aerated lagoon (wastewater stabilisation ponds) into 40 L containers from Mt Barker Wastewater treatment plant, South Australia. Then, transported and kept in a cold room (4°C) at Environmental Health laboratories, Flinders University before use.

Baby Cos lettuce samples were purchased from a local supermarket in Adelaide, South Australia. They were sold packaged in pairs, in clear polythene freezer bags (with the label 'wash before use'), and were transported to Environmental Health Laboratories, Flinders University in a cold pack (4°C). Lettuce samples were used in experiments within 45 minutes of purchase.

5.2.2 Lettuce contamination

Lettuces were contaminated with wastewater in the laboratory using the bucket submersion technique to simulate the wastewater spray-irrigated lettuce. A 50 L plastic bucket was used for lettuce submersion (the bucket was put into a larger tray to prevent spills), 9 lettuces were each, separately submerged into the bucket filled with 30 L wastewater for 30 s (all lettuces were flipped using autoclaved tongs after 15 s to ensure consistent contact with contaminated wastewater). Then, the samples were put onto a sterile strainer (Plate 5.1; wiped with 70% v/v ethanol -water) under which was a larger plastic tray to contain the drainage from the contaminated lettuce. The lettuce samples were drained for 4 minutes (each lettuce was flipped after 2 minutes to assure they were drained from both sides).

5.2.3 Experimental protocol

After contamination, 3 samples were analysed to determine the initial concentration of *E. coli* at the starting time (0 h), 6 samples were put into a 4 compartment storage box (19 L; 30 cm x 50 cm), one lettuce per compartment (12 cm x 30 cm). There was rolled, autoclaved chicken wire in the bottom of each compartment to prevent the recontamination and spoilage of the lettuce from any drained wastewater droplets. Two storage boxes were covered and wrapped in yellow biohazard bags, then stored in a cold room < 4°C (Plate 5.2)

or at 20°C in a temperature controlled incubator (Innova^{TM} 4340) (Plate 5.4 – 5.5). The wastewater (100 mL) used to contaminate the lettuce was dispensed into 20, 120 mL sealed tubes. Three wastewater samples were analysed to determine the initial *E. coli* concentration (0 h), and the remainder were put into a plastic container maintained under the same conditions (≤4°C or 20°C) as the lettuce samples (Plate 5.3).

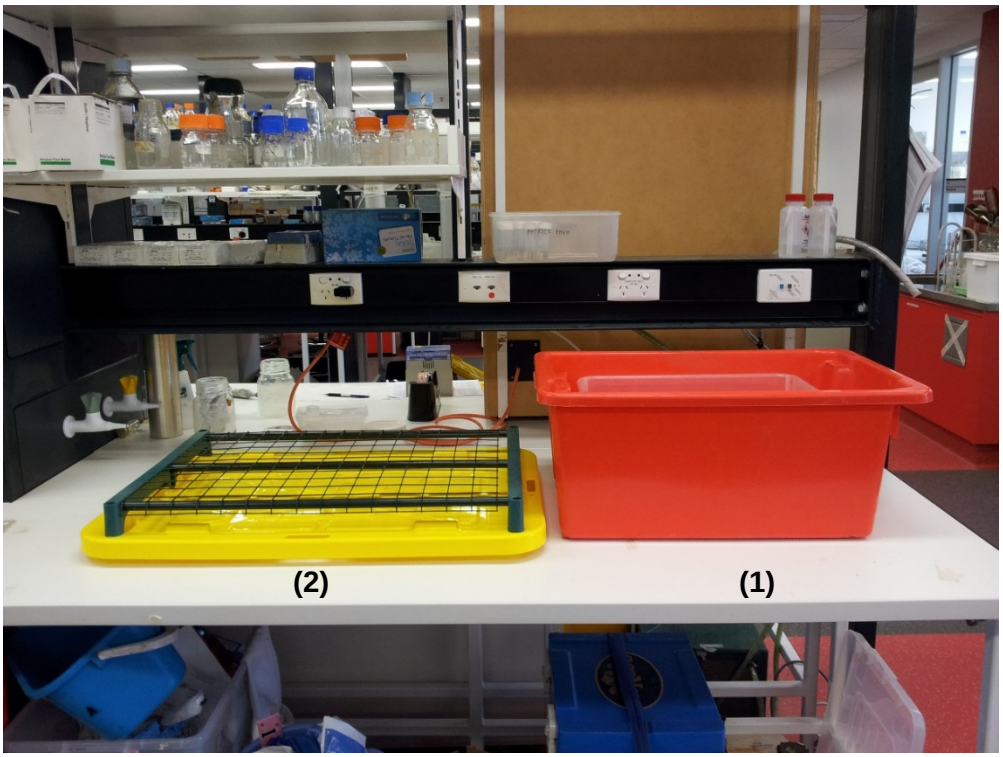

Plate 5.1 Baby Cos lettuces were submersed in the double contained (1) wastewater from the aerated lagoon of the Mt Barker and then allowed to drain (2) for 4 minutes.

Plate 5.2 Boxes used for the storage of wastewater contaminated lettuce.

Plate 5.3 Lettuce and wastewater samples kept at 4˚C

Plate 5.4 The refrigerated temperature controlled incubator set at 20°C

Plate 5.5 Lettuce and wastewater samples stored at 20°C

All samples were stored over 48 hours, and triplicates samples were enumerated for *E. coli* every 8 hours (8 h, 16 h, 24 h, 32 h, 40 h and 48 h). Three wastewater samples from 3 tubes were taken directly; however, for lettuce samples, at each sampling time, a quarter from each of 3 samples was cut and *E. coli* enumerated. Additional quarters from the same lettuce samples were cut at the later times; therefore, 3 lettuce heads were utilised in 32 hours after which, a quarter of lettuce samples were cut from the new 3 lettuce heads at time 40 and 48 h.

5.2.4 Quantification of *E. coli* in wastewater and lettuce samples

A quarter of lettuce from each sample, composited from the different part of the leaves, was cut into 25 g to which was added, in a stomacher bag, to 225 mL, 0.1% sterile buffered peptone water. The suspension was homogenised for 1 minute using a stomacher (Model 2X (IDEXX)). The supernatant from the homogenate was enumerated for *E. coli* using the Colilert®-18 MPN method and expressed as the most probable number (MPN) of *E. coli* per 100 g of lettuce (*E.coli* MPN/100 g).

5.2.5 Statistical analysis

The difference in *E. coli* concentration in lettuce and wastewater samples at different 8 hour-storage time intervals were analysed using One – Way ANOVA using SPSS (PASW Statistics 18) with the confidence level of 95 %. Significant differences between mean values of *E. coli* counts at each 8 hour-storage time were determined by Bonferroni *post-hoc* test.

5.3 RESULTS

5.3.1 Survival of *E. coli* on lettuces and in wastewater stored at 4°C

The 3 randomly selected samples of baby Cos lettuce enumerated for *E. coli* after purchase and before starting the contamination experiment all tested negative for *E. coli*. The changes in the populations of *E. coli* on lettuce and wastewater stored at 4°C are presented in Figs 5.1 – 5.2. The initial populations of *E. coli* on lettuce and in the wastewater were 3.85 $\log_{10}$ *E. coli* MPN/ 100 g and 4.49 $\log_{10}$ *E. coli* MPN/ 100 mL, respectively. The populations of *E. coli* on lettuce declined by 0.14 $\log_{10}$ *E. coli* MPN/ 100 g, but no significant differences were determined over the 48-hour storage period ($p > 0.05$). In

wastewater, the populations of *E. coli* decreased significantly after 32 hours ($p < 0.05$), then slightly declined recording a 0.21 $\log_{10}$ *E. coli* MPN/ 100 mL decrease at the end of the storage period.

5.3.2 Survival of *E. coli* on lettuces and in wastewater stored at 20°C

The populations of *E. coli* on lettuce and in wastewater stored at 20°C over 48 hours are given in Figs 5.3 – 5.4. The initial populations of *E. coli* on lettuce and in wastewater were 3.90 $\log_{10}$ *E. coli* MPN/ 100 g and 4.60 $\log_{10}$ *E. coli* MPN/ 100 mL, respectively. After 24 hours storage the population of *E. coli* on the lettuce decreased significantly ($p < 0.05$), when compared with the initial count. However, the *E. coli* count then increased significantly ($p < 0.05$) between 24 h and 48 h storage. Overall the population of *E. coli* on lettuce decreased by 0.05 $\log_{10}$ *E. coli* MPN/ 100 g from the initial count to the final count after 48 hours storage. In contrast, the *E. coli* count in wastewater, decreased significantly ($p < 0.05$) over the storage period recording a much larger $\log_{10}$ reduction of 0.73 compared to the lettuce stored at the same temperature.

A second experiment was performed at 20°C with lettuces contaminated with wastewater (as above) and stored for 4 days (96 hours) with the population changes in *E. coli* determined over the extended storage period. Additionally, the quality of lettuce samples was assessed visually, noting the development of a brown discolouration and unpleasant odour. The results of this experiment are shown in Figs 5.5 – 5.6.

The initial populations of *E. coli* on lettuce and wastewater were 3.91 $\log_{10}$ *E. coli* MPN/ 100 g and 4.55 $\log_{10}$ *E. coli* MPN/ 100 mL, respectively. The populations of *E. coli* on lettuce in this experiment behaved similarly to the previous experiment (above) for the first 48 hours. The count decreased over the first 24 hours of storage and then slightly increased. However, there was a significant decrease ($p < 0.05$) after 3 days ($p < 0.05$) compared with the initial count. After 4 days at 20°C, the overall decrease compared to the initial count was 0.33 $\log_{10}$ *E. coli* MPN/ 100 g. The reduction in the populations of *E. coli* in wastewater stored at 20°C over 4 days was significant ($p < 0.05$) and much larger, 1.32 $\log_{10}$ *E. coli* MPN/ 100 mL, than the decrease in *E. coli* count on the lettuce stored under the same conditions.

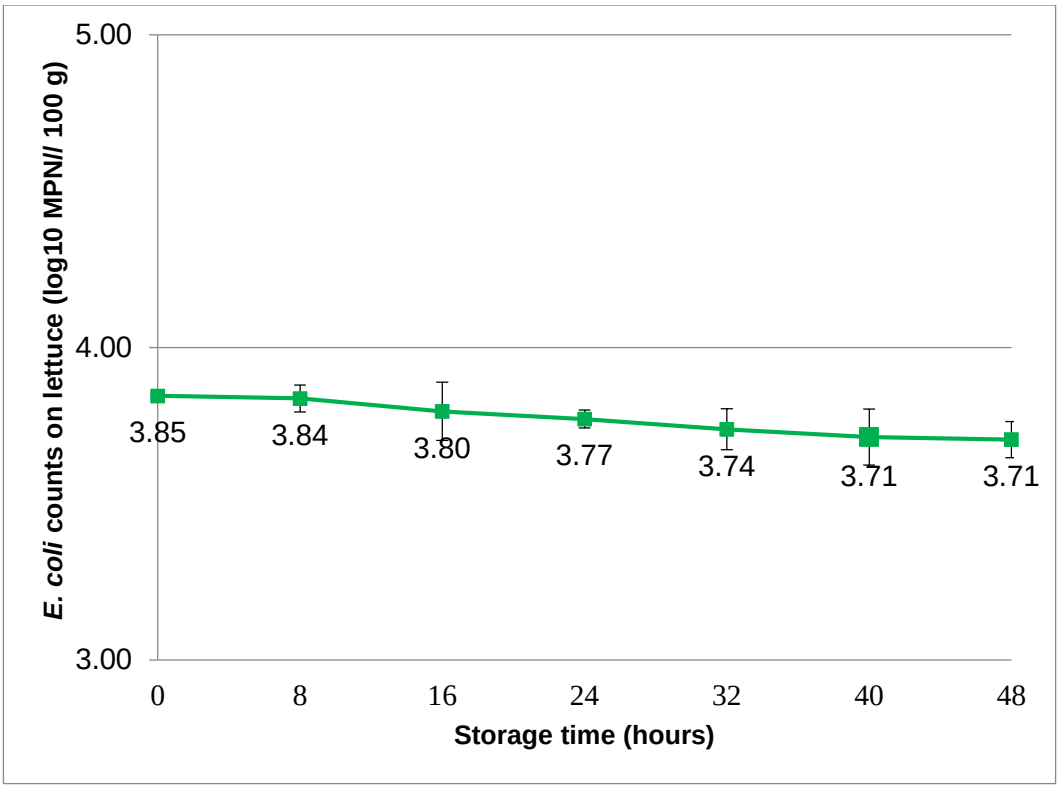

Figure 5.1 *E. coli* counts on lettuces *(E. coli* $\log_{10}$ MPN/ 100 g) contaminated with wastewater (4.49 $\log_{10}$ *E. coli* MPN/ 100 mL) and stored at 4°C for 48 hours.

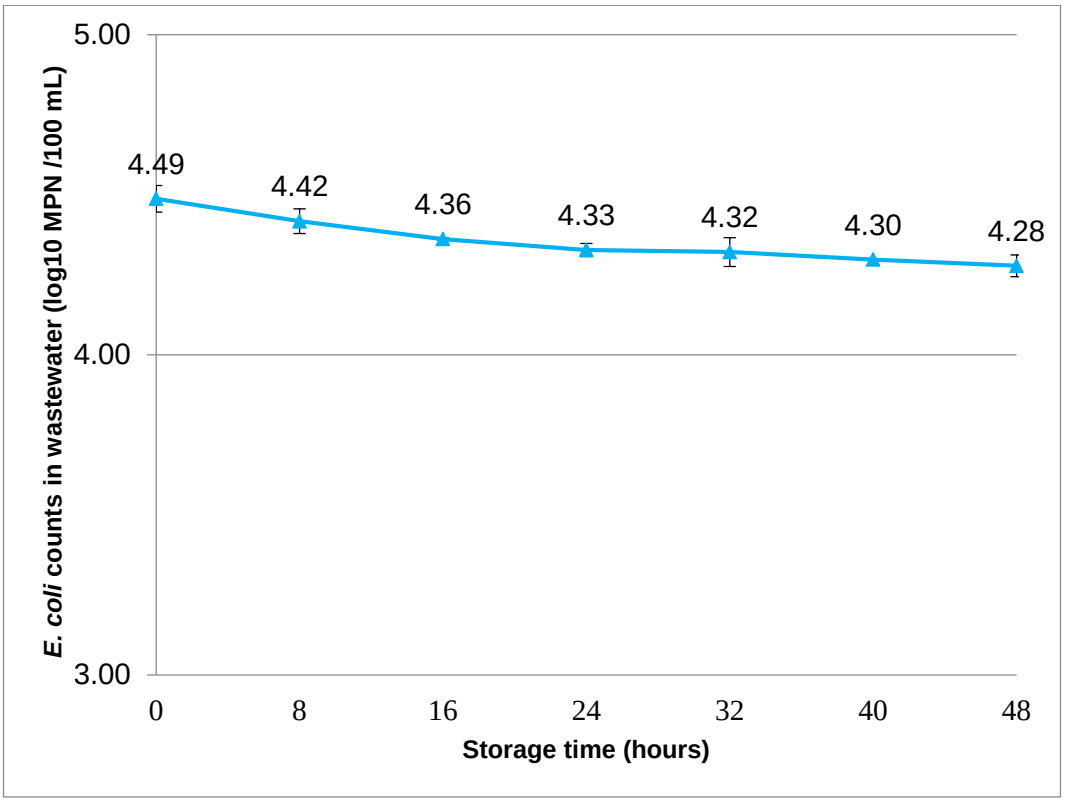

Figure 5.2 *E. coli* counts *(*$\log_{10}$ *E. coli* MPN/ 100 mL) in wastewater stored at 4°C for 48 hours.

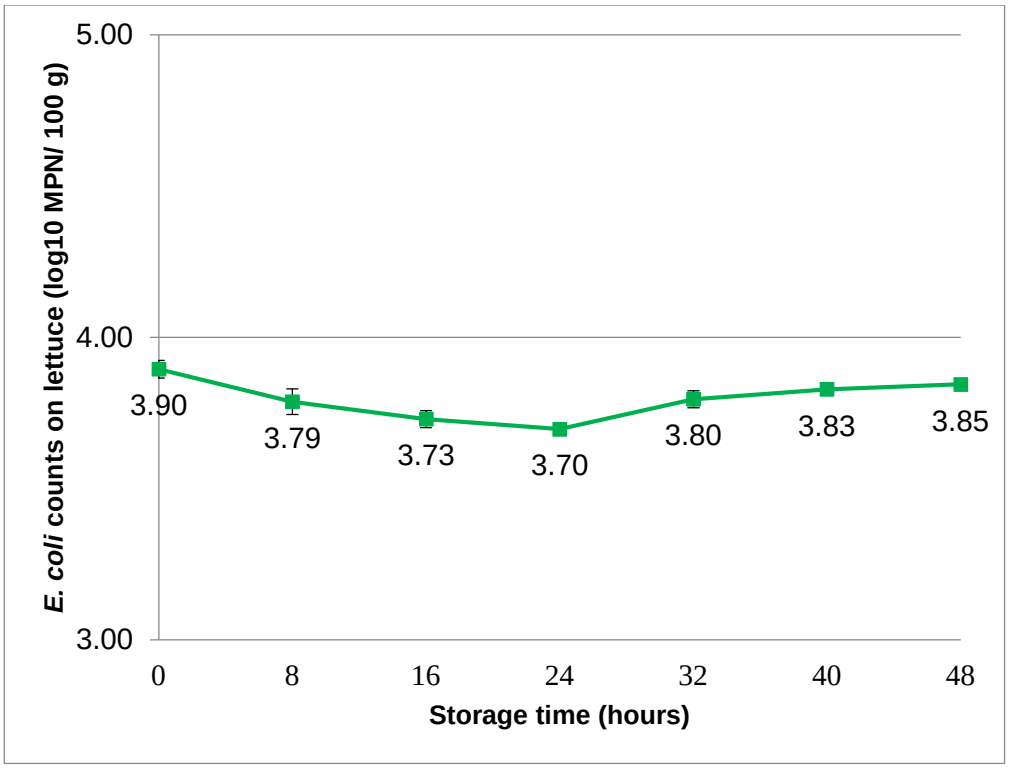

Figure 5.3 *E. coli* counts on lettuces *(E. coli* $\log_{10}$ MPN/ 100 g) contaminated with wastewater (4.60 $\log_{10}$ *E. coli* MPN/ 100 mL) and stored at 20°C for 48 hours.

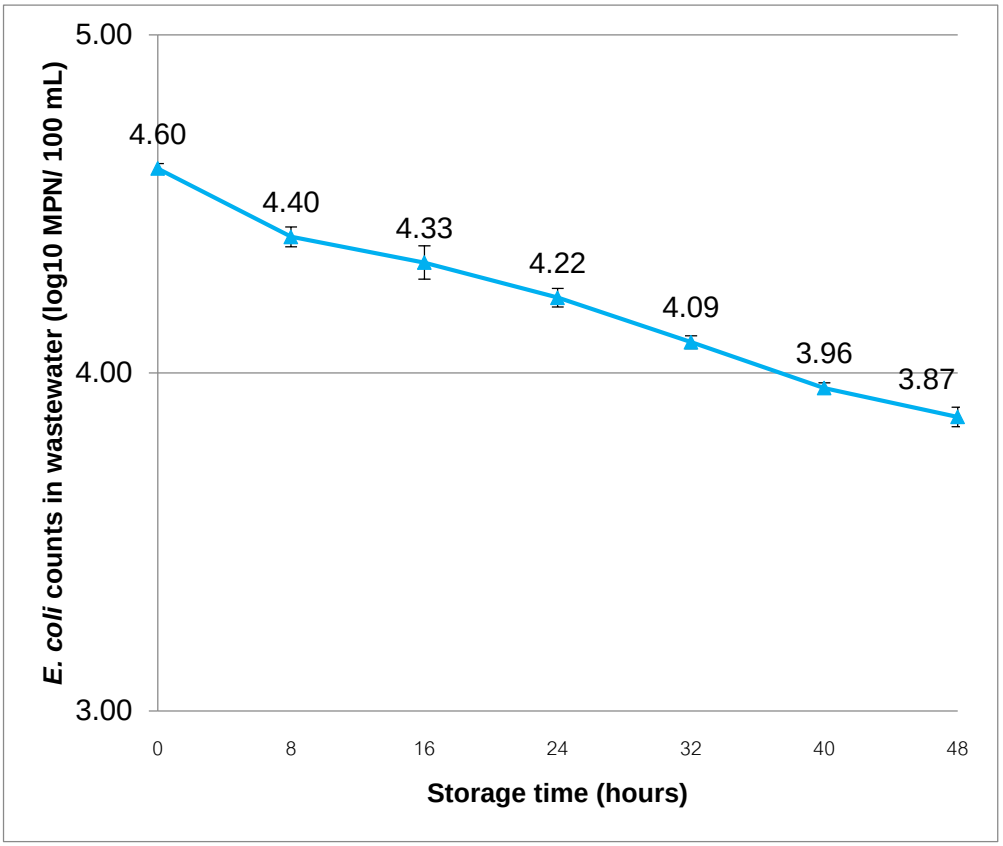

Figure 5.4 *E. coli* counts (log$_{10}$ *E. coli* MPN/ 100 mL) in wastewater stored at 20°C for 48 hours.

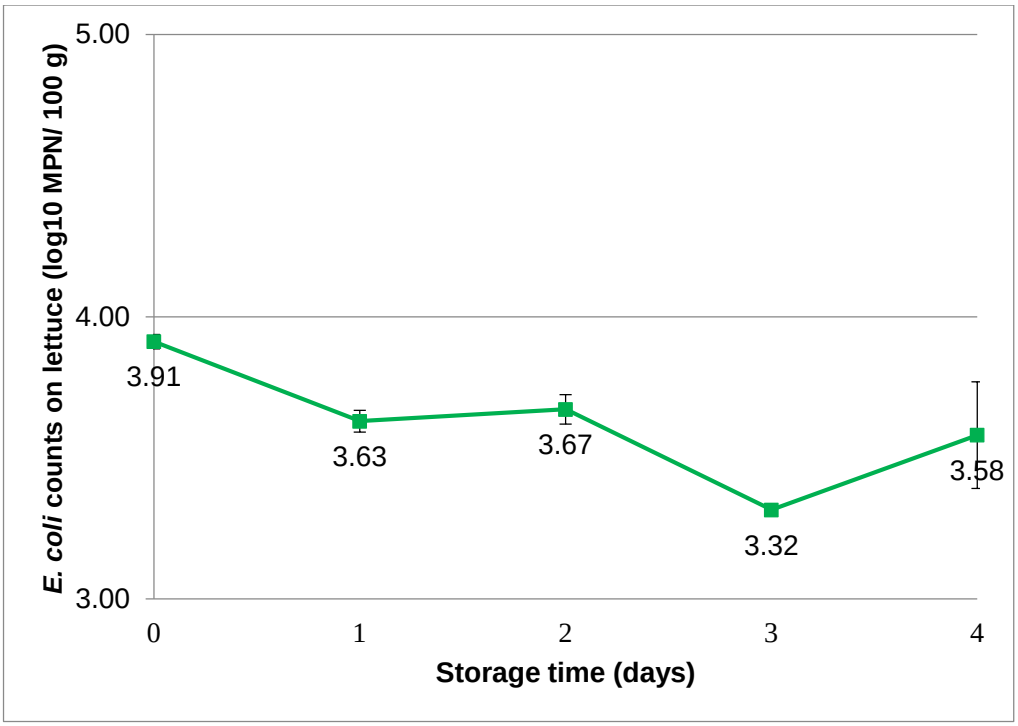

Figure 5.5 *E. coli* counts (log$_{10}$ *E. coli* MPN/ 100 g) in lettuce stored at 20°C for 4 days.

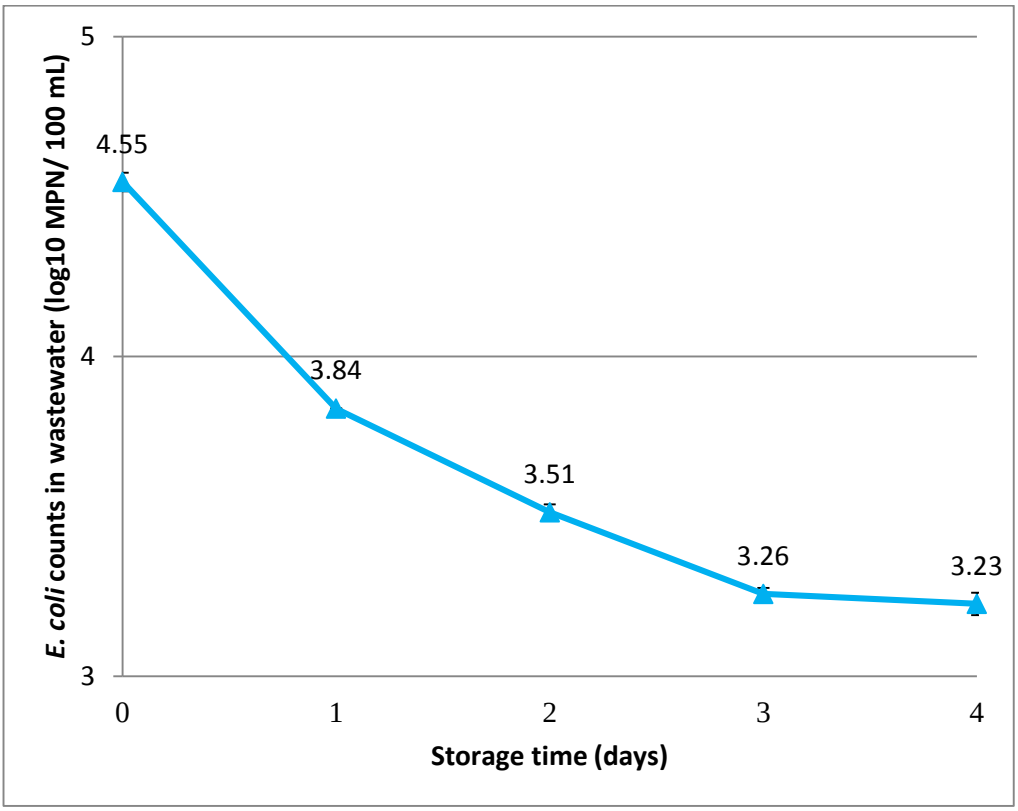

Figure 5.6 *E. coli* counts (log$_{10}$ *E. coli* MPN/ 100 mL) in wastewater stored at 20°C for 4 days

Plate 5.6 Visual quality of lettuces stored at 20^{o}C at D_0

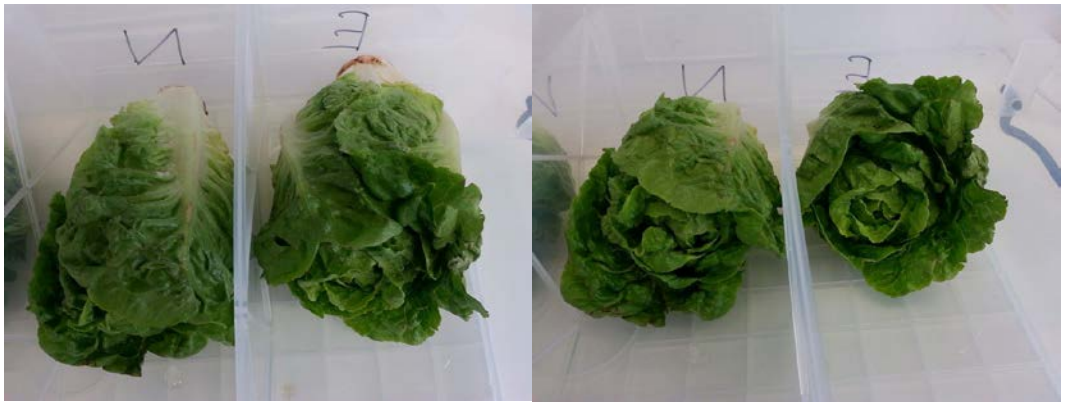

Plate 5.7 Visual quality of lettuces stored at 20^{o}C for 1 day

Plate 5.8 Visual quality of lettuces stored at 20^{o}C for 2 days

Plate 5.9 Visual quality of lettuces stored at 20ºC for 3 days

Plate 5.10 Visual quality of lettuces stored at 20ºC for 4 days

Plates 5.6 – 5.10 presents the observation of the changes in appearance on wastewater submersed lettuces stored at 20°C over 4 days (96 hours). The first change observed after 3 days (Plate 5.9) was that the external leaves appeared dehydrated, leaf brightness decreased, there was a slight decolourisation and the lettuce started developing a rotten smell. At the end of storage, the whole lettuce head presented dehydrated, browning and with a strong rotten smell (Plate 5.10).

Table 5.1 collates *E. coli* reduction in the wastewater (log_{10} reduction *E. coli*/ 100 mL) and on the lettuce (log_{10} reduction *E. coli* / 100 g) contaminated with the same wastewater stored at 4°C for 48 h and at 20°C for either 48 h or 96 h. Clearly, the reduction in *E. coli* numbers in wastewater was related to storage temperature, increasing with increasing temperature, and increasing with increased storage time at 20°C, whereas the reduction in *E. coli* numbers

was far less on lettuce contaminated with the same wastewater. Furthermore, there was no similar relationship with increased storage temperature as identified for the wastewater, although as for the wastewater the reduction in *E. coli* increased with increased storage time at 20°C.

Table 5.1 The reduction in *E. coli* in wastewater (log_{10} reduction *E. coli*/ 100 mL) and lettuce (log_{10} reduction *E. coli* /100 g) contaminated with the same wastewater stored at 4°C for 48 h and at 20°C for either 48 h or 96 h.

Storage temperature	**4°C**	**20°C**	
Storage time (h)	**48**	**48**	**96**
Lettuce (Log_{10} reduction *E. coli* / 100 g)	0.14	0.05	0.33
Wastewater (Log_{10} reduction *E. coli*/ 100 mL)	0.21	0.73	1.32

5.4 DISCUSSION

The aim of the experiment reported in this chapter was to better understand the effect of storage temperature on the survival of *E. coli* on wastewater irrigated lettuce. To the author's knowledge, there are no studies which have determined the survival of bacteria on wastewater irrigated fresh commodity lettuce. Consequently, the data obtained from these experiments were compared and discussed with other studies where pathogens were inoculated into either fresh or processed produce.

The results from the experiment conducted at 4°C were not unexpected. Storage of wastewater submersed lettuces at 4°C for 48 h had no significant effect on the survival and growth of *E. coli.* This is the storage temperature recommended to minimise the growth of spoilage organisms and pathogens and manage the risk of foodborne illness arising from transportation and storage fresh fruit and vegetables throughout the supply chain (USFDA, 2010, Rediers et al., 2009, Ukuku and Sapers, 2007). It is well documented that keeping vegetables at chill temperatures (5°C or less) can prevent their

physiological deterioration by decreasing the enzymatic activity and respiration rate of fresh produce as well as limiting the growth of pathogenic microorganisms (Luo et al., 2010, Matthews, 2014, Buchholz et al., 2010).

The populations of *E. coli* on lettuce declined by 0.14 $\log_{10}$ *E. coli* MPN/ 100 g over 48 hours, and the populations of *E. coli* in wastewater declined 0.21 $\log_{10}$ *E. coli* MPN/ 100 mL over storage period. Similar results were reported by Allwood et al. (2004), who showed that the populations of *E. coli* on lettuce and cabbage leaf stored at 4°C did not grow and the organism was detectable for 21 days. Piagentini et al. (1997) observed similar decreased of *S. hadar* in bagged shredded cabbage stored at 4°C for 10 days. Similar changes of other pathogens in salad crops kept at 4°C were also noted by Tian et al. (2012). They found that there was no significantly differences ($p < 0.05$) in the growth of *S.* Typhimurium, *S. aureus* and *E. coli* O157:H7 in romaine lettuce, iceberg lettuce and perilla leaves observed over storage time of 15 days.

In addition, many studies also have considered the effect of storing fresh produce at chill temperature of 5°C, and the results were very similar to those reported here. Luo et al. (2010) reported that *E. coli* O157:H7 did not grow on bagged shredded lettuce salads over an 8-day storage period. Similarly, *E. coli* O157:H7 on bagged baby spinach significantly decreased within 3 days of storage (Luo et al., 2009). Other studies have investigated salad crops stored under modified atmosphere packaging (MAP); again the results were similar to those reported here. Abdul-Raouf et al. (1993) showed that *E. coli* O157:H7 on shredded lettuce under MAP condition decreased 1.11 log10 CFU/ g (initial inoculum was $10^{5.34}$ CFU/ g) over 14-day storage time and Oliveira et al. (2010) revealed that *E. coli* O157:H7 and *Salmonella* spp. on shredded romaine lettuce under MAP decreased about 1 $\log_{10}$ unit after 10 days.

It is obvious that keeping salad crops at cool temperatures is a safe way to limit the growth of pathogens. The small reduction in numbers during storage at chill temperatures suggests that if the initial populations of pathogens were high, the final concentration of pathogens prior to consumption would also be high and could still be of concern to public health.

Obviously, from the results of this study and other studies considered above,

keeping fresh vegetable under 5°C is the best option to prevent bacterial growth during storage and transportation. In contrast, some pathogens can increase when fresh produce is stored at greater than 5°C. Numerous studies report that pathogens grow better when fresh produce is stored between 8 - 35°C. Studied pathogens including *E. coli* O157: H7 (Puerta-Gomez et al., 2013, Ding et al., 2012, Tian et al., 2012, Khalil and Frank, 2010, Carey et al., 2009, Luo et al., 2009, Doering et al., 2009, Kim and Harrison, 2008, Koseki and Isobe, 2005, Choi et al., 2011, Luo et al., 2010), *S*. Typhimurium (Puerta-Gomez et al., 2013, Tian et al., 2012), *S. Hadar* (Piagentini et al., 1997) and *S. aureus* (Tian et al., 2012). However, the results of the study reported here conducted at 20°C were quite different from those as a slight reduction of *E. coli* was noted.

The results conducted at 20˚C showed that the populations of *E. coli* on lettuce decreased by 0.05 $\log_{10}$ *E. coli* MPN/ 100 g over 48 hours, with a decrease followed by an increase in numbers within the second 24h of storage, however, overall there was no statistically significantly difference after 48h storage compared to initial concentration. The fluctuation in *E. coli* on lettuce stored at 20˚C for 48 hours, prompted another experiment whereby lettuce was stored at 20˚C for 4 days (96 hours). The results were also similar to those obtained for the 48 hour-storage experiment with the *E. coli* count fluctuating over the storage period >24 h. The reduction in *E. coli* numbers between initial (0 h) and final (96 h) count was 0.33 $\log_{10}$ *E. coli* MPN/ 100 g. In contrast, the changes in *E. coli* populations in wastewater were significantly different statistically between initial and final counts with decreases of 0.73 and 1.32 $\log_{10}$ *E. coli* MPN/ 100 mL recorded following storage for 48 hours and 96 hours (4 days), respectively.

Storage at a temperature of 20˚C allows wastewater contaminated lettuce to be kept for about 2 days as browning and a rotten smell were observed after 3 days storage. Some spoilage microorganisms on vegetables are very active at 20˚C such as *Erwinia* spp. which can cause the soft rot in vegetables (Barth et al., 2009). Therefore, postharvest cold chain management should be maintained to prevent the growth of spoilage microorganisms as well as pathogens.

Populations of microorganisms increase when kept at temperature higher than 4˚C. However, at higher temperature, it is not only pathogens that can grow, but also the native microorganisms on fresh produce. Carey et al. (2009) demonstrated that the indigenous microflora on romaine lettuce (in terms of total aerobic plate count) could grow at 15˚C over 9 day-storage time. There is variety of indigenous microflora on fresh produce, some of them can grow at higher temperatures and outcompete the pathogens for nutrients and space, resulting in a decrease in the number of pathogens (Cooley et al., 2006, Duffy et al., 1999). The competition between *E. coli* and native microflora may be the cause of the reduction of *E. coli* on wastewater submersed lettuce in this study. Although the level of indigenous microflora on lettuces is unknown in this study, they are assumed high. Fresh commodity Cos lettuce was contaminated with wastewater in this study, unlike some previous studies whereby minimally processed vegetables were spiked with pathogens (Rodríguez-Caturla et al., 2012, Khalil and Frank, 2010, Luo et al., 2009, Luo et al., 2010, Piagentini et al., 1997, Kärenlampi and Hänninen, 2004). Minimally processed vegetables are generally washed during the processing while the fresh commodity is not (Fig. 1.2) (Gorny et al., 2006). The most common effective sanitiser used in washing fresh cut produce is chlorine (hypochlorite) (Gil et al., 2009b), but others have been also used such as organic acids, peroxyacetic acid, ozone etc. (Warriner and Namvar, 2014a). These sanitisers can reduce not only pathogens on fresh produce but also the indigenous microflora (Sanz et al., 2002, Allende et al., 2009, McKellar et al., 2004, Luo, 2007). Therefore, the background populations of indigenous microflora on lettuce in this study might be high as they were not passed through the chemical washing process. Some of them might be able to compete with the growth of *E. coli*, resulting in the reduction of *E. coli* on wastewater submerged lettuce stored at 20˚C observed in this study. Thus, the background of indigenous microflora populations on lettuce could be one of factors that may affect the survival of *E. coli* on wastewater submersed lettuce stored at 20˚C reported here.

However, there are a few studies were the changes in populations of pathogens on fresh produce stored at 12-20˚C were similar to this study. Kim et al. (2006) reported that *Enterobacter sakazakii* on fresh produce including lettuce did not grow when kept at 12 and 25˚C for 14 days. Moreover, similar

results were found on *Campylobacter jejuni* on fresh produce. The decay rate of *C. jejuni* on lettuce stored at 20˚C (1.39 day^{-1}) was higher than at 7˚C (0.59 day^{-1}) over 72 hour-storage time (Kärenlampi and Hänninen, 2004). Guévremont et al. (2015) also found that *C. jejuni* on baby spinach decreased about 0.3 log unit when stored at 12˚C compared to 4˚C, but the ability of *C. jejuni* survival was also strain dependent (Kärenlampi and Hänninen, 2004, Guévremont et al., 2015).

This study is the first one to demonstrate the survival of *E. coli* in wastewater irrigated lettuce during storage at different temperatures. Further research is required to understand the influence of populations of native microflora on survival of indicator organisms and pathogens on wastewater contaminated lettuce. Furthermore, pathogens should be compared to *E. coli* both in wastewater and lettuce, particularly *Campylobacter* which is a reference pathogen for bacteria in wastewater reuse guidelines (WHO, 2006b).

CHAPTER 6

EFFICACY OF HOUSEHOLD WASHING METHODS TO REDUCE MICROBIAL LOAD ON WASTEWATER IRRIGATED LETTUCE LEAF SURFACES

6 EFFICACY OF HOUSEHOLD WASHING METHODS TO REDUCE MICROBIAL LOAD ON WASTEWATER IRRIGATED LETTUCE LEAF SURFACES

6.1 INTRODUCTION

Consumer's food handling and preparation at home is one of the important causes of foodborne diseases globally including handling of fresh fruit and vegetables. (Milton and Mullan, 2010, Taché and Carpentier, 2014). This step is very important and was included in the WHO guidelines (WHO, 2006b) as one of the multi approaches to reduce health risk from the consumption of wastewater irrigated salad crops. The multiple-barrier approach (Fig. 6.1) was established to minimise the health risk along the supply chain, the 'farm to fork' continuum, as conventional wastewater treatment alone cannot reduce the health risk to achieve the health-based target for the burden of waterborne disease, ≤10^{-6} Disability Adjusted Life Years (DALY). In addition, safe washing at home/ restaurant could be a Critical Control Point (CCP) if it is focused from the point of view of the Hazard Analysis and Critical Control Point (HACCP) system. A CCP is a step at which control can be applied and is essential to prevent or eliminate a food hazard or reduce it to an acceptable level (FAO, 1997). The washing step at home/ restaurant is the last step by which the pathogens on salad crops could be removed.

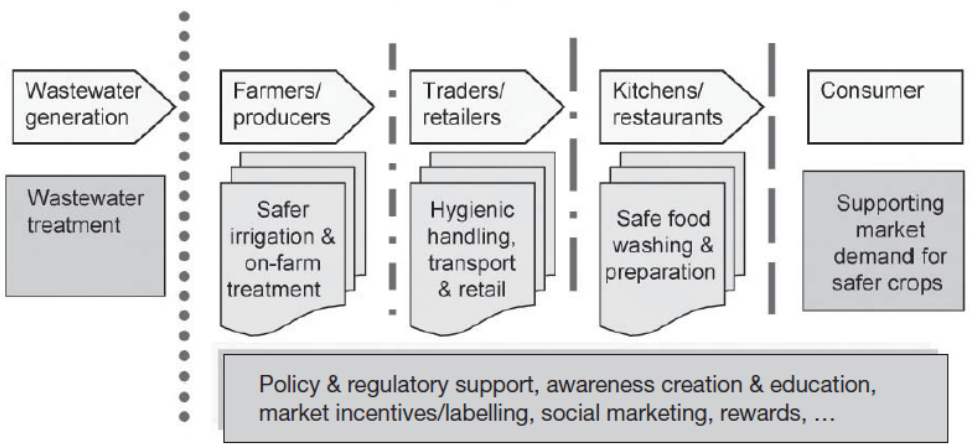

Figure 6.1 The multi-barrier approach in the wastewater food chain recommended by WHO (Ilic et al., 2010).

Although washing fresh produce at home plays an important role to reduce health risk before consumption, the guidelines or recommendations relevant to washing methods and procedures at the household level are relatively limited and diverse worldwide. USFDA (2011) advises that fruits and vegetables should be rubbed under plain running water without sanitisers applied while the Food Authority of New South Wales recommended that all fruits and vegetables should be washed in clean water and sanitised by soaking in 100 ppm free chlorine for 5 minutes or other commercial produce wash. Furthermore, household kitchen products are recommended in some countries, for example, salt water and vinegar are suggested to decontaminate fresh fruits and vegetables in Thailand (Department of Health, 2011).

The studies on the efficacy of sanitisers to decontaminate microorganisms from fresh produce have primarily focused on the food industry (Vijayakumar and Wolf-Hall, 2002b), while there is lack of information available for household settings (Nastou et al., 2012). Those that are available demonstrate the variety of media and methods applied in the home to reduce microbial load on fresh produce, including washing under running tap water (Kilonzo-Nthenge et al., 2006, Doménech et al., 2013), soaking in vinegar solution (Vijayakumar and Wolf-Hall, 2002b, Sengun and Karapinar, 2005, Chang and Fang, 2007, Allende et al., 2009), bleach (Vijayakumar and Wolf-Hall, 2002b, Doménech et al., 2013), washing with commercially produced washing solution (Kilonzo-Nthenge et al., 2006, Fishburn et al., 2012) and soaking in lemon juice solution (Vijayakumar and Wolf-Hall, 2002b, Sengun and Karapinar, 2005). However, the majority of washing protocols applied in these studies were not necessarily representative of likely real practice in home kitchens; for instance, small amounts (10– 25 g) of produce were used (Vijayakumar and Wolf-Hall, 2002b, Sengun and Karapinar, 2005, Chang and Fang, 2007, Doménech et al., 2013); samples were sanitised in small containers such as 50 and 100 mL solution jars (Vijayakumar and Wolf-Hall, 2002b, Sengun and Karapinar, 2005) or 40 mL tubes (Chang and Fang, 2007). Additionally, these studies were performed using vegetables ‘spiked’ with pathogens, rather than wastewater-irrigated crops, which are the focus of this thesis.

Amoah et al. (2007) uniquely, considered the efficacy of household washing methods to reduce the load of faecal coliform bacteria and helminth eggs in wastewater irrigated lettuce in Ghana. The common home washing methods used in cities in West Africa were surveyed and applied to sanitise lettuce in their study. Several methods were employed in their study, from using only tap water to chemicals such as salt and vinegar, and household bleach. The results showed that washing lettuce under running tap water for 2 minutes had the highest $\log_{10}$ reduction of 2.2, and could reduce helminth eggs from about 9 to 1 egg per 100 g of lettuce (wet weight). The results from their study are very useful to assist consumers to reduce the risk from the consumption of wastewater-irrigated lettuce at home. The study, however, required a more 'realistic' approach since the 50 g samples used in the study were submerged in a 1L solution in a bowl. More research is required, which reflects realistic practices within the home kitchen and focusses on the effectiveness of various decontamination processes applied to wastewater-irrigated vegetables. The objective of this study was to assess the efficacy of decontamination methods applicable to home settings to reduce *E. coli* on wastewater-irrigated lettuce.

6.2 MATERIALS AND METHODS

6.2.1 Lettuce contamination and preparation

Lettuces were contaminated with wastewater in the laboratory using the bucket submersion technique to simulate the wastewater spray-irrigated lettuce (the wastewater and lettuce samples collection were previously described in Chapter 5). A 50 L plastic bucket was used for lettuce submersion (the bucket was put into a larger tray to contain spillage), the bucket was filled with 30 L wastewater in to which 6 lettuces were individually submerged for 30 s (all lettuces were flipped using autoclaved tongs after 15s to ensure consistent contact with contaminated wastewater). Then, the samples were placed in to a sterile strainer under which was a larger plastic tray to contain the drainage from the contaminated lettuce. The lettuce samples were drained for 4 minutes (each lettuce was flipped after 2 minutes to ensure they were drained from both sides). Following submersion, three samples were analysed to determine the initial concentration of *E. coli* on submersed lettuces. The other three lettuces were further prepared for the decontamination treatments. These

lettuces were cut off at the base causing the leaves to separate, the core was discarded, all the leaves from each lettuce were placed into separate sterile aluminum trays.

6.2.2 Decontamination treatments

Ten treatments were investigated for decontaminating wastewater-irrigated lettuce leaf surfaces in this study as described in Table 6.1.

Table 6.1 The 10 home washing methods investigated for decontaminating wastewater grown lettuce.

Method	Code	Description
1	SOAK	Soak in tap water for 3 min
2	RUNW	Washing under running tap water (200 mL/ s) for 20 s
3	PRUN	Pre-soak in tap water for 3 min + washing under running tap water (200 mL/ s) for 20 s
4	PSWR	Pre-soak in tap water for 3 min + soak in tap water for 5 min + rinse with tap water (200 mL/ s) for 20 s
5	0.05Vin	Pre-soak in tap water for 3 min + soak in 0.05% white rice vinegar (Narcissus, China; Plate 6.3) for 5 min + rinse with tap water (200 mL/ s) for 20 s
6	0.5Vin	Pre-soak in tap water for 3 min + soak in 0.5% white rice vinegar (Narcissus, China)(Plate 6.3) for 5 min + rinse with tap water (200 mL/ s) for 20 S
7	P50Cl*	Pre-soak in tap water for 3 min + soak in 50 ppm Chlorine solution (commercial chlorine tablets, Foodsaf, UK)) (Plate 6.3; 2) for 5 min + rinse with tap water (200 mL/ s) for 20 s
8	50Cl*	Soak in 50 ppm Chlorine solution (commercial chlorine tablets, Foodsaf, UK)) (Plate 6.3) for 5 min + rinse with tap water (200 mL/ s) for 20 s
9	SWAS**	Soak in wash solution (Safeguard, Australia)) (Plate 6.3; 3) for 30 s + rinse with tap water (200 mL/ s) for

Method	Code	Description
		20 s
10	PWAS**	Pre-soak in tap water for 3 min + soak in wash solution (Safeguard, Australia)) (Plate 6.3) for 5 min + rinse with tap water (200 mL/ s) for 20 s

** Manufacturer instruction –2 tablets (3.25 g) in 40 L water would give available chlorine of 50 ppm.*

*** Manufacturer instruction – 2-4 pumps in a half sink of water (2.5 mL in 2.5 L water applied in this study), and soak for 30 s.*

Method 1, and 3-10 were performed in a 2.5 L plastic bowl (Plate 6.1); excess solution was removed from the leaves by spinning in a sterile salad spinner (Plate 6.2). Method 2 (RUNW), lettuce leaves were contained in a sterile colander when washing under running tap water (Plate 6.3). Tap water (22-24°C) with a free Cl_2 residual of 0.02-0.04 mg/ L was used for soaking, rinsing and preparing solutions. The lettuce leaves were soaked for a total of 3 min and flipped in the bowls (from the bottom to the top) using sterile tongs after initially soaking for 1.5 min (Method 1, 3-8, and 10).

Plate 6.1 Lettuce leaves in a 2.5 L solution bowl

Plate 6.2 A salad spinner used in this study (4WALLS, VIC, Australia)

Plate 6.3 Lettuce leaves were run under tap water in a colander

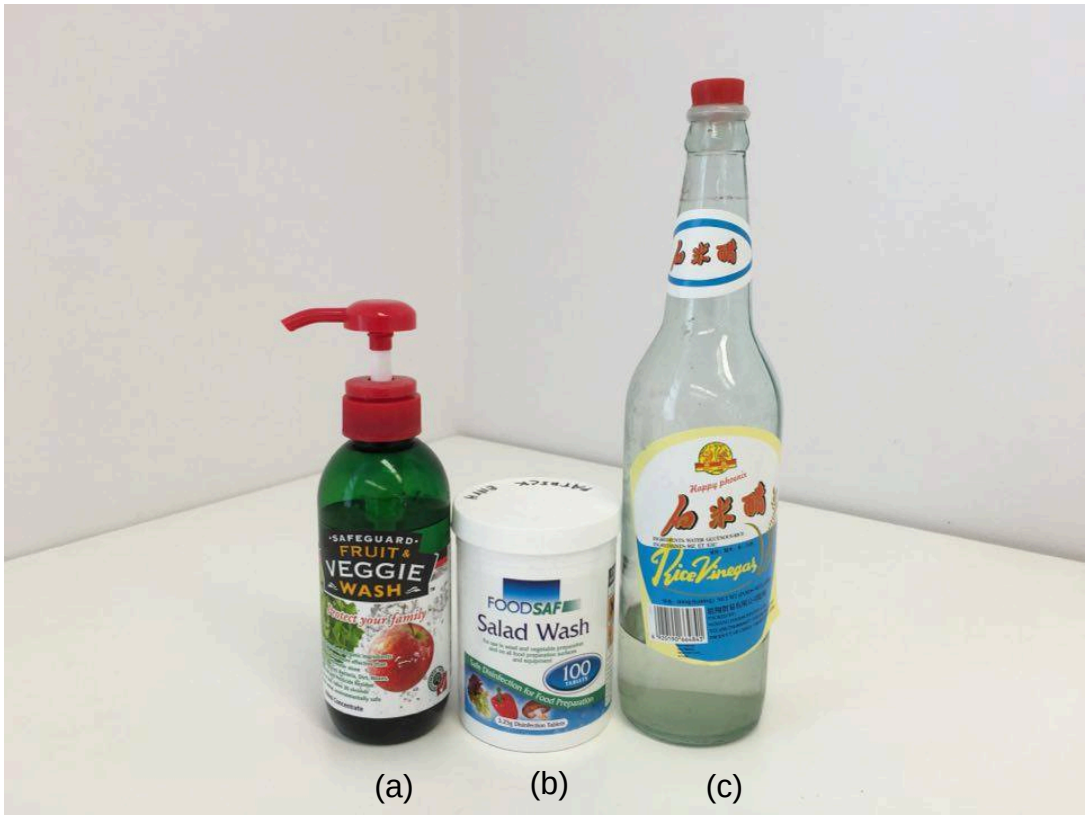

Plate 6.4 Sanitisers used in this study to soak lettuce samples from right to left; (a) Fruit& Veggie wash (b) Foodsaf Salad Wash, chlorine tablets (c) White rice vinegar

6.2.3 Enumeration of *E. coli* in lettuce samples after decontamination process

After spinning, 25 g of lettuce leaves were randomly picked from the spinner, aseptically placed in a stomacher bag to which was added 225 mL, 0.1% sterile buffered peptone water. The suspension was homogenised for 1 minute using a stomacher (Model 2X (IDEXX)). The supernatant from the homogenate was enumerated for *E. coli* using the Colilert®-18 MPN method and expressed as the most probable number (MPN) of *E. coli* per 100 g of lettuce (*E. coli* MPN/100 g). The efficacy of the washing methods is presented as $\log_{10}$ reduction (Eq. 6.1).

Efficacy of a washing method ($\log_{10}$ reduction) = E_{before} - E_{after} **Equation 6.1**

Where, E_{before} is the initial concentration of *E. coli* ($\log_{10}$ MPN/ 100 g) and E_{after} is the concentration of *E. coli* on lettuce after washing ($\log_{10}$ MPN/ 100 g).

6.2.4 Statistical analysis

The difference in *E. coli* concentration in lettuce following different washing methods was analysed using One – Way Analysis of ANOVA using SPSS (PASW Statistics 18) with the confidence level of 95 %. Significant differences between mean values of *E. coli* counts at each treatment were determined by Bonferroni *post-hoc* test.

6.3 RESULTS

The results of the efficacy of 10 different home washing methods to reduce *E. coli* on wastewater irrigated lettuce leaves are presented in Table 6.2. The initial concentration of *E. coli* in wastewater submersed lettuces ranged from 2,057 to 19,381.67 *E. coli* MPN/ 100 g. All 10 methods could reduce *E. coli* populations on the lettuce by 1.3 – 3.3 $\log_{10}$ reductions. Washing lettuce with Method 7 P50Cl (Pre-soaked in tap water for 3 min + soaked in 50 ppm Chlorine solution for 5 min + rinsed with tap water for 20 s) achieved the highest $\log_{10}$ reduction of 3.3 units. However, in the absence of chemical addition, Method 3 PRUN (Pre-soaked in tap water for 3 min + run under tap water for 20 s) achieved a 2.4 $\log_{10}$ reduction of *E. coli*.

Table 6.2 Log_{10} reduction of *E. coli* on lettuce treated with 10 washing methods

Method	Code	Initial *E. coli* counts (MPN/ 100 g)	*E. coli* counts after washing (MPN/ 100 g)	Log_{10} reduction
1	SOAK	2,057 ± 74.48	74.67 ± 20.21	1.4
2	RUNW	2,250.33 ± 54.85	109.67 ± 11.50	1.3
3	PRUN	2,250.33 ± 54.85	10 ± 0	2.4
4	PSWR	12,734.00 ± 1,214.17	92.67 ± 13.28	2.1
5	0.05Vin	2,231.67 ± 321.58	52 ± 0	1.6
6	0.5Vin	2,231.67 ± 321.58	34.33 ± 5.77	1.8
7	P50Cl	18,351.67 ± 885.66	10 ± 0	3.3
8	50Cl	18,351.67 ± 885.66	16.67 ± 5.77	3.0
9	SWAS	19,381.67 ± 833.69	147.67 ± 23.67	2.1
10	PWAS	13,756.33 ± 657.6	59.33 ± 6.35	2.4

The washing methods were performed at different times, consequently, the initial concentrations of *E. coli* on lettuce were different since the *E*. *coli* concentrations in wastewaters used were also different. The exceptions were Methods 2 & 3, 5 & 6, and 7 & 8, which were performed concurrently using the same wastewater. The statistical analysis of difference in efficacy of different methods was only performed for washing methods were the initial concentration of *E. coli* in lettuce was not significantly different, which were Methods 1, 2, 3, 5, 6, and the Methods 7, 8, 9. The results of the statistical analyses of the *E. coli* counts on lettuce after washing between different washing methods is shown in Table 6.3 and Table 6.4. Considering the differences of effectiveness of home washing methods 1, 2, 3, 5, and 6 (Table 6.3) to reduce *E. coli* on wastewater-irrigated lettuce, the effectiveness differed significantly when Method 3 PRUN (Pre-soaked in tap water for 3 min + run under tap water for 20 s) was compared to Methods 1 SOAK (Soaked in tap

water for 3 min), 2 RUNW (Running under tap water for 20 s), and 5 0.05Vin. (Pre-soaked in tap water for 3 min + soaked in 0.05% white rice vinegar; Narcissus, China for 5 min + rinsed with tap water for 20 s). In addition, from Table 6.4, the effectiveness differed significantly when Method 9 SWAS (Soaked in wash solution; Safeguard, Australia for 30 s + rinsed with tap water for 20 s) was compared to Methods 7 P50Cl (Pre-soaked in tap water for 3 min + soak in 50 ppm Chlorine solution; Foodsaf, UK for 5 min + rinsed with tap water for 20 s) and 8 50Cl (Soak in 50 ppm Chlorine solution; Foodsaf, UK for 5 min + rinsed with tap water for 20 s).

Table 6.3 Bonferroni *post-hoc* test presenting the statistical significance in the *E. coli* counts on lettuce after washing between Methods 1, 2, 3, 5, and 6.

Method	**The statistical significance (accepted at p < 0.05)**				
	1 SOAK	2 RUNW	3 PRUN	5 0.05Vin	6 0.5Vin
1 SOAK					
2 RUNW	NS				
3 PRUN	*	*			
5 0.05Vin	NS	*	*		
6 0.5Vin	NS	*	NS	NS	

* = *Statistically significant (p<0.05), NS = Not significant*

Table 6.4 Bonferroni *post-hoc* test presenting the statistical significance of the *E. coli*

counts on lettuce after washing between Methods 7, 8, and 9.

Method	The statistical significance (accepted at p < 0.05)		
	7 P50Cl	8 50Cl	9 SWAS
7 P50Cl			
8 50Cl	NS		
9 SWAS	*	*	

* = *Statistically significant (p<0.05), NS = Not significant*

6.4 DISCUSSION

A series of experiments were conducted to determine the efficacy of various home-washing methods to reduce *E. coli* load on wastewater irrigated lettuce leaves. Ten different methods were applied varying from using only tap water to household chemical (white rice vinegar) and commercial salad washes (chlorine tablets and a vegetables wash solution). All methods employed could reduce *E. coli* populations on the lettuce over the range of 1.3 – 3.3 $\log_{10}$ reductions. The highest $\log_{10}$ reduction of 3.3 was achieved by pre-soaking the lettuce in tap water for 3 min followed by soaking in 50 ppm chlorine solution for 5 min and finally rinsing with tap water for 20s (Method 7 P50Cl).

Soaking lettuce leaves in tap water for 3 min (Method 1 SOAK) achieved a 1.4 $\log_{10}$ reduction. Other studies have reported similarly, Amoah et al. (2007) found that faecal coliforms on wastewater irrigated leaves were reduced by 1.4 $\log_{10}$ when soaked in cold water (< 25°C) for 2 min. In addition, Nastou et al. (2012) demonstrated that soaking lettuce in cold water (19°C) for 5 min could reduce *L. monocytogenes* by 0.67 and 1.17 $\log_{10}$ with stirring and without stirring, respectively. Washing under running tap water for 20s (Method 2 RUNW) achieved the least $\log_{10}$ reduction of 1.3 in this study. A similar result was found by Fishburn et al. (2012), who reported that lettuce washed under running tap water (2 L/ min for 15 s) showed reductions in *Salmonella* spp., *E.*

coli O157:H7, and *L. monocytogenes* of 1.58, 1.69, and 1.49 log units, respectively. However, in the study reported here when soaking (Method 1) and running under tap water (Method 2) were combined (Method 3) the $\log_{10}$ reduction in *E. coli* increased to 2.4. The $\log_{10}$ reduction in *E. coli* achieved by Method 3 was significantly different statistically ($p < 0.05$) from soaking in tap water for 3 min (Method 1 SOAK), or running under tap water (Method 2 RUNW). This result suggests that soaking prior to rinsing by running tap water removes more contaminating microorganisms from crop surfaces than either intervention alone. Some of microorganisms will be in the soil and dirt attached to the produce, soaking prior to rinsing in order to remove any soil is needed to reduce microbial load on crops' surfaces. This result was consistent with the finding of Kilonzo-Nthenge et al. (2006), were the populations of *L. innocua* on lettuce leaves reduced by 1.37 – 1.45 $\log_{10}$ when run under tap water for 15 s, with the $\log_{10}$ reduction increasing to 1.74 – 1.85 when the lettuce were soaked prior to running under tap water.

Organic acids are also currently being used to sanitise fresh produce (Karapinar and Gönül, 1992). Vinegar (acetic acid) is an interesting alternative sanitiser since it is 'consumer friendly' and used to flavor and acidify the dressing for vegetable salads (Sengun and Karapinar, 2005). Its performance in reducing pathogenic microorganisms including *E. coli* O157:H7, *L. monocytogenes*, *S*. Typhimurium, and *S. sonnei* from pathogens spiked-fresh produce had previously been evaluated (Rhee et al., 2003, Wu et al., 2000). In this study, 0.05% and 0.5% (v/v) rice white vinegar, Method 5 and 6, were performed to reduce *E. coli* on wastewater-irrigated lettuce, resulting in the reduction of *E. coli* by 1.6 and 1.8 $\log_{10}$ respectively. The higher concentration of vinegar yielded greater reduction, although the difference was not statistically significant ($p > 0.05$). Previous studies have also shown that the pathogen reduction was higher when the concentration of the vinegar used was increased. Chang and Fang (2007) reported that soaking for 5 minutes in rice vinegar containing 0.05 and 0.5% acetic acid reduced *E. coli* O157:H7 on shredded lettuce by less than 1 $\log_{10}$ unit, however, the reduction increased to 3 $\log_{10}$ units when the vinegar concentration used was increased to 5% acetic acid. Amoah et al., 2007, also demonstrated that increased contact time with vinegar increased pathogen removal. However, in the study reported here,

soaking lettuce in vinegar solution (Method 5 and 6) was significantly ($p < 0.05$) less effective compared to washing under running tap water (Method 2). This was in contrast to the study reported by Kilonzo-Nthenge et al. (2006), where the $\log_{10}$ reduction of *L. innocua* on lettuce was greater when it was soaked for 2 min in 5% vinegar and rinsed for 15 s (1.77 – 1.98 log units), compared to running the lettuce under tap water for 15s (1.37 – 1.45 log units). The study reported here also used rice white vinegar (Narcissus, China) which had no recorded acetic acid content on the label, so the concentration of acetic acid in solutions were unknown. As discussed above, the effectiveness of vinegar to decontaminate pathogens on fresh produce depends on the concentration of acetic acid in the vinegar. The disinfection efficacy could be improved by increasing the concentration of vinegar in soaking solution, however, negative organoleptic effects may emerge for vegetables washed at higher concentrations of vinegar. The smell of vinegar on washed lettuce was noticeable in this study when they were washed using 0.05 and 0.5% vinegar solution, even after rinsing with tap water for 20s.

Two types of commercial salad wash were also used in this study; chlorine tablets (Foodsaf; Method 7 and 8) and salad wash solution (Safeguard; Method 9 and 10). There was no statistically significant difference ($p > 0.05$) in *E. coli* reduction between pre-soaking the lettuce for 3 minute followed by soaking in 50 ppm chlorine solution (Method 7) and soaking in 50 ppm chlorine solution without pre-soak (Method 8). Irrespectively, soaking in 50 ppm chlorine solution (Foodsaf; Method 7) recorded the highest $\log_{10}$ reduction (3.3 $\log_{10}$) of *E. coli* compared with all other 9 methods used in this study. Method 9 followed the manufacturer's instructions for its use, which suggests using 2-4 pumps of the solution in a half sink of water to soak fresh produce for 30 s (pre-soak prior washing was not mentioned). However, other results showed that pre-soaking prior to sanitizing and rinsing improved the efficacy of washing methods, consequently, Method 10 was also included in the study. This included pre-soaking, extended from 30 s to 5 min, prior to treatment with the commercial product Safeguard. The statistically significantly difference in the initial concentration of *E. coli* on lettuce between these 2 treatments (Methods 9 & 10) precluded them from statistical comparison of their performance relative to each other. However, pre-soaking the lettuce and at the extended

soaking time resulted in an increased reduction of *E. coli* (2.4 $\log_{10}$ reduction) compared with soaking in Safeguard alone (2.1 $\log_{10}$ reduction).

Chlorine is the most commonly used sanitiser in commercial fresh produce washing (Warriner and Namvar, 2014a). The recommended concentrations to decontaminate fresh fruit and vegetable range from 50 to 200 ppm with at least 1-2 min contact time (Beuchat, 1998); However, washing produce in 20 – 200 ppm free chlorine solution cannot remove pathogens completely, generally achieving a 1-3 $\log_{10}$ reduction (Aruscavage et al., 2006). Many studies have been conducted to demonstrate the effectiveness of chlorine solution in this range to remove pathogens in fresh produce, however, the results are diverse. Vijayakumar and Wolf-Hall (2002b) reported a 1.6 $\log_{10}$ reduction of *E. coli* on lettuce that were soaked in 180 ppm chlorine (NaOCl) solution for 10 min. Behrsing et al. (2000) evaluating Cos lettuce leaves treated with 50 and 100 ppm chlorine solution (CaOCl) for 5 min observed the reduction of *E. coli* on the leaves of 2.3 - 2.7 and 2.0 - 2.7 $\log_{10}$, respectively. Lang et al. (2004) investigated the performance of 200 ppm chlorine solution (NaOCl) to remove pathogens on lettuce leaves, the results showed that agitation (150 rpm for 5 min) of lettuce resulted in the reduction of *Salmonella*, *E. coli* O157:H7, *L. monocytogenes* of 1.09 - 1.85, 1.1 - 1.75, and 1.15 - 1.79 $\log_{10}$, respectively. Fishburn et al. (2012) also found that lettuce treated with 70 ppm chlorine solution (NaOCl) for 2 min caused a reduction in *Salmonella*, *E. coli* O157:H7, *L. monocytogenes* of approximately 2.05, 2.34 and 2.16 $\log_{10}$, respectively. However, the chlorine tablets used in the study reported here contained sodium dichloroisocyanurate (NaDCC; Foodsaf, UK) which is a different compound from the chlorine used in those studies considered above, which used either sodium hypochlorite (NaOCl) or calcium hypochlorite (CaOCl). NaDCC is also known as sodium dichloro-s-triazinetrione which has been approved by the WHO for the treatment of drinking water at household level and in emergency situations (Clasen and Edmondson, 2006). In aqueous solution, it releases chlorinated and non-chlorinated isocynurates and free available chlorine (FAC) as hypochlorous acid (HOCl), a well-known oxidising and antimicrobial agent. However, unlike other forms of chlorine compounds, NaDCC has the advantage of leaving no taste or odour when it has been used to treat water (Clasen and Edmondson, 2006). A few studies have been done

to demonstrate the effectiveness of NaDCC to decontaminate fresh produce. Nicholl and Prendergast (1998) found that the numbers of total aerobic mesophiles were reduced 2.08 $\log_{10}$ when shredded lettuce were soaked in 320 ppm NaDCC for 30 min. Nascimento et al. (2003) evaluated the effectiveness of NaDCC (200ppm for 15 mins) to sanitise lettuce leaves and reported decimal reductions of 3.23, >3.08, >1.95, and >0.26 $\log_{10}$ CFU/g for the total aerobic mesophilic count, yeast and mould, total coliforms and *E. coli*, respectively. Amoah et al. (2007) used NaDCC (Foodsaf chlorine tablets, UK) to remove faecal coliforms in wastewater irrigated lettuce leaves and found that the faecal coliforms reduction was 2.3 $\log_{10}$ when soaked in 100 ppm solution for 5 min. Faour-Klingbeil et al. (2016) recently reported that exposure to NaDCC (250 ppm soaked for 15 min) was effective at reducing *S*. Typhimurium on parsley, achieving a 1.92 – 3.12 $\log_{10}$ reduction.

Decontamination process using NaDCC tablets (Foodsaf; Method 7 and 8) was also more effective in this study, significantly ($p < 0.05$) reducing *E. coli* on wastewater irrigated lettuce leaves compared with another commercial salad wash solution (Safeguard; Method 9). Safeguard Fruit and Veggie Wash (WA, Australia), formulation included organic aloe vera, citrus essential oil, olive leaf extract, emulsifier (from fruit and berries), glycerin (from vegetable source), and purified water. This method could reduce *E. coli* on lettuce by 2.1 $\log_{10}$. Commercial produce wash solutions typically contain plant extract as a main ingredient. These kind of products were developed due to increasing consumer demand, looking for user friendly, alternative and perceived less 'toxic' washing technologies. These products not only have antimicrobial properties, but also can remove soil and wax from the surface of produce (Tang, 2010). Kim et al. (2011) evaluated the feasibility of 12 plants extracts to reduce *S*. Typhimurium, *E. coli* O175: H7 and *L*. *monocytogenes* on lettuce leaf surfaces. The study found that clove extract (*Syzygium aromoticum*) showed the highest effectiveness compared with other extracts; achieving, 2 and 3 $\log_{10}$ reductions of *S.* Typhimurium and *E. coli* O175: H7 when treated with 5% and 10% clove extracts. In addition, Singh et al. (2002) determined the effectiveness of thyme essential oil against *E. coli* O175: H7 on shredded lettuce and found that the oil could reduce *E. coli* O175: H7 on shredded lettuce by 1.91 and 2.33 $\log_{10}$ reductions when 1and 10 mL/ L were applied.

Results from this study suggest that 10 various home washing methods could reduce *E. coli* populations on the wastewater irrigated lettuce by 1.3 – 3.3 $\log_{10}$ reduction. Pre-soaking lettuce for 3 min prior soaking in 50 ppm NaDCC solution and spinning for 15s had the highest efficacy for reduction of bacterial contamination on the surface of lettuce leaves. However, where chemical sanitisers are not available or are too expensive for consumers to use, such as in poor rural communities, pre-soaking lettuce for 3 min and running under tap water for 20s, followed by removal of excess water (spinning for 15s) had the potential to reduce *E. coli* load on wastewater irrigated lettuce surfaces by 2.4 $\log_{10}$.

CHAPTER 7

EXPOSURE MODEL FOR THE CONSUMPTION OF WASTEWATER IRRIGATED LETTUCE

7 EXPOSURE MODEL FOR THE CONSUMPTION OF WASTEWATER IRRIGATED LETTUCE

7.1 INTRODUCTION

Human health risks from the consumption of wastewater irrigated salad crops have been investigated by using either epidemiological studies or Quantitative Microbial Risk Assessment (QMRA) approach as described in Chapter 1. From those epidemiological studies, using wastewater in agricultural irrigation had potentially adverse health effects in humans, not only consumers but also farmers, their families, and surrounded communities where wastewater irrigation being applied. (WHO, 2006b, Blumenthal and Peasey, 2002, Shuval et al., 1986). Additionally, consumer risks relate specifically to consumption of crops irrigated with untreated wastewater and consumed uncooked (Shuval et al., 1984). QMRA is another method to assess human risk by estimating exposure to infectious microorganisms. Generally, it is defined as four steps: hazard identification, dose-response assessment, exposure assessment, and risk characterisation, which is increasingly the technique used to manage food safety risk, as well as the safe use of wastewater in agriculture (Haas et al., 1999, WHO, 2006b).

Numerous studies have been published using QMRA to estimate the risk associated with the consumption of wastewater irrigated vegetables, but using different base assumptions for estimating exposure of the crops to pathogens (Forslund et al., 2010, Hamilton et al., 2006, Mara et al., 2007, Petterson et al., 2001, Sales-Ortells et al., 2015, Pavione et al., 2013, Forslund et al., 2012). Some studies estimated the microbial load on the crops through wastewater retained on the leaf surfaces which was based on the assumption of Shuval et al. (1997) that all pathogens contained in wastewater would attach to the crop' surfaces (Hamilton et al., 2006, Mara et al., 2007, Petterson et al., 2001). While others tried to enumerate pathogens directly from the crop' surfaces (Bastos et al., 2008, Pavione et al., 2013, Aiello et al., 2012, Forslund et al., 2012). Then, the estimated risk of those studies are generally presented as infection risk and compared to the health-based target of a tolerable additional disease burden of $\leq 10^{-6}$ DALYs pppy set by WHO (2006).

In addition, those QMRA studies mostly refer to conventional wastewater treatment as the ultimate measure to reduce health risk from water safety perspective. However, in food safety risk management, the microbiological risk could either be increased or decreased throughout the production chain, which influences or may influence the microbial risk. Furthermore, the risk could be minimised by multi-barrier approaches both at the farm, post-harvest, and in the home as discussed in Chapter 6. Therefore, in this chapter the exposure assessment from wastewater irrigated lettuce consumption attempts to combine those two different perspectives, wastewater quality and food handling, in order to develop a better estimation of microbial risk in salad crops where irrigating wastewater is an important source of pathogen contamination. It involves a variety of pathways in line with the principle of the Hazard Analysis and Critical Control Points (HACCP) from "farm to fork". Previous Chapters presented the levels of *E. coli* contaminating lettuce following wastewater irrigation, the potential effect of temperature on microbial quality along the supply chain and finally, interventions in the home environment which could reduce microbial load. This information allowed an exposure assessment associated with the consumption of wastewater irrigated lettuce to be conducted.

7.2 MATERIALS AND METHODS

7.2.1 Exposure assessment

7.2.1.1 Probabilistic exposure model for E. coli in wastewater-irrigated lettuce

There are many factors associated with the microbial risk from the consumption of wastewater irrigated salad crops as considered in the previous chapters. To simplify the scope of the probabilistic exposure model, and to gain a better understanding of the issues, a flow diagram of sprinkler-field wastewater grown Cos lettuce was developed as presented in Fig. 7.1. The two-level factorial experimental design (2^4) was chosen to reflect all possible combinations of the 4 main factors, to estimate the exposure levels of *E. coli* on wastewater irrigated lettuce at the time of consumption. The two alternate levels of each factor are defined symbolically as (-) and (+), and refer to the low and high level of each factor. The factors included in this chapter were chosen from the results and observations presented in the previous chapters.

The four main factors were defined as follows:

1) Effect of rainfall on wastewater *E. coli* concentration: (-) = no rain; (+) = rainfall
2) Withholding period: (-) = 0 day; (+) = 2 days
3) Supply chain: (-) = Farmers' market with no temperature control (20^{o}C); (+) = Supermarket with temperature control (4^{o}C).
4) Decontamination process: (-) = No washing applied; (+) = Washing applied

The decontamination process included as Factor 4 was pre-soaking the lettuce in tap water for 3 min followed by soaking in 50 ppm chlorine solution for 5 min and finally rinsing with tap water for 20s identified, as Washing Method 7 (Chapter 6), as being the most effective at reducing *E. coli* contamination on wastewater irrigated lettuce leaves. Therefore, 2^4 scenarios were built to estimate the level of *E. coli* contamination on wastewater irrigated Cos lettuce at the point of consumption as presented in Table 7.1.

7.2.1.2 Model input variables

The sophistication of the development of the risk model to estimate the microbial risk from the consumption of wastewater-irrigated Cos lettuce in this chapter was in the evaluation of the variability and uncertainty in values for model inputs, which was addressed using 10,000-iterations Monte Carlo simulation (ModelRisk 5, Vose Software BVBA, Ghent, Belgium, 2013), and by using the 2-level Factorial design. The parameters inputs were obtained from the findings of previous chapters together with some additional information as summarised in Table 7.2. A full description of input variables are described below in section 7.2.1.3.

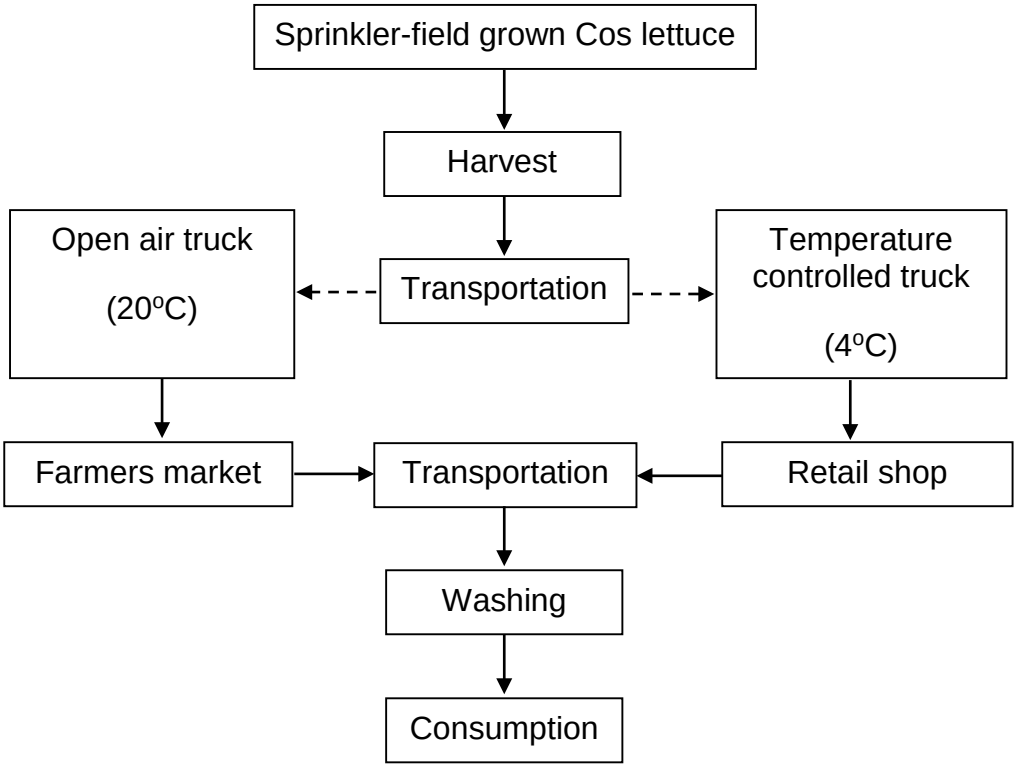

Figure 7.1 Wastewater-irrigated Cos lettuce production chain used in this study

Table 7.1 The 2^4 quantitative risk model scenarios

Factor	Rainfall	Withholding period	Supply chain	Decontamination process
Scenario	**X1**	**X2**	**X3**	**X4**
1	–	–	–	–
2	+	–	–	–
3	–	+	–	–
4	+	+	–	–
5	–	–	+	–
6	+	–	+	–
7	–	+	+	–
8	+	+	+	–
9	–	–	–	+
10	+	–	–	+
11	–	+	–	+
12	+	+	–	+
13	–	–	+	+
14	+	–	+	+
15	–	+	+	+
16	+	+	+	+

Table 7.2 Model input parameters

Code	Input	Unit	Value	Factor*	Distribution/ Calculation	References
	Pre-harvest					
A	Wastewater *E. coli* concentration	Log_{10} MPN/ 100 mL	Normal(2.33,0.23) Normal (3, 0.082)	No Rainfall	- Boolean & distribution	Field data (Appendix B)
B	Weight	g	Normal(290.23,52.65)		Distribution	Chapter 4
C	Water retained	%	ExtValueMax(20.94,3.57)		Distribution	Chapter 4
D	Water retained	g	B*(C/100)		Calculation	
E	Total weight (lettuce & water)	g	B + C		Calculation	
F	*E. coli* on lettuce	MPN	(C/100)*10^A		Calculation assuming density waste water = 1 g/cc	
G	*E. coli* concentration on lettuce	MPN/ 100 g	F/(E*100)		Calculation	
H	Log_{10}(MPN/ 100 g) after watering	Log_{10}MPN/ 100 g	LOG10(G)	No withholding (0 d)	Calculation	
J	Log_{10}(MPN/ 100 g) after drying	Log_{10}MPN/ 100 g	LOG10(F/B*100)	Withholding (2 d)	Calculation	

Code	Input	Unit	Value	Factor*	Distribution/ Calculation	References
K	*E. coli* inactivation rate (field)	day^{-1}	Pert(-1.7, -0.38, -0.34)		Distribution	Chapter 3
M	Withholding period	days	0 2	No withholding Withholding	Boolean fixed	
N	*E. coli* on lettuce at harvest	log_{10} MPN/ 100 g	H+(K*M) J+(K*M)	No withholding Withholding	Boolean calculation	
	Postharvest					
L	temperature (°C)		4 20	Supermarket Farmers market	Boolean fixed	
O	time (days)		Uniform(1,3)		Distribution	
P	*E. coli* inactivation rate, storage	day^{-1}	0 Normal(-0.078, 0.0053)	Farmers market Supermarket	Boolean & distribution	Chapter 5
Q	*E. coli* counts on lettuce prior to washing	log_{10} MPN/ 100 g	N + (P*O)		Calculation	
	Washing					
R	Log reductions for washing step		0 Normal (-3.26, 0.02)	No washing Washing method 7	Fixed Distribution	Chapter 6

Code	Input	Unit	Value	Factor*	Distribution/ Calculation	References
S	*E. coli* counts on lettuce after washing	log_{10} MPN/ 100 g	Q + R		Calculation	
T	Total log reductions from harvest to end washing		S-H		Calculation	
	Exposure					
U	Leaf weight (g)		Normal(17.2, 0.5)		Distribution	Appendix C
V	Exposure MPN (assuming 1 leaf consumed)	MPN	(U/100)*(10^S)		Calculation	
X	Exposure Log10 MPN	Log_{10} MPN per one leaf consumed	LOG10(V)		Calculation	

*Factor set when running factors analysis using Yates method

7.2.1.3 The description of input variables for the exposure model

This section summarises the data and information used to develop the input variables for the QMRA model of Cos lettuce irrigated using waste water. Two groups of variables are described. The first group are the design variables around which the factorial design model is based. Each of these variables is set at either of two levels as required to make up the 16 combinations of the 2^4 design. The second group of variables are common across the 16 combinations. They are the inputs necessary to run the complete mathematical structure of the model but are not considered explicitly in assessing their relevance to the QMRA model outputs.

Design variables

X1: *E*. *coli* concentration in irrigating wastewater which was influenced by rainfall (log_{10} (MPN/ 100 mL))

Examination of daily rainfall information for Mount Barker (Bureau of Meteorology Station number, 023733) for February and March 2014 suggested a correlation to the measured wastewater pond *E*. *coli* concentration (Appendix B). A hypothesis was that pond inflows due to rainfall may resuspend solids from the sediment increasing the *E*. *coli* concentration in the wastewater in the pond. It was noted that when rainfall had occurred in the day prior to pond water sampling the concentration was elevated compared to days where no rainfall was recorded.

Data for each group was pooled, logarithmically transformed and the mean and standard deviation calculated giving the following fitted distributions:

X1(-): Normal (2.33, 0.23); X1(+): Normal (3.00, 0.082)

X2: Withholding period (days)

A withholding period of two days was chosen as a typical time that Cos lettuce could remain unwatered without adverse impacts on quality.

X2(-): 0 days; X2(+): 2 days

X3: Supply chain

Two supply chains were considered in the exposure model. The first pathway was through a rural farmers market where Cos lettuce was sold directly to consumers without refrigeration. It was assumed that there was no growth or inactivation of *E*. *coli* in this pathway because of the short transportation time (within 2 hours). The second pathway was through a supermarket where the Cos lettuce was refrigerated during retail display prior to purchase. Some inactivation of *E*. *coli* was expected due to the low refrigeration temperatures.

X3(-): Farmers' market with no temperature control; X3(+): Supermarket with temperature control

X4: Decontamination process – log reductions (log_{10} (MPN/100g))

Lettuces can be consumed unwashed directly after purchase or after a decontamination process has been used to reduce surface contamination. An examination of the log reductions achieved by the many decontamination protocols considered in this thesis (Chapter 6) suggested that Method 7 was the best for inactivating *E*. *coli* on lettuce, and the data was fitted to a normal distribution. This method resulted in -3.26 log reduction with a standard deviation of 0.02. The low level of X4 was 'no washing' which provides no inactivation of *E*. *coli*.

X4(-): 0; X4(+): Normal (-3.26, 0.02)

Other variables

Cos lettuce weight

The Cos lettuces' weight in the challenge trials were the weight of Cos lettuce samples (n = 24) before submersion in the wastewater bucket (Chapter 4). The mean and standard deviation was calculated as 290.23 g and 52.65 g, respectively.

Water retained

Cos lettuces were submerged in wastewater and the total amount of water retained after draining was calculated by difference (based on the experiments reported in Chapter 4). The distribution of results for the water retained was found to be highly skewed. A Normal distribution was not deemed to be appropriate for this data. Vose ModelRisk distribution fitting methods were used to determine the most appropriate distribution for these data. After considering the fit statistics and the suitability of the distributions an Extreme value distribution with parameter values of 20.94 and 3.97 was chosen.

E. coli inactivation rate, field

The field trials for the survival of *E. coli* on wastewater irrigated lettuce demonstrated that the inactivation was rapid. Results presented in Chapter 3 showed that *E. coli* could not be recovered from lettuces beyond two days post-watering.

First-order inactivation rates were determined using the data presented in Chapter 3. These data were combined to develop parameters for a Pert distribution with minimum, most likely and maximum values of -1.7, -0.38 and -0.34 $\log_{10}$ (MPN/100g)/day.

Postharvest storage time

The specific data was not fitted to a distribution for this variable. However, the quality of lettuce deteriorates during storage, especially at ambient temperatures. A Uniform distribution with minimum and maximum values selected of one and three days respectively was chosen to reflect the uncertainty in this variable. .

E. coli inactivation rate, storage

Challenge trial data (Chapter 5) was statistically analysed to determine the first-order inactivation rate of *E. coli* on the surface of lettuce stored a 4°C. A Normal distribution with a mean and standard deviation of -0.078 and 0.0052 was developed. The units are $\log_{10}$ (MPN/100g)/day.

Leaf weight

In order to understand Cos lettuce leaf weight, an additional study was performed to account for different consumption weight by people. The individual leaves were removed and weighed from the outer most layers to the core. A 'hockey-stick' nonlinear regression model was fitted to the leaf weight-leaf order data based on the method of Grossman et al. (2000).

The major finding of this study was that the largest outer leaves were the same weight and independent of the total weight of the lettuce. The heaviest lettuce simply had more layers of the larger sized leaves. The average weight of the outer layer leaves was 17.2 g. A Normal distribution was chosen with a mean of 17.2 g and a standard deviation of 0.5 g. The standard deviation was selected to reflect the variability seen in the weight of individual outer leaves. This distribution therefore represents a "worst-case" for a single leaf consumption scenario. More details of this additional study are provided in Appendix C

7.2.2 Factor analysis

Factor analysis was performed using the Yates method (Lawson and Erjavec, 2001) to show which factors are important and drive the microbial risk from the consumption of wastewater irrigated Cos lettuce in order to provide important information to risk managers or food safety regulators for risk-based decision making. 10-iterations were performed for each simulation using ModelRisk 5 (Vose Software BVBA, Ghent, Belgium, 2013). The outputs of each scenario, which was the level of *E. coli* contamination on wastewater irrigated Cos lettuce at the point of consumption were entered into a Yates spreadsheet (Appendix D) to perform factors analysis using the Yates method (Microsoft Excel, Microsoft Corp., USA, 2013).

The four factors included for this analysis were: rainfall (X1), withholding period (X2), supply chain (X3), and decontamination process (X4). Main effects and interaction effects were determined as summarised in the Yates order table (Table 7.3). From Table 7.3, the main effects are X1, X2, X3; and X4; 2-way interaction effects are X1X2, X1X3, X2X3, X1X4, X2X4, and X3X4;3-way interaction effects are X1X2X3, X1X2X4, X1X3X4, and X2X3X4; 4-way

interaction effect which is the highest order of interaction is X1X2X3X4, and (1) is the mean value for all 16 runs.

Table 7.3 **Yates order for factors analysis**

Scenario	Code	Factor			
		X1	**X2**	**X3**	**X4**
1	(1)	-	-	-	-
2	X1	+	-	-	-
3	X2	-	+	-	-
4	X1x2	+	+	-	-
5	X3	-	-	+	-
6	X1X3	+	-	+	-
7	X2X3	-	+	+	-
8	X1X2X3	+	+	+	-
9	X4	-	-	-	+
10	X1X4	+	-	-	+
11	X2X4	-	+	-	+
12	X1X2X4	+	+	-	+
13	X3X4	-	-	+	+
14	X1X3X4	+	-	+	+
15	X2X3X4	-	+	+	+
16	X1X2X3X4	+	+	+	+

7.3 RESULTS

7.3.1 Exposure model

Sixteen scenarios based on four factors, namely rainfall, withholding period, supply chain, and decontamination process were built to estimate the level of *E. coli* on wastewater irrigated Cos lettuce at the point of consumption. The amount of lettuce consumed was obtained by assuming one leaf of Cos lettuce, based on common recipes for making sandwiches and wraps (per single serve) (FSANZ, 2016). The distribution of Cos lettuce leaves' weight is presented in Appendix C. The estimated number of *E. coli* on wastewater irrigated Cos lettuce consumed was presented as $\log_{10}$ MPN per one leaf consumed.

The frequency distribution of *E. coli* on wastewater irrigated Cos lettuce at the point of consumption estimated from 16 scenarios are presented in Figs. 7.2 – 7.18. In each case, the panel shows a histogram of the quantitative model prediction for the consumption of wastewater irrigated Cos lettuce as *E. coli* $\log_{10}$ MPN per one leaf consumed. The scale in each plot runs between -5.5 - 2.5. The maximum value of $\log_{10}$ MPN per one leaf consumed obtained from scenario 2 (+ - - -; mean = 1.5 $\log_{10}$ MPN), when rainfall affects the microbial quality of irrigating wastewater, no withholding period, transported to farmers market without temperature control, and no washing applied before consumption. While the minimum value came from scenario 15 (- + + +; mean = -3.69 $\log_{10}$ MPN), when there was no rainfall affecting the microbial quality of irrigating wastewater, lettuce was harvest after 2 days from the last irrigation, transported to retail shops with refrigerated vehicles, and the decontamination step (Method 7) applied before consumption.

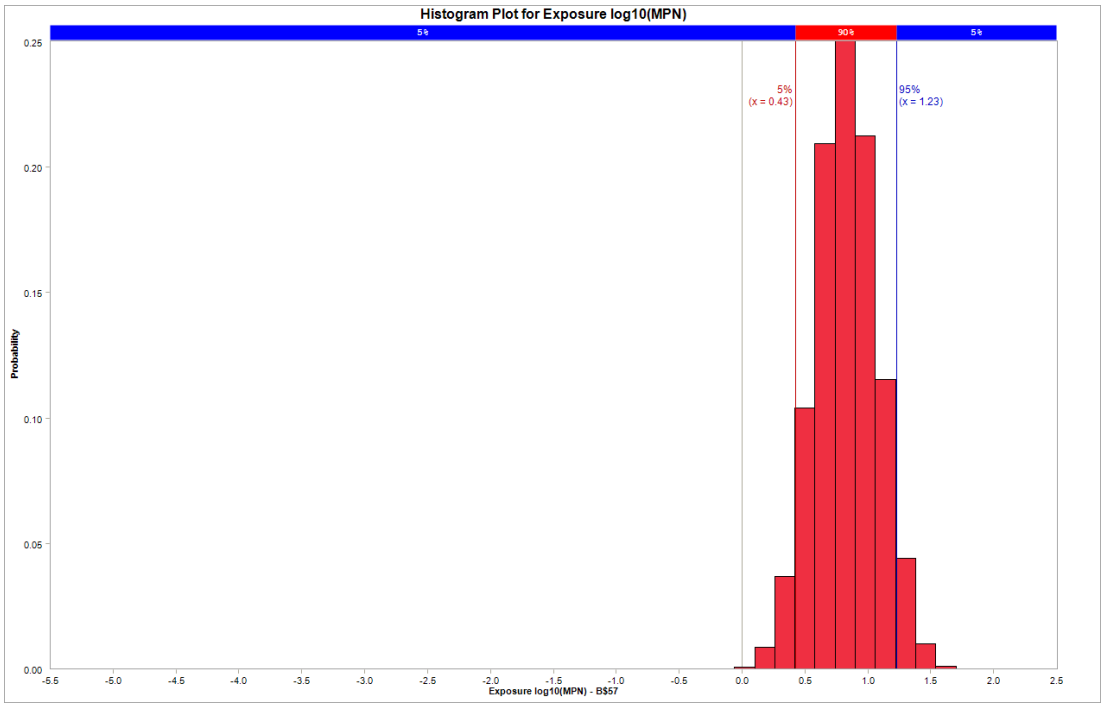

Figure 7.2 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 1 (- - - -); mean = 0.83

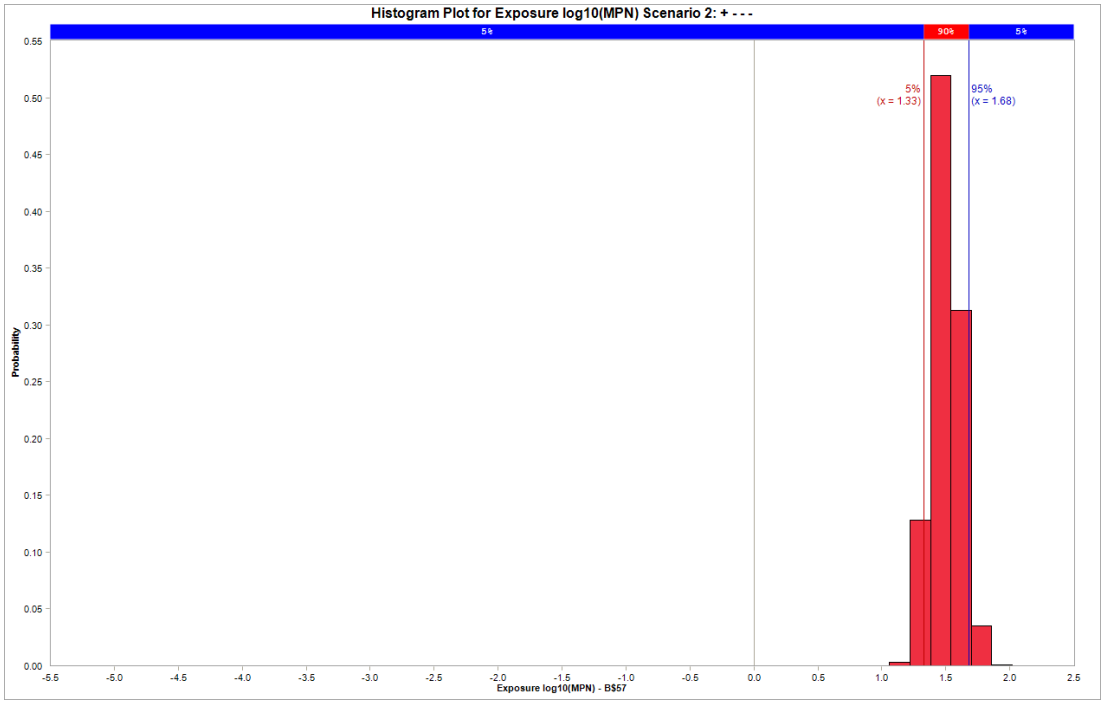

Figure 7.3 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 2 (+ - - -); mean = 1.50

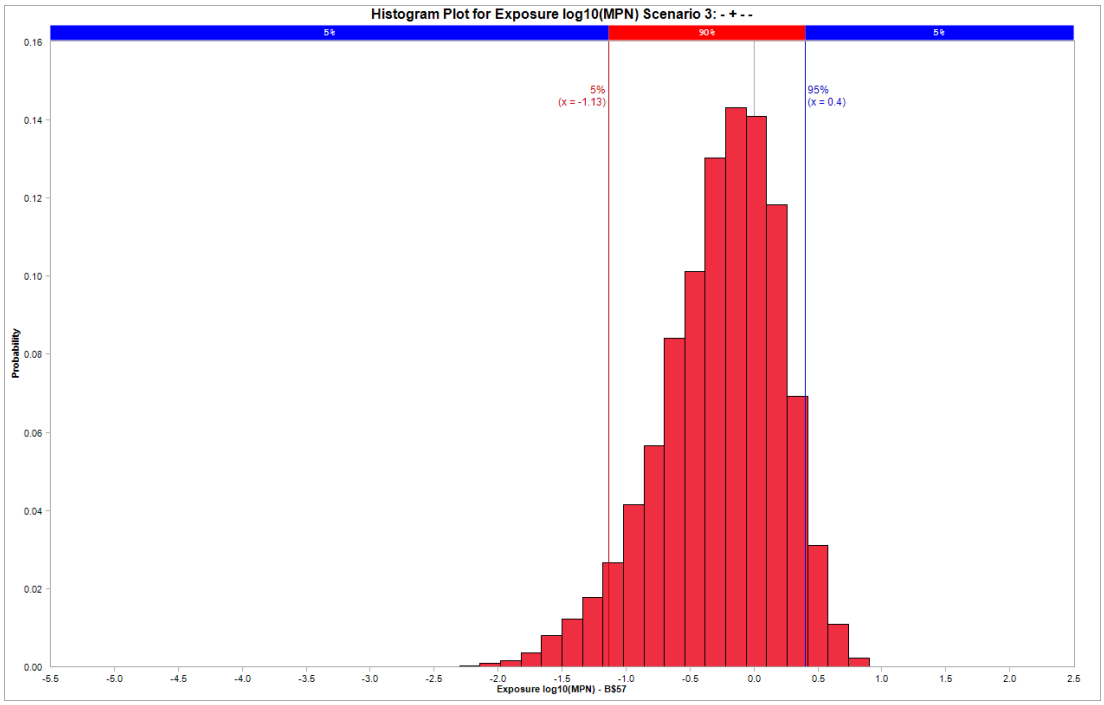

Figure 7.4 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 3 (- + - -); mean = -0.26

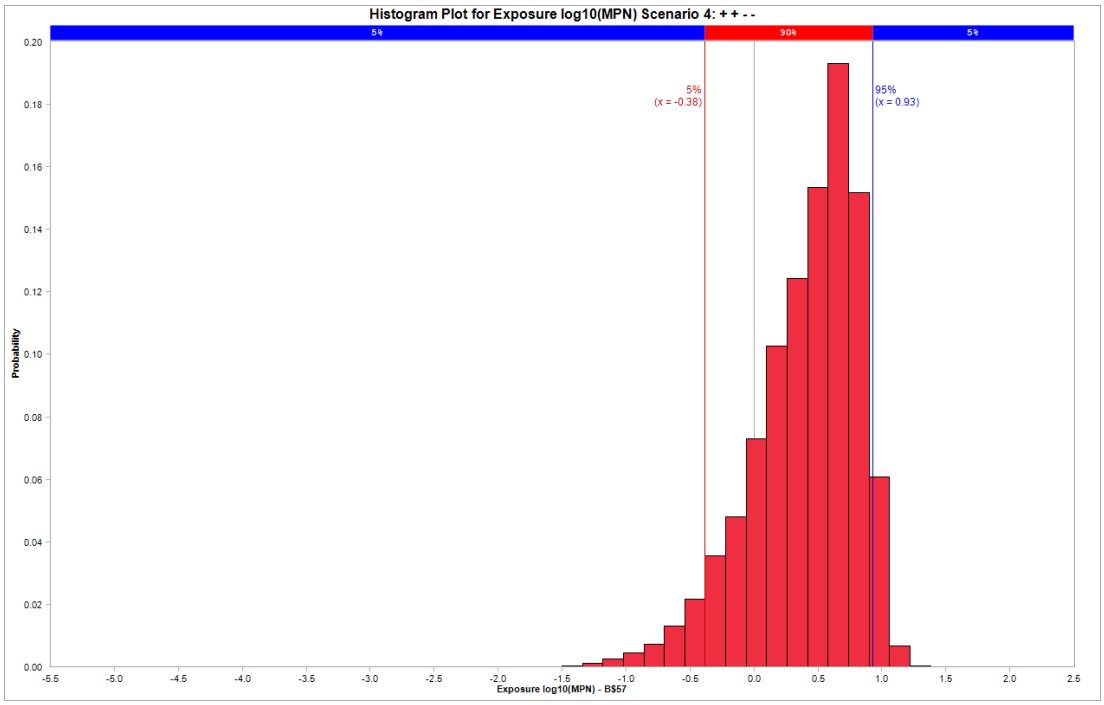

Figure 7.5 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 4 (+ + - -); mean = 0.41

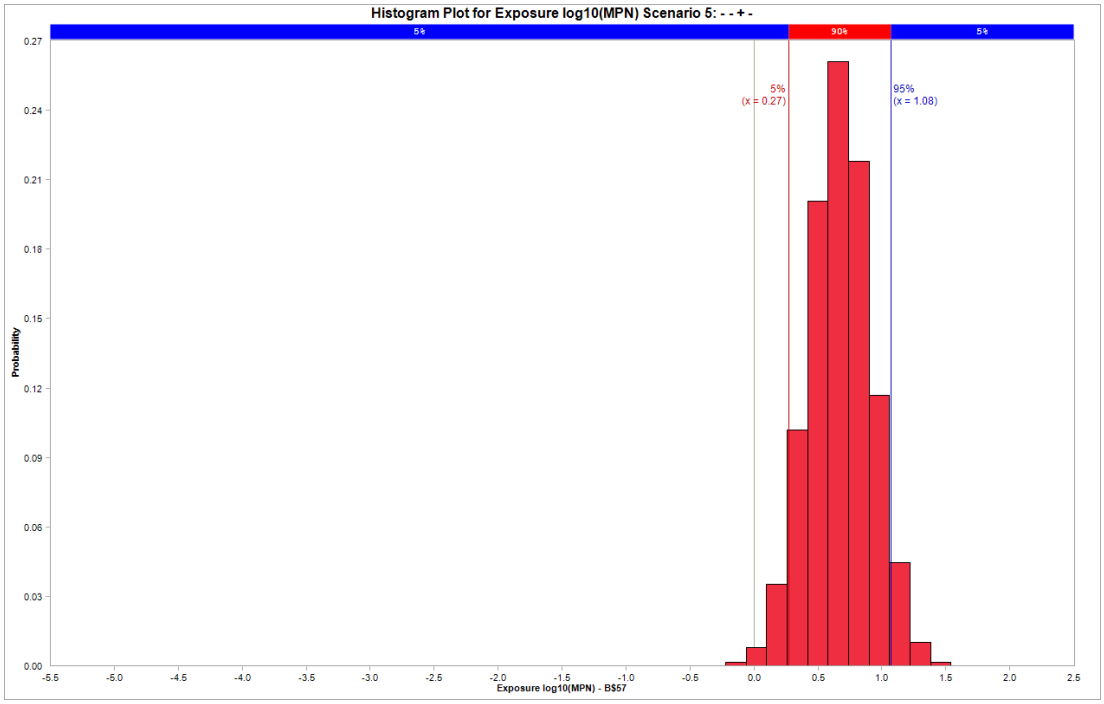

Figure 7.6 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 5 (- - + -); mean = 0.67

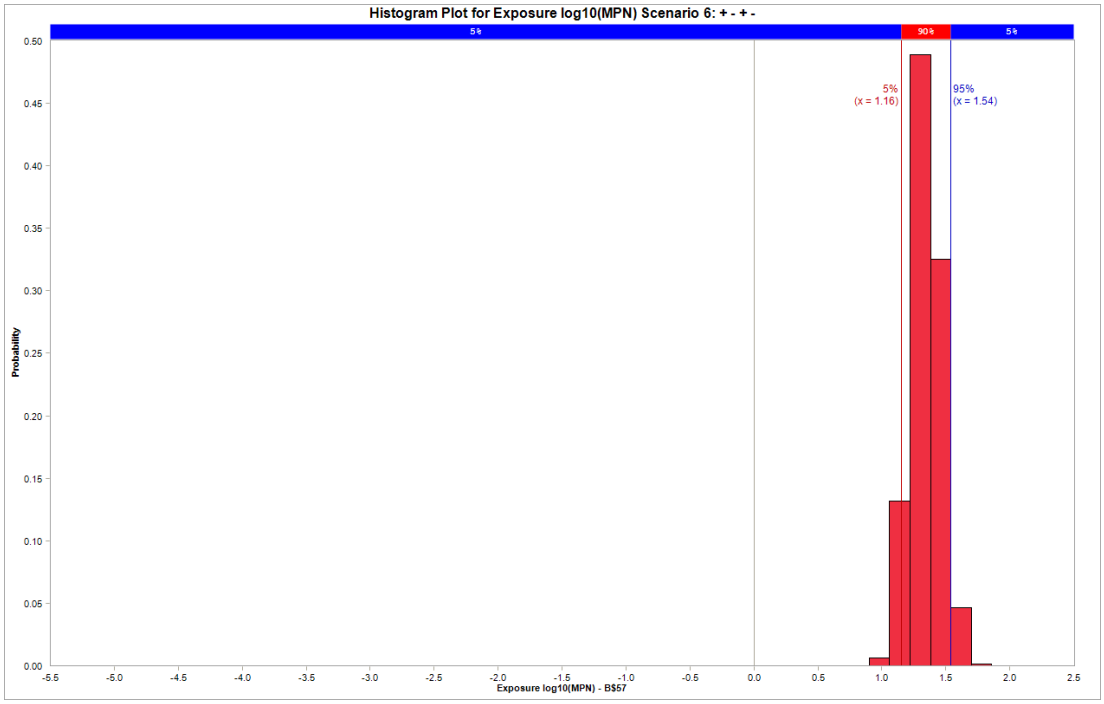

Figure 7.7 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 6 (+ - + -); mean = 1.34

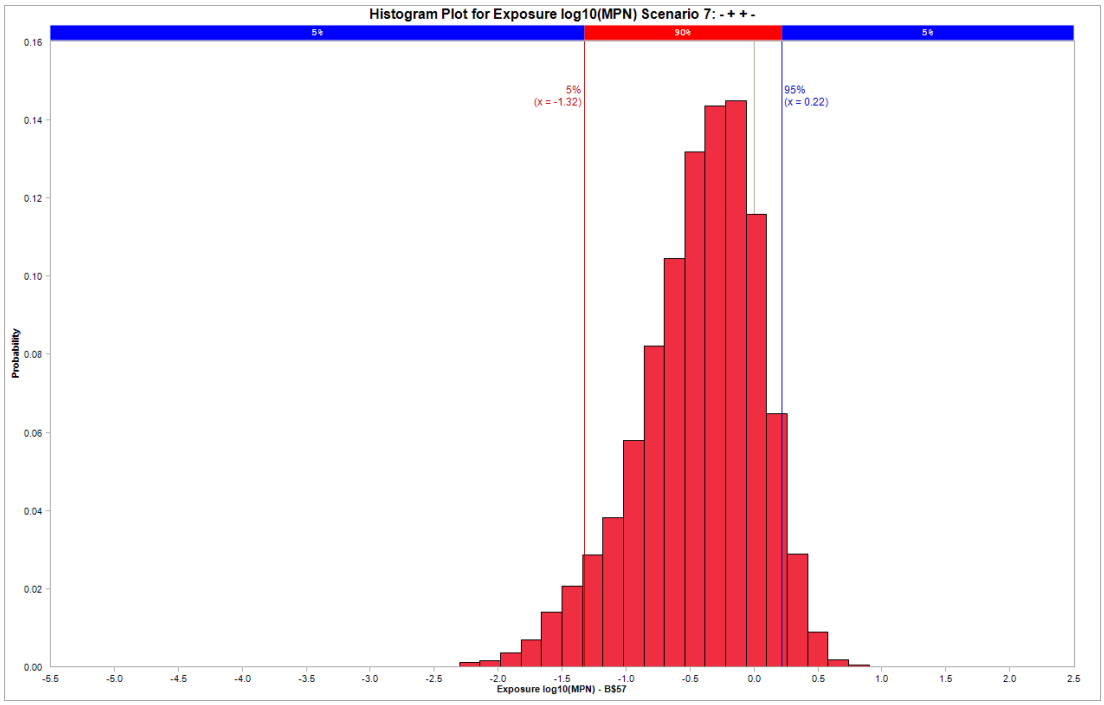

Figure 7.8 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 7 (- + + -); mean = -0.43

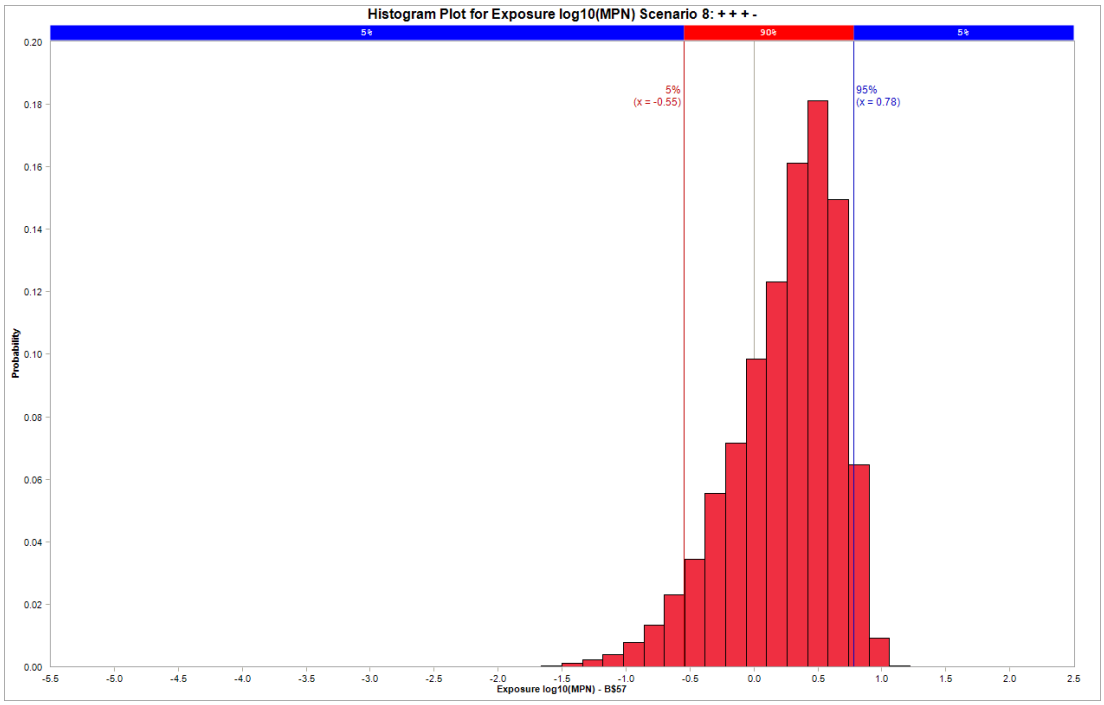

Figure 7.9 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 8 (+ + + -); mean = 0.25

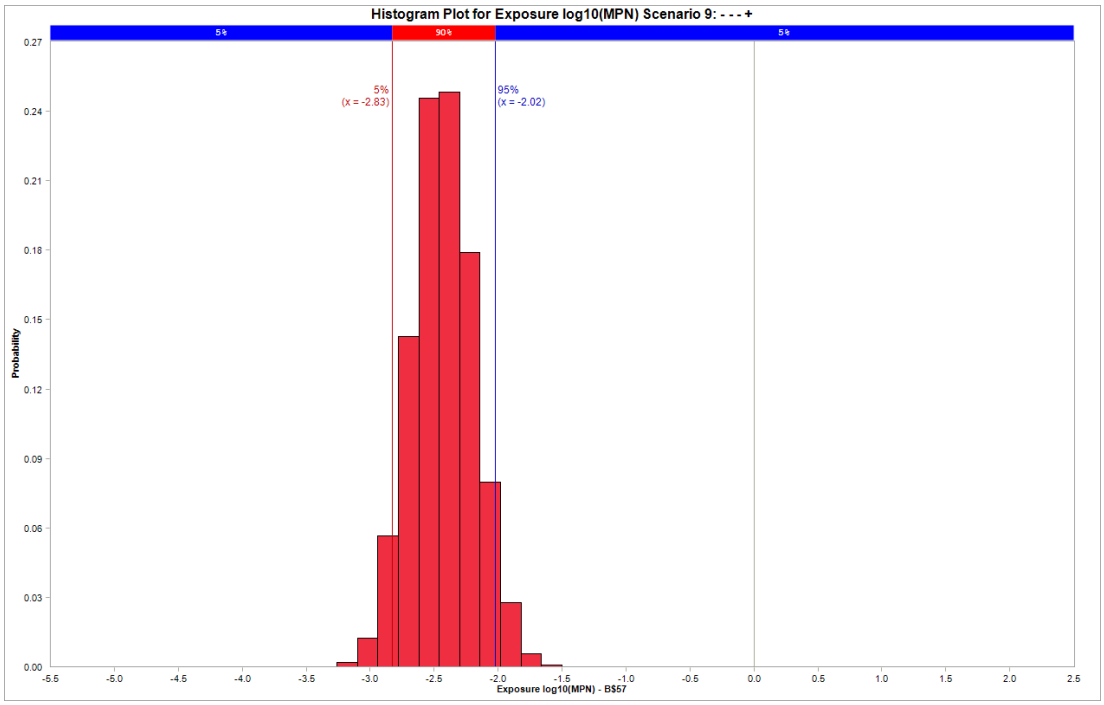

Figure 7.10 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 9 (- - - +); mean = -2.43

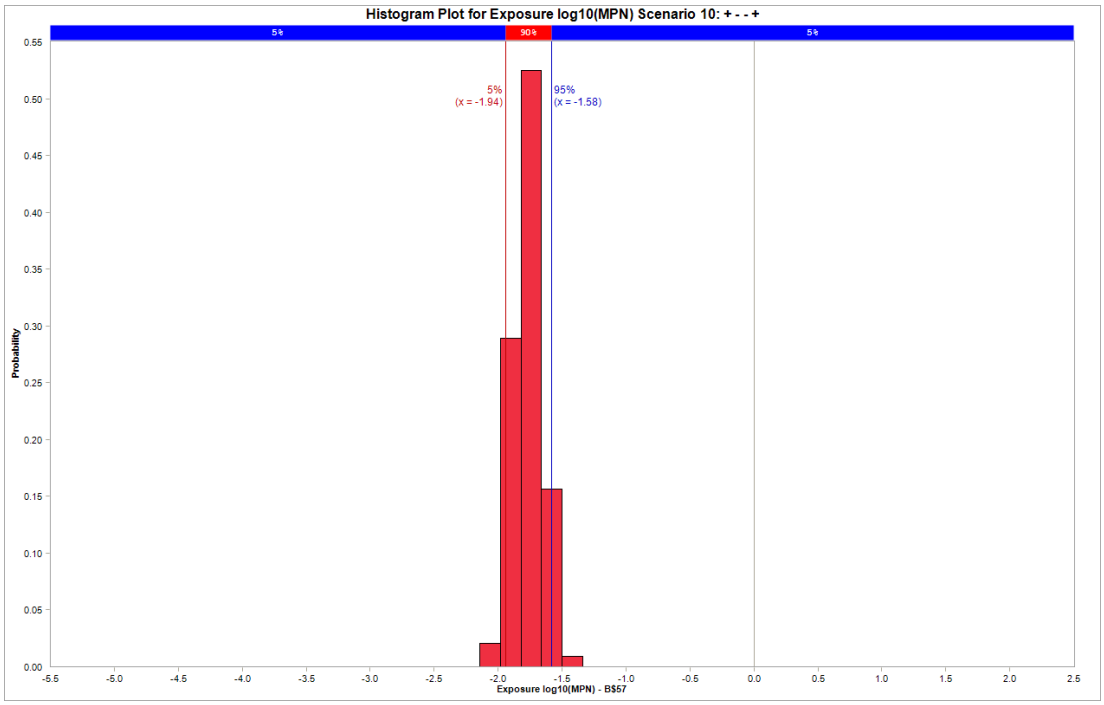

Figure 7.11 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 10 (+ - - +); mean = -1.76

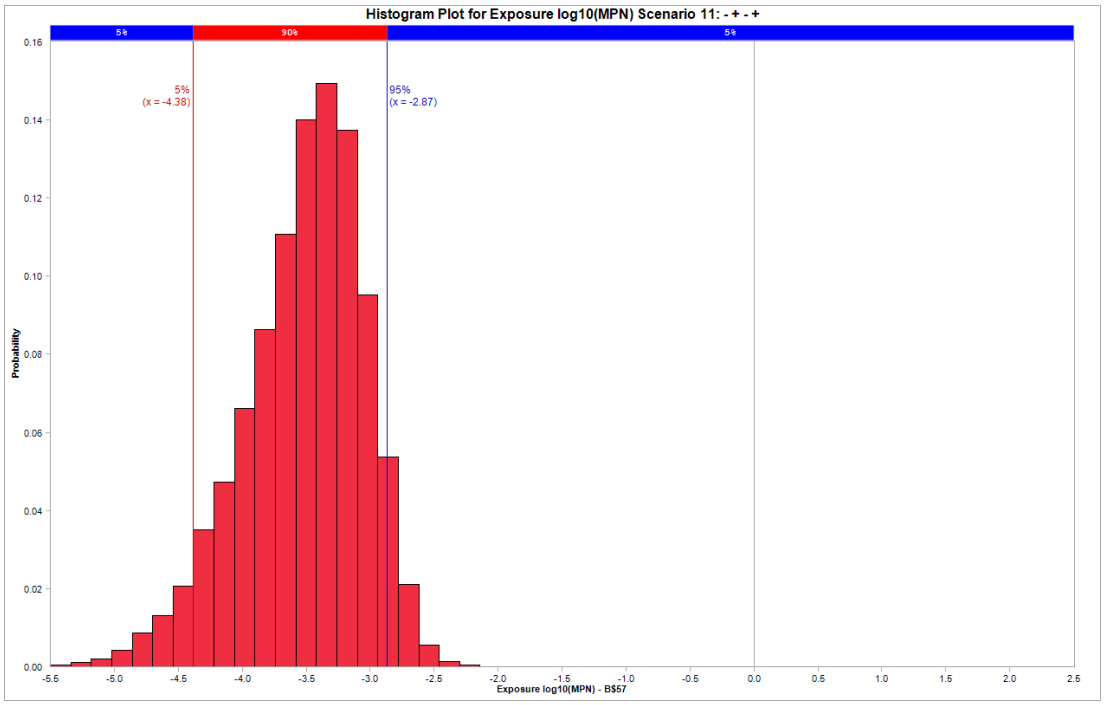

Figure 7.12 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 11 (- + - +); mean = -3.52

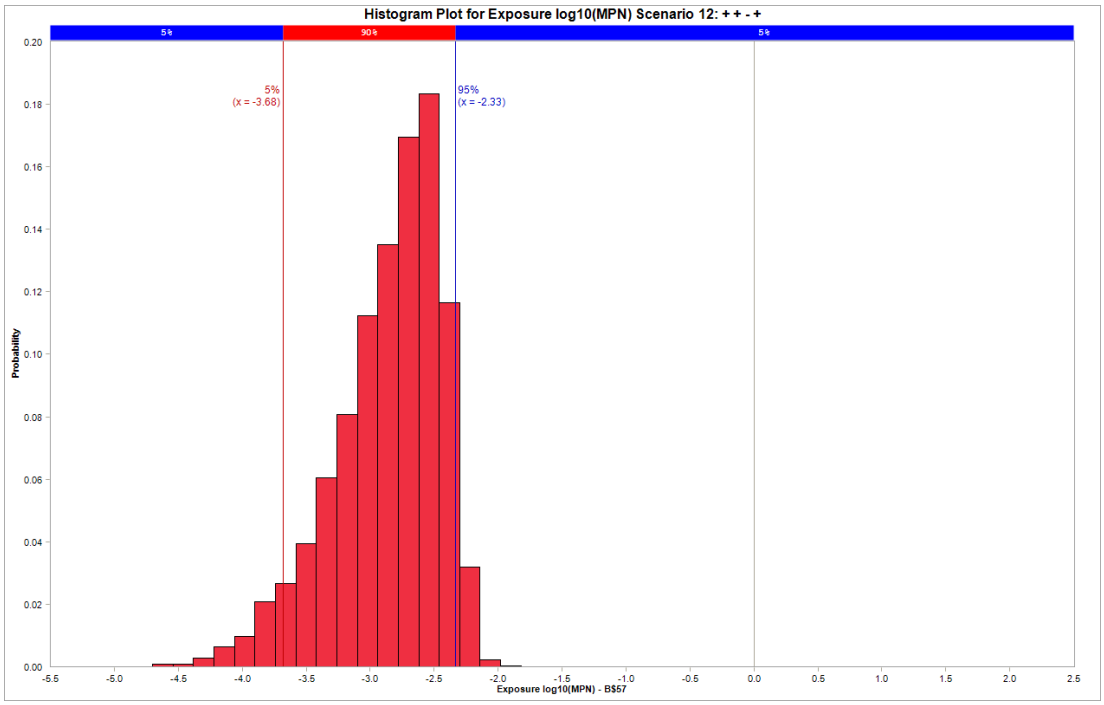

Figure 7.13 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 12 (+ + - +); mean = -2.86

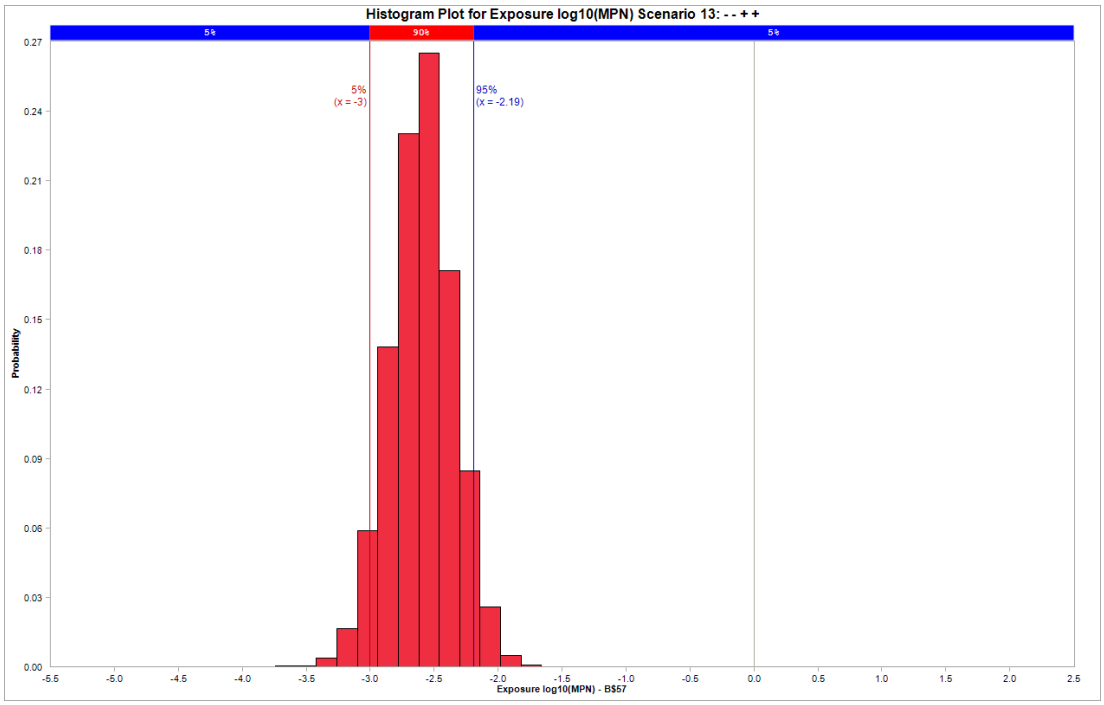

Figure 7.14 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 13 (- - + +); mean = -2.59

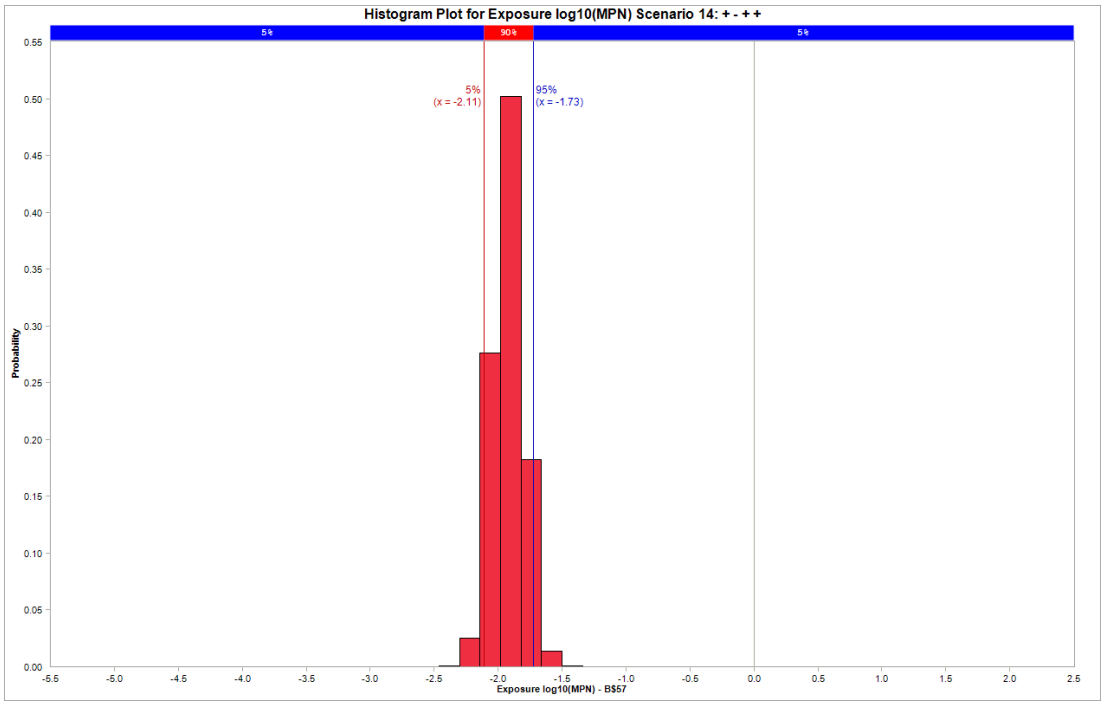

Figure 7.15 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 14 (+ - + +); mean = -1.92

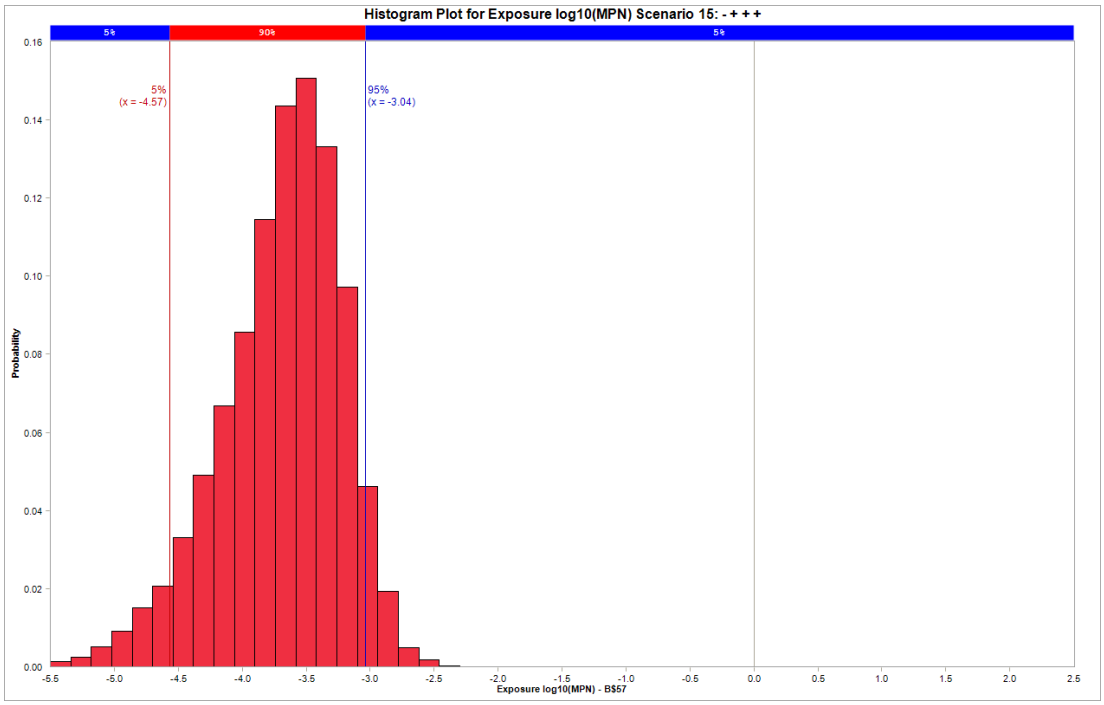

Figure 7.16 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 15 (- + + +); mean = -3.69

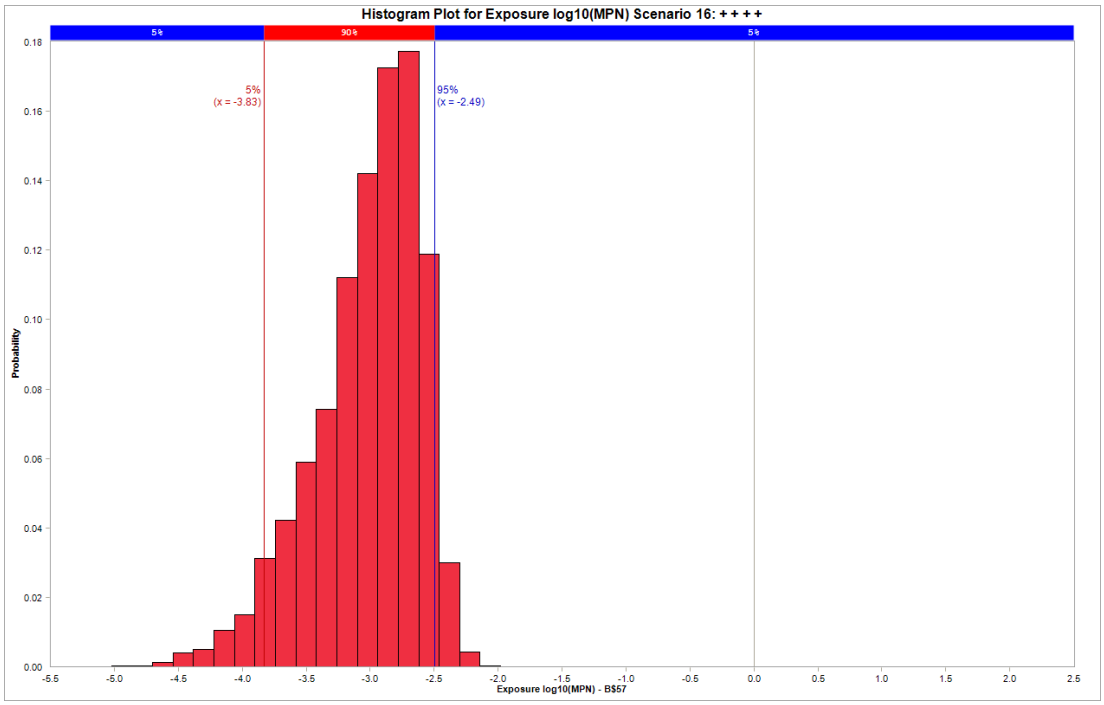

Figure 7.17 Distribution of *E. coli* concentration at time of consumption (log_{10} MPN per one leaf consumed) from Scenario 16 (+ + + +); mean = -3.02

Obviously, from scenario 9 – 16 (Figs. 7.10 to 7.17) when a washing step was employed, the estimated *E.coli* $\log_{10}$ MPN per one leaf consumed of wastewater irrigated Cos lettuce at the consumption point were lower (mean = -3.69 to -1.76) when compared with Scenario 1 – 8 (Figs 7.2 to 7.9) where a washing step was not included (mean = -0.43 to 1.50). Fig. 7.18 better describes this phenomenon, where each figure demonstrates the changes of *E. coli* concentration in different stages of each Scenario. In this figure, stage 1, 2, 3, and 4 are the *E. coli* concentration ($\log_{10}$ MPN/ 100 g) after watering, at harvest, prior to washing, and after washing, respectively. Clearly, when the washing step (Method 7) was applied (Fig. 7.18, (9)-(16)), the *E. coli* concentration decreased to -1.00 to -2.93 $\log_{10}$ MPN/ 100 g.

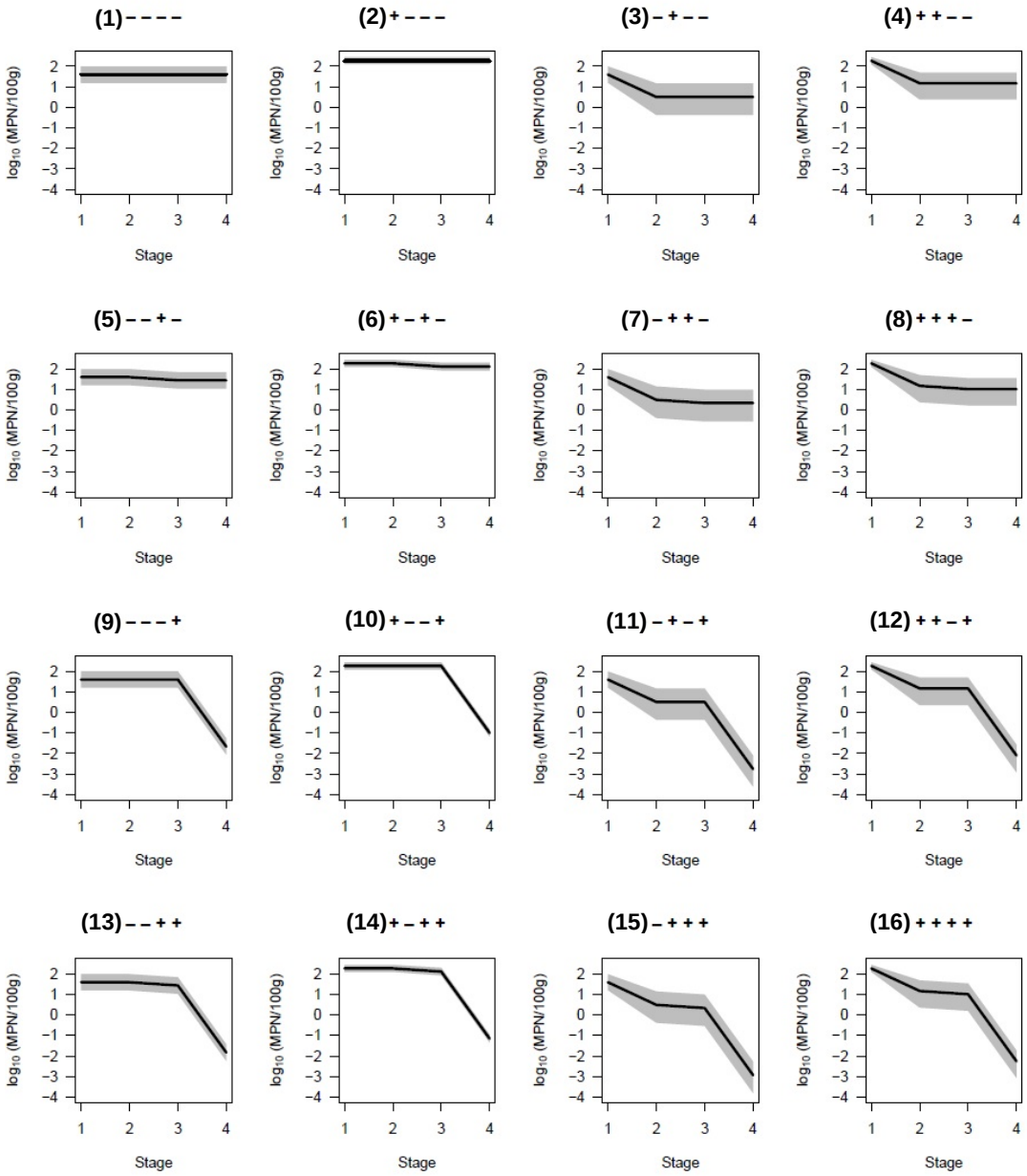

Figure 7.18 The changes of *E. coli* concentration (log10 MPN/ 100 g) on wastewater irrigated Cos lettuces at different stages

7.3.2 Factors analysis

The benefit of two-level factorial design used in this chapter was to allow risk modeler to calculate main effects and interaction effects of four factors; rainfall (X1), withholding period (X2), supply chain (X3), and decontamination process (X4) for the 2^4 Factorial design, which consequently was a consideration of 16 scenarios of microbial risk from the consumption of wastewater irrigated Cos lettuce. For these scenarios, main effects are X1, X2, X3, and X4, 2-way interaction effects are X1X2, X1X3, X2X3, X1X4, X2X4, and X3X4, 3-way

interaction effects are X1X2X3, X1X2X4, X1X3X4, and X2X3X4, 4-way interaction effect which is the highest order of interaction is X1X2X3X4, and (1) is the mean value for all 16 runs. The calculation of the main effects and interaction effects was done using the computational table shown in Appendix D with 10-iterations for each simulation, and the plot of effects for this experiment is demonstrated in Fig. 7.19. A horizontal line with an intercept at zero was added to identify which factors (main effects and/ or interaction effects) were important. Main effects and/ or interaction effects which don't cross the line mean they are important variables in this study.

From Fig. 7.19, there is insufficient evidence to indicate that supply chain (X3) had any effect on the level of *E. coli* contamination at the point of consumption. All the three other factors; rainfall, withholding period, and decontamination process (X1, X2, and X4) did significantly affect the amount of *E. coli* contamination at the point of consumption. Moreover, factor X4 (washing) was obviously very important to reduce the amount of *E. coli* at the point of consumption (estimate effect -3.50 to -3.05 $\log_{10}$ MPN per one leaf consumed); while, X1 (rainfall) could affect the amount of *E. coli* on lettuce at the point of consumption to a high level (estimate affect 0.78 – 0.26 $\log_{10}$ MPN per one leaf consumed).

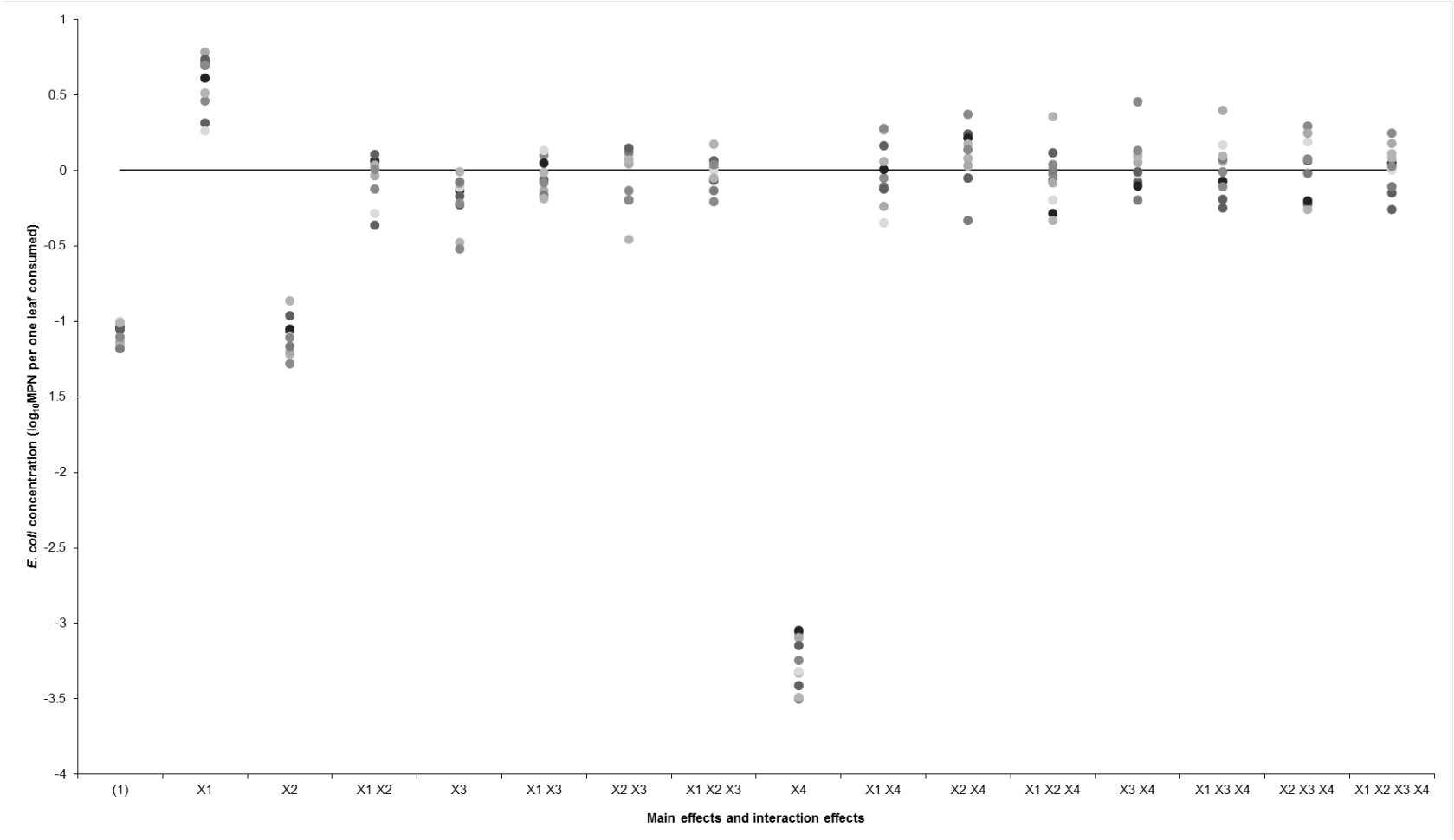

Figure 7.19 Plot of effects on the level of *E. coli* on wastewater irrigated Cos lettuce at the consumption point

7.4 DISCUSSION

Quantitative Microbial Risk Assessment (QMRA) is a powerful tool which is widely used for estimating human health risk related to wastewater reuse in agriculture, including the microbial risk from consumption of wastewater irrigated salad crops. The aim of this work was to provide supporting information for risk management, and to those food safety regulators or wastewater management authorities regarding the health risk with different exposure scenarios. However, it is also generally accepted that QMRA is site-specific, and there are number of variabilities and uncertainties which should be included. In the case of health risk from agricultural wastewater irrigation, it could be seen from two different points of view; water safety management and food safety management. In this research, exposure assessment from consuming fresh vegetables, following irrigation by partially treated wastewater was assessed. Traditionally, the initial perspective was that of wastewater safety management with freshwater scarcity as the driving force. However, the approach used in this study was more focused on the safety of consumers, which is addressing the issue from the viewpoint of food safety management. In this chapter, probabilistic simulation was used to estimate the level of *E. coli* at the point of consumption, taking into account the effect of rainfall on the *E. coli* concentration of irrigating wastewater, withholding period (the time between the last irrigation and harvest), the supply chain which has different style of transportation (to retail shops with temperature controlled and to farmers market with no temperature control), and the effect of decontamination processes to reduce the microbial load prior consumption at home. The aim was to identify the important factors which could be prevented or instituted, mitigated, and controlled, to enable effective communication of the scientific-based evidence to risk managers and food safety regulators.

Sixteen scenarios were constructed based on 2-level factorial experimental design with 4 factors. The results showed that the highest value of *E.coli* $\log_{10}$ MPN/ consumption was obtained from Scenario 2 (+ - - -; mean = 1.5), when the microbial quality of irrigating wastewater was poor due to the effect of the rainfall, lettuce was harvested after the last irrigation with no withholding period, transported to farmers market without temperature control, and no

decontamination step employed prior consumption at home. While the lowest value came from Scenario 15 (- + + +; mean = -3.69 log_{10} MPN), when no rainfall affects the microbial quality of irrigating wastewater, lettuce was harvested after a 2 day withholding period, transported to retail shops in refrigerated vehicles, and an effective washing step (Method 7, Chapter 6) was applied before consumption at home. As the risk estimate reported here used a different approach from many of previous QMRA studies associated with wastewater irrigation, it is difficult to compare the risk estimation from this study with those of other researchers. The majority of previous QMRA studies about the health risk from the consumption of wastewater irrigated salad crops estimated the risk in terms of infection risk pppy (per person per year) and compared with the benchmark acceptable risk level $\leq 10^{-6}$ DALYs pppy set by the WHO (2006) (Barker-Reid et al., 2010, Bastos et al., 2008, Mara et al., 2007, Seidu et al., 2008, Mara and Sleigh, 2010b, Pavione et al., 2013), while the exposure estimation in this study was presented as the degree of *E. coli* contamination at the point of consumption (*E. coli* log_{10}MPN/ consumption). However, when QMRA is applied to food risk analysis, comparing risk estimates to acceptable risk level benchmarking is uncommon, the expression of risk outcomes is also variable (Keuckelaere et al., 2015). For example, Doménech et al. (2013) determined the health risk of *L. moonocytogenes* from the consumption of contaminated lettuces, and expressed the risk as the probability of illness per serving. Ding et al. (2013) examined the health risk from the consumption of *L. moonocytogenes* contaminated lettuces in Korea, and the risk was presented as contamination level at the consumption point and the probability of listeriosic illness pppy.

The QMRA approach used in this study is the first study to use 2-level factorial experimental design to construct the scenarios. This approach analyses all the possible combinations of factors which influence the microbial risk from different pathways/ scenarios. Therefore, this approach could provide clear evidence on how relevant factors could be managed to mitigate the risk. For example, scenario 15 (- + + +; mean = -3.69 log_{10} MPN) obtained the lowest *E. coli* exposure at the consumption point. From this scenario, it was clear that the risk is lowest when good pre- and postharvest management was applied throughout from “farm to fork’ viewpoint. Pre-harvest, there was no rainfall to

affect the microbial quality of irrigating wastewater at the production site, and lettuce was harvested 2 days after the last irrigation was applied, and postharvest, the lettuce was transported to retail shops with refrigerated vehicles at < 4^{o}C, and decontamination (washing) process was performed prior to consumption at home.

The degree of contamination of *E. coli* on lettuce at the production site was derived from the linear regression between *E. coli* concentration of the irrigating wastewater and *E. coli* concentration on composite Cos lettuce leaves presented in Chapter 4. The data regarding *E. coli* concentration in wastewater during a 6-week growing period was used to estimate the concentration of *E. coli* on the lettuce (Appendix B). This method was the indirect method to estimate the level of *E. coli* contamination on the produce when the concentration of *E. coli* in irrigating wastewater was known; however, from the finding of Chapter 4, this method was comparable to the direct method where *E. coli* was quantified directly from the lettuce's leaves. Moreover, the consumption data, or amount of lettuce consumed was also different from other studies. Shuval et al. (1997) and Mara et al. (2007) assumed that people ate 100 g lettuce per person every 2 days for 150 days a year, while Petterson et al. (2001) assumed 100 g per each consumption event. Some use the location specific consumption data relative to the studied site, for instance, Bastos et al. (2008) used the consumption data from Brazil as defined by the amount of lettuce consumed in low and high income families, which was 0.19 and 1.1 g lettuce per person per day. In the study reported here, the exposure models were probabilistic, so point estimates for consumption quantity should be avoided. The distribution of Cos lettuce leaves' weight (Appendix C) was chosen to represent the amount of lettuce consumed based on common practices for making sandwiches and wraps (one leaf per serving) (FSANZ, 2016).

To obtain a better understanding about the microbial risk from the consumption of wastewater irrigated lettuce, this study included the data of the survival of microorganisms along the production chain including both transportation and decontamination at home (the findings from Chapter 5 and Chapter 6). From factors analysis using the Yates method, the decontamination (washing)

process at home prior consumption was identified as one of the important factors which could influence the level of microbial contamination on Cos lettuce at the point of consumption. Pathogen removal at home is an important step to reduce the microbial load on fresh produce and could be set as a critical control point (CCP) of a "HACCP" approach. In other QMRA studies associated with wastewater irrigation in agriculture, a washing step in a household setting was not included. However, some studies assume a 2-3 $\log_{10}$ pathogen reduction between harvest and consumption, by either washing or postharvest die-off (Pavione et al., 2013, Shuval et al., 1997). Moreover, to estimate the infection risk of rotavirus and *Ascaris* in Ghana when wastewater was applied to irrigate lettuces, Seidu et al. (2008) used a 3 $\log_{10}$ reduction to represent the performance of the pathogen removal process when preparing salad at home, this number was based on the combined effect of washing with water and a disinfection process suggested in the WHO guidelines (WHO, 2006b). In this Chapter, the $\log_{10}$ reduction from washing step used in this model was obtained by determination of the most effective home washing method (Chapter 6), which was pre-soaking lettuce for 3 min prior soaking in 50 ppm NaDCC solution and spinning for 15s, which achieved 3.3 $\log_{10}$ reduction. Significantly, including this step into the risk scenarios (Scenario 9 – 16), showed that the estimated exposure to *E. coli* ($\log_{10}$ MPN) from wastewater irrigated lettuce at consumption were lower (mean = -3.69 to -1.76) than any scenario for which this washing step was omitted (Scenario 1 – 8; mean = -0.43 to 1.50). The result from this study unequivocally confirmed that an effective decontamination (washing) process was an important step to reduce the microbial risk from the consumption of wastewater irrigated lettuce; it is an absolutely, essential step which should be encouraged and employed to minimise the risk to consumers.

A withholding period, is also an important factors which influence the risk. Hamilton et al. (2006) also suggested the withholding period as a risk mitigation measure from the consumption of wastewater irrigated salad crops. The inactivation data of 2 day withholding period used here in the models were obtain from the field data reported in Chapter 3, where *E. coli* was not detect (< 10 MPN/ 100 g) in all lettuce samples after 2 days of the last irrigation during the sunny week. However, the impact of the withholding period on the survival

of microorganism on the crops also relies on the environmental condition at the production site, the microbial quality of irrigating wastewater, and the persistence of the pathogens (Hamilton et al., 2006).

The last factor which was an important driver of the microbial risk in the studied scenarios was the effect of rainfall on the *E. coli* concentration in irrigating wastewater. There is no clear evidence about the correlation of rainfall events and the microbial quality of wastewater in wastewater treatment ponds system. However, in recreational marine water quality, rainfall events caused the resuspension of sediments in to overlying water, resulting in increased numbers of faecal indicator organisms (Craig et al., 2004, Le Fevre and Lewis, 2003). In addition, several studies have shown the correlation between increased rainfall and the concentration of faecal indicator microorganisms in rivers and watersheds (Hunter et al., 1992, Shehane et al., 2005, Dorner et al., 2007, Schilling et al., 2009). In this study, the concentration of *E. coli* in wastewater used to irrigate crops increased after rainfall events occurred. Therefore, this may influence the microbial safety of vegetables, irrigated, grown and harvested during rainfall periods. Using other sources of irrigating water to watering crops during or after rainfall events could be an alternative option to prevent the contamination of the produce, especially for greenhouse-grown vegetables where watering needs to be performed regularly, even when there is precipitation.

One of the challenges to applying QMRA to estimate the risk from the consumption of wastewater irrigated crops is the complexity of the production pathways. Complex production chains also increase the complexity of the risk assessment due to the ability of microbial hazards to either multiply or be inactivated at each step throughout the production chain. Although the 2^4 factorial experimental design used in this study could demonstrate the whole picture of production chain, including all the combinations of related factors, there is a limitation of this exposure assessment according to the complexity of the pathway, for instance, any potential washing process at the production site, and the inactivation/ growth of microorganisms during storage at retail shops and at home were not included in this models. However, the QMRA approach performed in this study provided a better picture of the microbial risk

when all the combination factors from different pathways influencing the risk were included. The results from this study suggest that wastewater irrigation should be avoided, or directly used after rainfall events. Withholding periods after the last irrigation for harvest should be considered, although this is site-specific. Most importantly, the effective washing step should be performed prior the consumption. These three factors should be highlighted as key risk mitigation measures to reduce consumer exposure to microbial pathogens from wastewater irrigated lettuce.

CHAPTER 8

GENERAL DISCUSSION

8 GENERAL DISCUSSION

Wastewater irrigation in agriculture is increasingly applied globally due to the pressure related to the scarcity of freshwater, population growth, and a growing water demand for producing food. Worldwide, the agricultural sector is the largest user of water and wastewater, accounting for 70 % of the average water consumption (FAO, 2010). However, epidemiological studies demonstrate that there are adverse health impacts resulting from using wastewater irrigation in agriculture (WHO, 2006b, Blumenthal and Peasey, 2002, Shuval et al., 1986). One of the public health concerns is microbial risk from exposure to pathogens associated with the consumption of wastewater-irrigated crops. A number of studies have identified that using raw wastewater/ partially treated wastewater results in pathogen contamination of crops at harvest including *Clostridium* spp. (Rai and Tripathi, 2007), *Vibrio* spp.(Rai and Tripathi, 2007), *Salmonella* spp.(Rai and Tripathi, 2007, Ait Melloul et al., 2001, Bastos and Mara, 1995), protozoa (Armon et al., 2002, Amahmid et al., 1999), and helminths (Keraita et al., 2007, Nikaido et al., 2010, Ensink et al., 2007, Amahmid et al., 1999). However, microbial risk in fresh produce not only occurs at preharvest, but also via postharvest handling. To minimise microbial risk from a 'farm-to-fork' perspective, microbial safety of fresh produce at postharvest should be a focus as well as at preharvest. The objective of this research was to examine the microbial quality of wastewater-irrigated lettuce from the production site, transport/storage, and handling at home in order to obtain a better estimation of microbial risk from the consumption of wastewater irrigated salad crops.

A field based experiment examined the level of *E. coli* contamination on lettuces following spray and drip irrigation, using partially treated domestic wastewater. Spray irrigation resulted in a higher hazard than drip irrigation, since there was no *E. coli* detected in any of the drip-irrigated lettuce samples from the field study. However, the final microbial load on lettuce was also influenced by the microbial quality of the irrigating water, the withholding period (the time between the last irrigation and harvest) and climate conditions such as rainfall and sunlight. Resuspension of contaminated sediment, effected by rainfall into the wastewater treatment pond, into the wastewater used for

irrigation may cause a significant increase in exposure risk, in contrast, the disinfection process on wastewater-irrigated lettuce leave surfaces by UVA and UVB from sunlight at the production site may also reduce the risk. The interaction of these climatic factors requires further investigation.

Pathogen transfer or the degree of microbial contamination of the crops during irrigation is one of the key issues for QMRA applied to wastewater irrigation of fresh produce (Keuckelaere et al., 2015). Some studies estimated the microbial load, indirectly, from the volume of wastewater retained by the lettuce and the *E. coli* concentration of the wastewater in which they were submerged (Shuval et al., 1997, Petterson et al., 2001, Hamilton et al., 2006, Mara et al., 2007), others tried to quantify the number of microbial load directly from the crop surfaces (Bastos et al., 2008, Aiello et al., 2012, Forslund et al., 2012, Pavione et al., 2013). Notably, this study was the first to confirm that the direct enumeration method, was comparable with indirect method to estimate the level of contamination of pathogens following irrigation. Moreover, the indirect method (using a bucket submersion technique in a laboratory) was also comparable to the spray irrigation method in the field. This was concluded since there was no statistical difference between the *E. coli* concentration of wastewater irrigated Oak leaf lettuce following submersion in wastewater containing 1,299.7 *E. coli* MPN/ 100 mL in a laboratory based experiment compared with lettuce harvested, following six weeks of cultivation in the field experiment, which had last been irrigated with wastewater with a similar *E. coli* concentration (962.6 MPN/ 100 mL) in the irrigating wastewater. It was concluded that microbial contamination of lettuce resulting from the laboratory submersion technique was equivalent to that recorded from experimental sites on the day of spray irrigation. Hence, the submersion technique is an acceptable surrogate method to assess initial contamination from spray irrigation practiced on farms, and it can reasonably be used to conduct a conservative risk assessment associated with consuming wastewater irrigated lettuce. However, as was noted above, the microbial quality of crops at harvest was also dependent on the holding period after the last irrigation since *E. coli* was below the limit of detection on lettuces two days after irrigation ceased.

Further laboratory experiments using the indirect, bucket submersion method,

modified from Shuval et al., 1997, as a surrogate for field spray irrigation, demonstrated that different varieties of lettuce retained different volumes of wastewater. Oak leaf lettuce retained the highest volume of wastewater, followed by Cos and Iceberg lettuce. Moreover, using the same technique the *E. coli* concentration on lettuces was shown to be significantly ($p < 0.01$) correlated with the *E. coli* concentration of the wastewater in which they were submersed. High concentrations of *E. coli* in the irrigating wastewater resulted in a high level of contamination of the irrigated lettuce. Moreover, the significant linear regressions relating wastewater concentrations to the concentration of *E. coli* on the submersed lettuce could be used with caution for the *pre-assessment* of the likely contamination of produce following irrigation with wastewater for which *E. coli* count is known.

The spatial variability of *E. coli* contamination of lettuce was also evaluated and shown to be dependent on the concentration in the wastewater. There were no statistical differences in the *E. coli* concentration determined on outer, inner and composite samples of lettuce leaves following submersion in wastewaters with *E. coli* concentrations of 10^2 and 10^3 *E. coli* MPN/ 100 mL, however, the outer leaves had higher *E. coli* concentration compared to either inner or composite leaf samples of lettuce when submersed in wastewaters with an *E. coli* concentration of 10^4 MPN/ 100 mL wastewater. Therefore, discarding the outer leaves of lettuce before washing should be recommended as a risk mitigation measure associated with the consumption of wastewater irrigated lettuce.

Survival of microorganisms at postharvest was also investigated in this research. Wastewater-irrigated lettuces were stored at 4°C, representative of refrigerated-controlled transportation, and at 20°C representing open-air or non-temperature controlled transportation frequently used in developing countries. When stored at 4°C for 48h, there was no significant effect on the survival and growth of *E. coli* on wastewater submersed lettuces. In contrast, the results obtained at 20°C showed that the populations of *E. coli* on lettuce increased firstly, decreasing afterwards by 0.05 $\log_{10}$ *E. coli* MPN/ 100 g over 48 hours, and by 0.33 $\log_{10}$ *E. coli* MPN/ 100 g when stored for 96 hours. However, storage at a temperature of 20°C for more than 48 hours resulted in

deterioration of the visual quality of the lettuce, as they turned brown, and also developed a rotten smell. Data for the survival of microorganisms on wastewater-irrigated lettuce postharvest is very limited. The findings from this study were the first to report the behavior of *E. coli* on wastewater-irrigated lettuce leaves stored at 4°C and 20°C. These data were included in a subsequent QMRA conducted in this study.

Alternative decontamination processes suitable for application in the home setting were also evaluated. The effectiveness, for the removal *E. coli* from wastewater-submersed lettuce leaves, of ten different home washing methods was compared. The methods considered in this study varied from using only tap water to the application of sanitisers, including white rice vinegar, salad wash solution, and commercial chlorine tablets. The results demonstrated that ten different home washing methods could reduce the *E. coli* load on the wastewater irrigated lettuce by 1.3 – 3.3 $\log_{10}$. Pre-soaking lettuce for 3 min followed by soaking in 50 ppm NaDCC solution and spinning for 15s achieved the highest reduction of *E. coli* populations on the surface of lettuce leaves. However, in locations where chemical sanitisers are not available, or unaffordable, pre-soaking lettuce for 3 min and running under tap water for 20s, prior to the removal of excess water (spinning for 15s) was the most effective method, reducing the *E. coli* load on wastewater irrigated lettuce surfaces by 2.4 $\log_{10}$. Amoah et al. (2007), uniquely, considered the effectiveness of household washing methods to reduce the load of faecal coliform bacteria and helminth eggs on wastewater irrigated lettuce in Ghana. In contrast, the study reported here evaluated more potential decontamination methods and considered techniques which could reasonably be employed at the household level. Furthermore, many previous QMRA studies considering the public health risk associated with the consumption of wastewater irrigated vegetables did not include postharvest removal/ inactivation, some even concluding it was negligible, others justifying its exclusion as part of a worst-case scenario assessment (Barker-Reid et al., 2010, Bastos et al., 2008, Forslund et al., 2012). Notwithstanding, the $\log_{10}$ reduction values obtained in this study (1.3 – 3.3 $\log_{10}$ reduction) were consistent with the 3 $\log_{10}$ reduction proposed in the WHO (2006b) guidelines and considered representative of the performance of the decontamination process when preparing salad at home.

Seidu et al. (2008) included the (WHO, 2006b) value when estimating the infection risk of rotavirus and *Ascaris* in Ghana associated with consumption of wastewater irrigated lettuce. The findings of the decontamination study reported here were included in a probabilistic exposure assessment from the consumption of wastewater irrigated lettuce.

A probabilistic exposure model to estimate the microbial dose from the consumption of wastewater irrigated Cos lettuce was conducted. Sixteen different exposure scenarios, from a 2-level factorial experimental design, were simulated based on four factors, namely, the effect of rainfall on the concentration of *E. coli* in irrigating wastewater, lettuce withholding period post-irrigation (no withholding time and 2 day-withholding period), supply chain - transported to farmers market without temperature control and transported to retail shops with temperature control, and the effect of decontamination process before consumption at household level. The risk estimation was presented as the concentration of *E. coli* ($\log_{10}$ MPN/ one lettuce leaf consumed) at the point of consumption. Results from the probabilistic simulation showed that the lowest exposure was obtained from Scenario 15, where the microbial quality of irrigating wastewater was not adversely affected by rainfall, a 2 day-withholding period following irrigation and before harvest was applied, the lettuce was transported to retail shops with under temperature control at 4°C and lettuce leaves were decontaminated before consumption at home. This scenario could be considered as the best practice model for controlling the microbial risk along the production chain from fresh produce irrigated with wastewater.

The 2-level factorial experimental design used in this study allowed the risk assessor, using Yates analysis, to determine the effects and interaction effects of the 4 factors, which impacted the risk. The results demonstrated that 3 factors; rainfall, withholding period, and washing step at home were the significant factors influencing the microbial risk, especially the washing step at home which should be considered a Critical Control Point (CCP) within a HACCP approach for the wastewater irrigated vegetable production chain.

The exposure assessment of wastewater irrigated lettuce presented here uniquely provided data for, the relationship between direct and indirect

methods for assessing wastewater contamination of lettuce post irrigation; differential wastewater retention by three varieties of lettuce; the spatial variation of contamination between lettuce leaf locations; a significant relationship between the *E. coli* concentration of the wastewater and the subsequent concentration on the irrigated lettuce; the temperature dependent survival of *E. coli* on wastewater irrigated lettuce during storage and transport and the relative effectiveness of washing methods to decontaminate *E.coli* contaminated lettuce in the home environment. A single faecal contamination indicator, *E. coli* was used in this study. Further, similar research is required on the survival and the effectiveness of household decontamination processes for other human bacterial pathogens, especially *Campylobacter,* which is a reference pathogen for bacteria in wastewater reuse guidelines (WHO, 2006b), and *Salmonella* and *E. coli* O157:H7 which are the major common pathogens associated with disease outbreaks from the consumption of fresh produce (Olaimat and Holley, 2012).

The findings of this research have highlighted the key risk mitigation measures both at pre- and postharvest. At the production site, low microbial contamination wastewater should be used to water crops, but avoided when rainfall occurs or after the rainfall events. The crops should be harvested at least 2 days after the last irrigation. At postharvest, harvested lettuce should be transported and stored at ≤ 4°C. Essentially, before consumption at home, the outer leaves should be discarded before washing and ideally sanitising (e.g. 500 ppm NaDCC based chlorine). These measures should be implemented and maintained to minimise the microbial risk from the consumption of wastewater irrigated lettuce from a ‘farm to fork’ perspective.

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APPENDIX

APPENDIX A

Makkaew P., Miller M., Fallowfield H. J. & Cromar N. J. (2016) Microbial risk in wastewater irrigated lettuce: Comparing *Escherichia coli* contamination from an experimental site with a laboratory approach. *Water Science and Technology*, 74(3), 749-755.

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APPENDIX B

Table B-1 *E. coli* concentration in wastewater (MPN/ 100 mL) and amount of rainfall (mm) during the growing period

Week	Sample	*E. coli* concentration	Mean	S.D.	*Rainfall
1	1	435.2	462.5	47.3	0
	2	517.2			
	3	435.2			
2	1	770.1	984.7	216.6	5.0
	2	1203.3			
	3	980.8			
3	1	107.6	141.0	30.4	0
	2	148.3			
	3	167			
4	1	108.1	210.0	99.8	0
	2	307.6			
	3	214.2			
5	1	162.8	177.8	27.8	0
	2	209.8			
	3	160.7			
6	1	1046.2	962.6	72.4	9.2
	2	920.8			
	3	920.8			

*Source: www.http://www.bom.gov.au/sa/?ref=hdr

APPENDIX C

In order to understand Cos lettuce leaf weight an additional study was performed where individual leaves were removed and weighed from the outer most layer to the core. The summary of this study is described below.

Material and methods

1. Three baby Cos lettuce were purchased from a local supermarket in Canberra, ACT (02/0715).

2. The total lettuce weight and individual weight were measured using a digital kitchen scale (+/- 1 g). This measurement was conducted at FSANZ, ACT.

3. A fitted distribution of the leaf weight-leaf order data was determined.

Results

The summary of the weight of Cos lettuces is presented in Table C-1, and the detail of the individual weight is shown in Table C-2.

Table C-1 The summary of Cos lettuces' weight (n=3)

Sample	Total weight (g)	Leaf weigh (g)	Leaf numbers
1	204	193	23
2	213	203	23
3	250	242	26

Table C-2 Leaves' weight of Cos lettuce

Leaf order (From outer leaves to inner leaves)	Weight of Cos lettuce samples (g)		
	Sample 1	Sample 2	Sample 3
1	15	17	15
2	20	17	17
3	17	16	19
4	17	18	17
5	16	19	17
6	19	16	19
7	14	15	18
8	11	14	14
9	11	12	18
10	10	11	16
11	11	9	14
12	7	7	11
13	7	8	11
14	6	6	8
15	4	4	5
16	4	3	5
17	2	1	3
18	2	2	3
19	1	1	2
20	<1	1	1
21	<1	<1	<1
22	<1	<1	<1
23	<1	<1	<1
24	-	-	<1
25	-	-	<1
26	-	-	<1
Lettuce core	11	10	8
Total weight	**204**	**213**	**250**

A ‘hockey-stick’ nonlinear regression model was fitted to the leaf weight-leaf order data based on the method of Grossman *et al*. (2000).

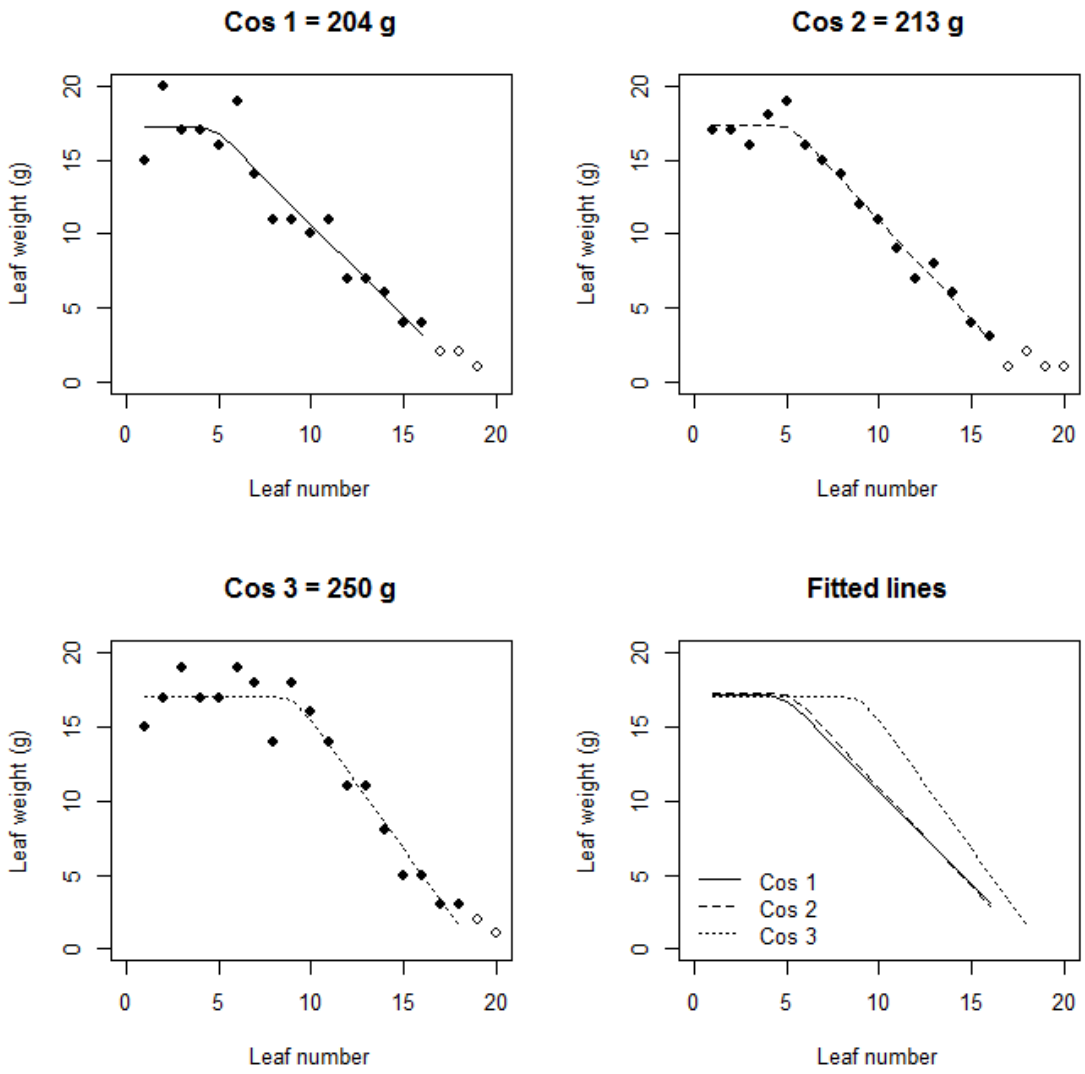

Figure C-1 A ‘hockey-stick’ nonlinear regression for the leaf weight-leaf order data (The open circles in the plots are results with weights of less than or equal to 2 g, they were dropped these due to the limited accuracy of the scales)

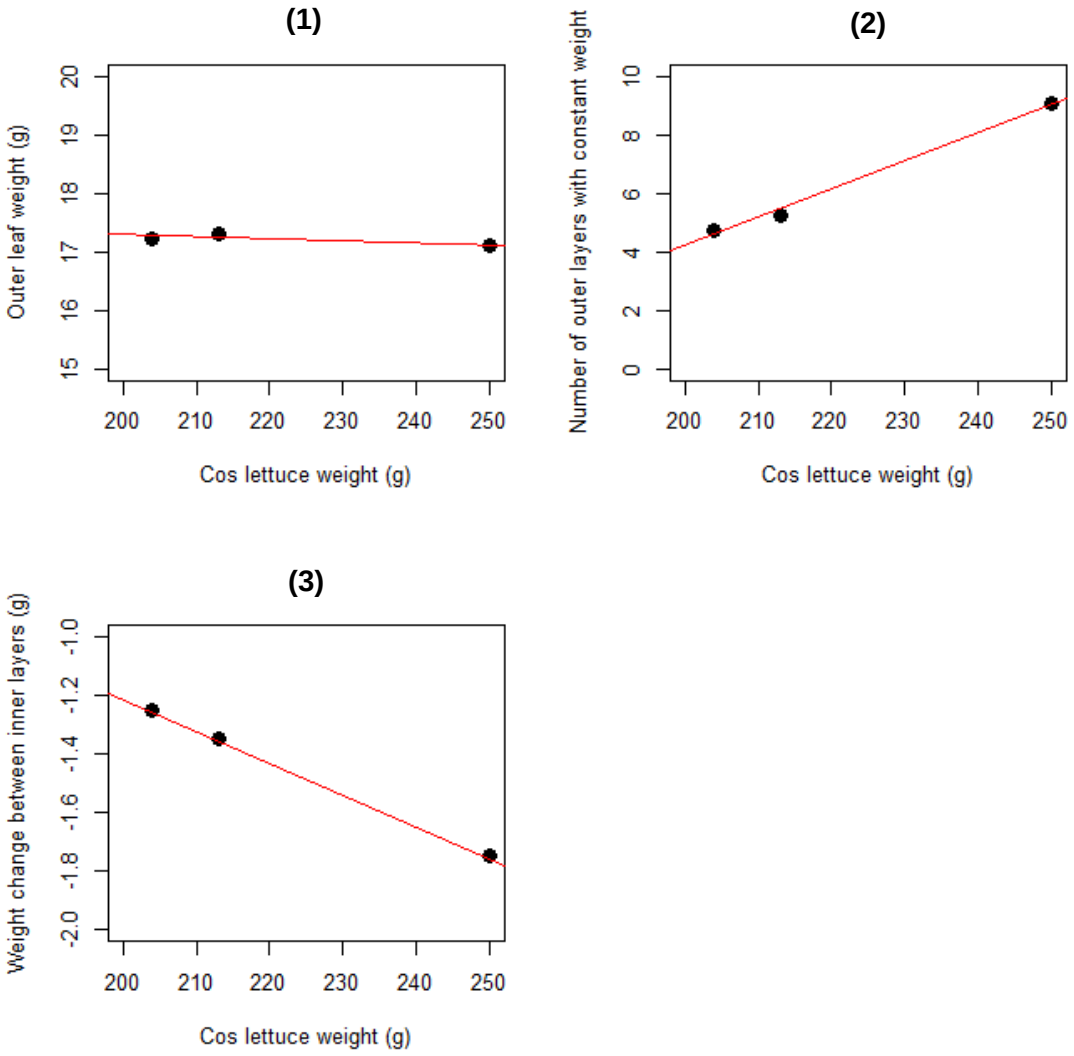

Figure C-2 The relationship between total Cos lettuce weight and (1) Outer leaf weight (2) Number of outer layers with constant weight (3) Weight change between inner layers

Conclusion

The major finding of this study was that the largest outer leaves were the same weight and independent of the total weight of the lettuce. The heaviest lettuce simply had more layers of the larger sized leaves. The average weight of the outer layer leaves was 17.2 grams. A Normal distribution was chosen with a mean of 17.2 grams and a standard deviation of 0.5 grams. The standard deviation was selected to reflect the variability seen in the weight of individual outer leaves. This distribution therefore represents a “worst-case” for a single leaf consumption scenario.

APPENDIX D

The computational tables in Yates spreadsheet for the Yates analysis from 10-iteration simulation.

Sample 1

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	1.1437917	2.8203893	2.9784144	5.348624	-16.600382	16	-1.0375239	average
2	X1	+	—	—	—	1.6765976	0.1580252	2.3702095	-21.949006	2.4961088	8	0.3120136	X1
3	X2	—	+	—	—	0.325616	2.4692241	-10.362994	0.5980084	-8.5155201	8	-1.06444	X2
4	X1 X2	+	+	—	—	-0.1675908	-0.0990146	-11.586012	1.8981005	-2.9006126	8	-0.3625766	X1 X2
5	X3	—	—	+	—	1.0552708	-4.7812706	0.0395992	-5.2306028	-1.8312228	8	-0.2289028	X3
6	X1 X3	+	—	+	—	1.4139533	-5.5817236	0.5584092	-3.2849173	-0.5009968	8	-0.0626246	X1 X3
7	X2 X3	—	+	+	—	-0.1493707	-4.5507739	1.4589536	-1.1849683	-1.589886	8	-0.1987357	X2 X3
8	X1 X2 X3	+	+	+	—	0.0503561	-7.0352382	0.4391468	-1.7156443	0.5299801	8	0.0662475	X1 X2 X3
9	X4	—	—	—	+	-2.9276947	0.5328059	-2.6623641	-0.6082049	-27.29763	8	-3.4122038	X4
10	X1 X4	+	—	—	+	-1.853576	-0.4932067	-2.5682387	-1.2230179	1.3000921	8	0.1625115	X1 X4
11	X2 X4	—	+	—	+	-2.9832793	0.3586824	-0.800453	0.51881	1.9456855	8	0.2432107	X2 X4
12	X1 X2 X4	+	+	—	+	-2.5984443	0.1997268	-2.4844643	-1.0198068	-0.530676	8	-0.0663345	X1 X2 X4
13	X3 X4	—	—	+	+	-2.6417638	1.0741187	-1.0260126	0.0941254	-0.614813	8	-0.0768516	X3 X4
14	X1 X3 X4	+	—	+	+	-1.9090101	0.3848349	-0.1589557	-1.6840113	-1.5386168	8	-0.1923271	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.3708157	0.7327537	-0.6892838	0.8670569	-1.7781367	8	-0.2222671	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-3.6644225	-0.2936069	-1.0263606	-0.3370768	-1.2041337	8	-0.1505167	X1 X2 X3 X4

Sample 2

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.9123521	2.6972166	3.1840552	4.0346742	-18.559565	16	-1.1599728	average
2	X1	+	—	—	—	1.7848645	0.4868386	0.850619	-22.594239	5.5771874	8	0.6971484	X1
3	X2	—	+	—	—	0.1796655	1.9252215	-10.547887	1.7094155	-9.7628208	8	-1.2203526	X2
4	X1 X2	+	+	—	—	0.3071731	-1.0746025	-12.046352	3.867772	0.359652	8	0.0449565	X1 X2
5	X3	—	—	+	—	0.659274	-4.8496726	1.00002	-5.2102019	-3.8319005	8	-0.4789876	X3
6	X1 X3	+	—	+	—	1.2659475	-5.6982148	0.7093955	-4.5526189	-0.1016652	8	-0.0127082	X1 X3
7	X2 X3	—	+	+	—	-0.5886623	-4.1711375	1.8394063	-1.2489561	-3.6449804	8	-0.4556225	X2 X3
8	X1 X2 X3	+	+	+	—	-0.4859402	-7.8752142	2.0283656	1.6086082	1.3720472	8	0.1715059	X1 X2 X3
9	X4	—	—	—	+	-2.8249861	0.8725123	-2.210378	-2.3334362	-26.628913	8	-3.3286142	X4
10	X1 X4	+	—	—	+	-2.0246865	0.1275076	-2.9998239	-1.4984643	2.1583565	8	0.2697946	X1 X4
11	X2 X4	—	+	—	+	-3.3686608	0.6066735	-0.8485423	-0.2906245	0.657583	8	0.0821979	X2 X4
12	X1 X2 X4	+	+	—	+	-2.329554	0.102722	-3.7040766	0.1889593	2.8575643	8	0.3571955	X1 X2 X4
13	X3 X4	—	—	+	+	-2.2502099	0.8002996	-0.7450047	-0.789446	0.8349719	8	0.1043715	X3 X4
14	X1 X3 X4	+	—	+	+	-1.9209276	1.0391067	-0.5039514	-2.8555344	0.4795838	8	0.059948	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-4.7871488	0.3292823	0.2388071	0.2410533	-2.0660884	8	-0.2582611	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-3.0880654	1.6990833	1.369801	1.1309939	0.8899406	8	0.1112426	X1 X2 X3 X4

Sample 3

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	1.549221	3.1533036	3.864172	3.8353505	-16.830098	16	-1.0518811	average
2	X1	+	—	—	—	1.6040826	0.7108684	-0.0288215	-20.665448	3.6751866	8	0.4593983	X1
3	X2	—	+	—	—	-0.0179182	2.0688358	-10.202036	2.0348121	-10.251816	8	-1.281477	X2
4	X1 X2	+	+	—	—	0.7287866	-2.0976573	-10.463413	1.6403744	1.0056402	8	-0.125705	X1 X2
5	X3	—	—	+	—	0.4507679	-4.032156	0.8015665	-6.6089282	-4.1543708	8	-0.5192963	X3
6	X1 X3	+	—	+	—	1.6180679	-6.1698796	1.2332457	-3.6428881	0.7990138	8	0.0998767	X1 X3
7	X2 X3	—	+	+	—	-1.0818015	-4.4791242	0.6365199	-0.4095111	-1.0914988	8	-0.1364373	X2 X3
8	X1 X2 X3	+	+	+	—	-1.0158558	-5.9842887	1.0038545	-0.5961291	-1.6346879	8	-0.204336	X1 X2 X3
9	X4	—	—	—	+	-2.2695378	0.0548617	-2.4424352	-3.8929935	-24.500799	8	-3.0625999	X4
10	X1 X4	+	—	—	+	-1.7626182	0.7467048	-4.166493	-0.2613773	-0.3944377	8	-0.0493047	X1 X4
11	X2 X4	—	+	—	+	-3.1497399	1.1672999	-2.1377236	0.4316792	2.9660401	8	0.370755	X2 X4
12	X1 X2 X4	+	+	—	+	-3.0201396	0.0659457	-1.5051645	0.3673346	-0.186618	8	-0.0233272	X1 X2 X4
13	X3 X4	—	—	+	+	-2.5452282	0.5069196	0.6918431	-1.7240578	3.6316162	8	0.453952	X3 X4
14	X1 X3 X4	+	—	+	+	-1.933896	0.1296003	-1.1013542	0.632559	-0.0643446	8	-0.0080431	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.1884055	0.6113322	-0.3773193	-1.7931973	2.3566168	8	0.2945771	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-2.7958832	0.3925224	-0.2188098	0.1585095	1.9517068	8	0.2439634	X1 X2 X3 X4

Sample 4

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	1.2285624	2.5508418	1.9804471	3.833401	-16.712227	16	-1.0445142	average
2	X1	+	—	—	—	-1.3222795	-0.5703947	1.8529539	-20.545628	4.8896577	8	0.6112072	X1
3	X2	—	+	—	—	-0.7250154	1.906454	-9.7913043	2.4201646	-8.4270217	8	-1.0533777	X2
4	X1 X2	+	+	—	—	0.1546207	-0.0535001	-10.754324	2.4694932	0.5133829	8	0.0641729	X1 X2
5	X3	—	—	+	—	0.7467187	-4.1727307	0.9733531	-5.0811906	-1.090513	8	-0.1363141	X3
6	X1 X3	+	—	+	—	-1.1597353	-5.6185736	1.4468114	-3.3458311	0.3686855	8	0.0460857	X1 X3
7	X2 X3	—	+	+	—	-0.5436475	-4.4271679	1.2871329	1.4066972	0.7071371	8	0.0883921	X2 X3
8	X1 X2 X3	+	+	+	—	0.4901474	-6.3271561	1.1823602	-0.8933143	0.0666771	8	0.0083346	X1 X2 X3
9	X4	—	—	—	+	-2.5487901	-0.0937171	-3.1212365	-0.1274933	-24.379029	8	-3.0473787	X4
10	X1 X4	+	—	—	+	-1.6239406	0.8796361	-1.9599541	-0.9630198	0.0493286	8	0.0061661	X1 X4
11	X2 X4	—	+	—	+	-2.9904285	0.4130166	-1.4458429	0.4734583	1.7353595	8	0.2169199	X2 X4
12	X1 X2 X4	+	+	—	+	-2.6281451	-1.0337948	-1.8999882	-0.1047727	-2.3000115	8	-0.2875014	X1 X2 X4
13	X3 X4	—	—	+	+	-2.5918611	-0.9248495	0.785919	-1.1612824	-0.8355265	8	-0.1044408	X3 X4
14	X1 X3 X4	+	—	+	+	-1.8353069	-0.3622834	0.6207782	-0.4541453	-0.578231	8	-0.0722789	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.3764811	-0.7565542	-0.5625661	-0.1651408	-1.6154277	8	-0.2019285	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-2.9506751	-0.425806	-0.3307482	-0.2318179	-0.3969586	8	-0.0496198	X1 X2 X3 X4

Sample 5

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.8558528	2.4458918	2.8351299	5.2976149	-15.94604	16	-0.9966275	average
2	X1	+	—	—	—	1.590039	0.3892381	2.462485	-21.243654	2.0947166	8	0.2618396	X1
3	X2	—	+	—	—	-0.0847611	2.4555383	-10.345656	2.4424405	-8.7690333	8	-1.0961292	X2
4	X1 X2	+	+	—	—	0.4739992	0.0069467	-10.897999	-0.3477239	-2.2668156	8	-0.283352	X1 X2
5	X3	—	—	+	—	0.8949545	-3.8292074	1.2929465	-4.5052452	-0.9249878	8	-0.1156235	X3
6	X1 X3	+	—	+	—	1.5605838	-6.5164484	1.149494	-4.2637881	1.0427562	8	0.1303445	X1 X3
7	X2 X3	—	+	+	—	-0.238459	-4.6607258	-0.7669663	-0.3571903	0.7187561	8	0.0898445	X2 X3
8	X1 X2 X3	+	+	+	—	0.2454057	-6.2372729	0.4192424	-1.9096253	0.0084563	8	0.001057	X1 X2 X3
9	X4	—	—	—	+	-1.9634147	0.7341861	-2.0566537	-0.3726449	-26.541269	8	-3.3176587	X4
10	X1 X4	+	—	—	+	-1.8657927	0.5587604	-2.4485915	-0.5523429	-2.7901644	8	-0.3487706	X1 X4
11	X2 X4	—	+	—	+	-2.8259301	0.6656293	-2.687241	-0.1434525	0.2414571	8	0.0301821	X2 X4
12	X1 X2 X4	+	+	—	+	-3.6905183	0.4838647	-1.5765471	-1.1862087	-1.5524349	8	-0.1940544	X1 X2 X4
13	X3 X4	—	—	+	+	-2.6720273	0.097622	-0.1754257	-0.3919378	-0.179698	8	-0.0224622	X3 X4
14	X1 X3 X4	+	—	+	+	-1.9886985	-0.8645883	-0.1817646	1.110694	1.3296612	8	0.1662076	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-2.9865933	0.6833287	-0.9622102	-0.0063389	1.5026318	8	0.187829	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-3.2506796	-0.2640863	-0.947415	0.0147952	0.0211341	8	0.0026418	X1 X2 X3 X4

Sample 6

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.3651538	2.0030539	1.9401993	3.3183253	-18.116096	16	-1.132256	average
2	X1	+	—	—	—	-1.6379001	-0.0628546	1.378126	-21.434422	6.2457077	8	0.7807135	X1
3	X2	—	+	—	—	-0.9443303	2.1287134	-10.646689	-4.0821587	-9.6184069	8	-1.2023009	X2
4	X1 X2	+	+	—	—	0.8814757	-0.7505874	-10.787733	2.163549	-0.2800964	8	-0.0350121	X1 X2
5	X3	—	—	+	—	0.7256585	-3.8624626	3.0985524	-4.9452093	-0.703117	8	-0.0878896	X3
6	X1 X3	+	—	+	—	1.4030549	-6.7842263	0.9836063	-4.6731976	-1.0504286	8	-0.1313036	X1 X3
7	X2 X3	—	+	+	—	-0.5283986	-4.5181494	0.5495157	0.181873	0.3569374	8	0.0446172	X2 X3
8	X1 X2 X3	+	+	+	—	-0.2221888	-6.2695833	1.6140333	-0.4619694	-0.4252359	8	-0.0531545	X1 X2 X3
9	X4	—	—	—	+	-2.1887328	1.2727463	-2.0659085	-0.5620734	-24.752747	8	-3.0940934	X4
10	X1 X4	+	—	—	+	-1.6737299	1.825806	-2.8793008	-0.1410436	-1.9186097	8	-0.2398262	X1 X4
11	X2 X4	—	+	—	+	-3.4093696	0.6773965	-2.9217637	-2.1149461	0.2720117	8	0.0340015	X2 X4
12	X1 X2 X4	+	+	—	+	-3.3748567	0.3062098	-1.7514339	-1.0645175	-0.6438424	8	-0.0804803	X1 X2 X4
13	X3 X4	—	—	+	+	-2.6579529	0.5150029	0.5530597	-0.8133924	0.4210297	8	0.0526287	X3 X4
14	X1 X3 X4	+	—	+	+	-1.8601965	0.0345129	-0.3711867	1.1703298	3.1794636	8	0.397433	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.5429301	0.7977564	-0.48049	-0.9242464	1.9837221	8	0.2479653	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-2.7266532	0.8162769	0.0185205	0.4990105	1.4232569	8	0.1779071	X1 X2 X3 X4

Sample 7

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.4947946	1.9953781	2.0369317	4.564469	-18.886683	16	-1.1804177	average
2	X1	+	—	—	—	1.5005834	0.0415537	2.5275372	-23.451152	5.7189905	8	0.7148738	X1
3	X2	—	+	—	—	-0.5422518	1.9540465	-11.179661	3.3004761	-9.3211712	8	-1.1651464	X2
4	X1 X2	+	+	—	—	0.5838055	0.5734907	-12.27149	2.4185143	0.3101633	8	0.0387704	X1 X2
5	X3	—	—	+	—	0.6905233	-3.9933212	2.131846	-3.3343802	-0.6012231	8	-0.0751529	X3
6	X1 X3	+	—	+	—	1.2635232	-7.1863403	1.1686301	-5.986791	-1.3099404	8	-0.1637426	X1 X3
7	X2 X3	—	+	+	—	-0.0110698	-4.738859	1.3826194	0.1428989	0.9725156	8	0.1215644	X2 X3
8	X1 X2 X3	+	+	+	—	0.5845605	-7.532631	1.0358949	0.1672644	-1.0538872	8	-0.1317359	X1 X2 X3
9	X4	—	—	—	+	-2.2018763	1.0057888	-1.9538244	0.4906055	-28.01562	8	-3.5019526	X4
10	X1 X4	+	—	—	+	-1.791445	1.1260572	-1.3805558	-1.0918285	-0.8819618	8	-0.1102452	X1 X4
11	X2 X4	—	+	—	+	-4.0792642	0.5729998	-3.193019	-0.9632159	-2.6524108	8	-0.3315513	X2 X4
12	X1 X2 X4	+	+	—	+	-3.1070761	0.5956303	-2.793772	-0.3467245	0.0243655	8	0.0030457	X1 X2 X4
13	X3 X4	—	—	+	+	-2.7270263	0.4104313	0.1202685	0.5732685	-1.582434	8	-0.1978042	X3 X4
14	X1 X3 X4	+	—	+	+	-2.0118327	0.9721881	0.0226304	0.3992471	0.6164914	8	0.0770614	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.9266661	0.7151936	0.5617568	-0.097638	-0.1740215	8	-0.0217527	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-3.6059649	0.3207013	-0.3944924	-0.9562492	-0.8586111	8	-0.1073264	X1 X2 X3 X4

Sample 8

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.6584684	2.204957	2.416075	4.1883071	-16.779071	16	-1.048692	average
2	X1	+	—	—	—	1.5464886	0.2111181	1.7722321	-20.967378	5.8993625	8	0.7374203	X1
3	X2	—	+	—	—	-0.1390592	1.7143767	-10.133834	3.4382373	-7.7167745	8	-0.9645968	X2
4	X1 X2	+	+	—	—	0.3501773	0.0578554	-10.833544	2.4611252	0.8586496	8	0.1073312	X1 X2
5	X3	—	—	+	—	0.4345438	-3.8347868	1.3772567	-3.6503601	-1.3435533	8	-0.1679442	X3
6	X1 X3	+	—	+	—	1.2798328	-6.2990471	2.0609807	-4.0664144	-0.6020178	8	-0.0752522	X1 X3
7	X2 X3	—	+	+	—	-0.5789181	-4.6156952	1.8734335	-0.0283809	1.1994239	8	0.149928	X2 X3
8	X1 X2 X3	+	+	+	—	0.6367736	-6.2178492	0.5876917	0.8870305	-0.5412712	8	-0.0676589	X1 X2 X3
9	X4	—	—	—	+	-2.1110658	0.8880202	-1.9938389	-0.6438429	-25.155685	8	-3.1444607	X4
10	X1 X4	+	—	—	+	-1.7237211	0.4892365	-1.6565212	-0.6997104	-0.9771121	8	-0.122139	X1 X4
11	X2 X4	—	+	—	+	-3.892568	0.845289	-2.4642603	0.683724	-0.4160543	8	-0.0520068	X2 X4
12	X1 X2 X4	+	+	—	+	-2.4064792	1.2156917	-1.6021541	-1.2857418	0.9154114	8	0.1144264	X1 X2 X4
13	X3 X4	—	—	+	+	-2.5076989	0.3873447	-0.3987836	0.3373176	-0.0558674	8	-0.0069834	X3 X4
14	X1 X3 X4	+	—	+	+	-2.1079963	1.4860888	0.3704027	0.8621063	-1.9694658	8	-0.2461832	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.2029191	0.3997026	1.098744	0.7691864	0.5247886	8	0.0655986	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-3.0149301	0.1879891	-0.2117136	-1.3104576	-2.079644	8	-0.2599555	X1 X2 X3 X4

Sample 9

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	1.1823766	2.6062388	3.1385544	5.8827462	-16.151349	16	-1.0094593	average
2	X1	+	—	—	—	1.4238621	0.5323157	2.7441918	-22.034096	4.1134312	8	0.5141789	X1
3	X2	—	+	—	—	-0.3498834	2.411149	-11.173512	-1.8274382	-6.9072312	8	-0.8634039	X2
4	X1 X2	+	+	—	—	0.882199	0.3330428	-10.860584	2.2859931	0.2595081	8	0.0324385	X1 X2
5	X3	—	—	+	—	1.2349459	-4.7533816	1.4735679	-4.1520294	-0.0814343	8	-0.0101793	X3
6	X1 X3	+	—	+	—	1.1762031	-6.4201305	0.3538703	-2.7552019	-1.4790645	8	-0.1848831	X1 X3
7	X2 X3	—	+	+	—	-0.0397852	-4.8860654	1.32268	1.4619529	0.5741128	8	0.0717641	X2 X3
8	X1 X2 X3	+	+	+	—	0.3728279	-5.9745183	0.9633131	-1.2024447	-0.3900274	8	-0.0487534	X1 X2 X3
9	X4	—	—	—	+	-2.8738181	0.2414855	-2.0739231	-0.3943626	-27.916842	8	-3.4896052	X4
10	X1 X4	+	—	—	+	-1.8795635	1.2320824	-2.0781063	0.3129283	0.4585549	8	0.0573194	X1 X4
11	X2 X4	—	+	—	+	-3.3742779	-0.0587428	-1.6667489	-1.1196976	1.3968275	8	0.1746034	X2 X4
12	X1 X2 X4	+	+	—	+	-3.0458525	0.4126131	-1.088453	-0.3593669	-2.6643976	8	-0.3330497	X1 X2 X4
13	X3 X4	—	—	+	+	-2.8180149	0.9942546	0.9905969	-0.0041832	0.7072909	8	0.0884114	X3 X4
14	X1 X3 X4	+	—	+	+	-2.0680505	0.3284254	0.4713559	0.5782959	0.7603308	8	0.0950413	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.0939335	0.7499643	-0.6658291	-0.519241	0.5824791	8	0.0728099	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-2.8805848	0.2133488	-0.5366156	0.1292136	0.6484545	8	0.0810568	X1 X2 X3 X4

Sample 10

Run	Code	X1	X2	X3	X4	Value	(1)	(2)	(3)	(4)	divisor	estimate	effect
1	(1)	—	—	—	—	0.9663504	2.3739141	2.7897861	4.1789751	-17.612602	16	-1.1007876	average
2	X1	+	—	—	—	1.4075637	0.415872	1.389189	-21.791577	5.5627227	8	0.6953403	X1
3	X2	—	+	—	—	0.0359335	2.2141722	-10.724087	1.668008	-8.8798808	8	-1.1099851	X2
4	X1 X2	+	+	—	—	0.3799385	-0.8249832	-11.06749	3.8947147	0.0428662	8	0.0053583	X1 X2
5	X3	—	—	+	—	0.8768368	-4.5132425	0.7852183	-4.9971974	-1.7439996	8	-0.218	X3
6	X1 X3	+	—	+	—	1.3373354	-6.2108448	0.8827897	-3.8826833	-0.6679392	8	-0.0834924	X1 X3
7	X2 X3	—	+	+	—	-0.6236372	-4.4412044	2.3301126	-0.1354157	-1.568592	8	-0.196074	X2 X3
8	X1 X2 X3	+	+	+	—	-0.201346	-6.6262854	1.564602	0.178282	0.3220088	8	0.0402511	X1 X2 X3
9	X4	—	—	—	+	-2.8497401	0.4412134	-1.9580421	-1.4005971	-25.970552	8	-3.246319	X4
10	X1 X4	+	—	—	+	-1.6635024	0.344005	-3.0391554	-0.3434025	2.2267066	8	0.2783383	X1 X4
11	X2 X4	—	+	—	+	-3.6773598	0.4604985	-1.6976023	0.0975714	1.1145141	8	0.1393143	X2 X4
12	X1 X2 X4	+	+	—	+	-2.5334849	0.4222912	-2.185081	-0.7655106	0.3136977	8	0.0392122	X1 X2 X4
13	X3 X4	—	—	+	+	-2.5565915	1.1862378	-0.0972084	-1.0811133	1.0571946	8	0.1321493	X3 X4
14	X1 X3 X4	+	—	+	+	-1.8846129	1.1438749	-0.0382073	-0.4874787	-0.863082	8	-0.1078853	X1 X3 X4
15	X2 X3 X4	—	+	+	+	-3.7594544	0.6719786	-0.0423629	0.059001	0.5936346	8	0.0742043	X2 X3 X4
16	X1 X2 X3 X4	+	+	+	+	-2.866831	0.8926234	0.2206448	0.2630077	0.2040067	8	0.0255008	X1 X2 X3 X4

Combined table for creating a plot graph Fig. 7.19

Code	Estimate									
(1)	-1.0375239	-1.1599728	-1.0518811	-1.0445142	-0.9966275	-1.132256	-1.1804177	-1.048692	-1.0094593	-1.1007876
X1	0.3120136	0.6971484	0.4593983	0.6112072	0.2618396	0.7807135	0.7148738	0.7374203	0.5141789	0.6953403
X2	-1.06444	-1.2203526	-1.281477	-1.0533777	-1.0961292	-1.2023009	-1.1651464	-0.9645968	-0.8634039	-1.1099851
X1 X2	-0.3625766	0.0449565	-0.125705	0.0641729	-0.283352	-0.0350121	0.0387704	0.1073312	0.0324385	0.0053583
X3	-0.2289028	-0.4789876	-0.5192963	-0.1363141	-0.1156235	-0.0878896	-0.0751529	-0.1679442	-0.0101793	-0.218
X1 X3	-0.0626246	-0.0127082	0.0998767	0.0460857	0.1303445	-0.1313036	-0.1637426	-0.0752522	-0.1848831	-0.0834924
X2 X3	-0.1987357	-0.4556225	-0.1364373	0.0883921	0.0898445	0.0446172	0.1215644	0.149928	0.0717641	-0.196074
X1 X2 X3	0.0662475	0.1715059	-0.204336	0.0083346	0.001057	-0.0531545	-0.1317359	-0.0676589	-0.0487534	0.0402511
X4	-3.4122038	-3.3286142	-3.0625999	-3.0473787	-3.3176587	-3.0940934	-3.5019526	-3.1444607	-3.4896052	-3.246319
X1 X4	0.1625115	0.2697946	-0.0493047	0.0061661	-0.3487706	-0.2398262	-0.1102452	-0.122139	0.0573194	0.2783383
X2 X4	0.2432107	0.0821979	0.370755	0.2169199	0.0301821	0.0340015	-0.3315513	-0.0520068	0.1746034	0.1393143
X1 X2 X4	-0.0663345	0.3571955	-0.0233272	-0.2875014	-0.1940544	-0.0804803	0.0030457	0.1144264	-0.3330497	0.0392122
X3 X4	-0.0768516	0.1043715	0.453952	-0.1044408	-0.0224622	0.0526287	-0.1978042	-0.0069834	0.0884114	0.1321493
X1 X3 X4	-0.1923271	0.059948	-0.0080431	-0.0722789	0.1662076	0.397433	0.0770614	-0.2461832	0.0950413	-0.1078853
X2 X3 X4	-0.2222671	-0.2582611	0.2945771	-0.2019285	0.187829	0.2479653	-0.0217527	0.0655986	0.0728099	0.0742043
X1 X2 X3 X4	-0.1505167	0.1112426	0.2439634	0.0496198	0.0026418	0.1779071	-0.1073264	-0.2599555	0.0810568	0.0255008