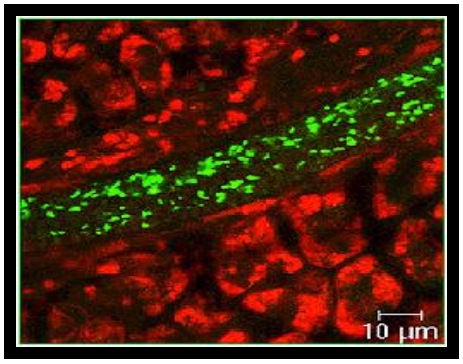
ENHANCED RECOVERY AND MOLECULAR TECHNIQUES FOR DETERMINING BACTERIAL COLONISTS AND VIABLE PATHOGENS ON THE COMPLEX PHYLLOPLANE MATRIX

- BY -

ARINDER KAUR SIHOTA

BSC PHARMACOLOGY (HONS)



A confocal scanning laser micrograph of fluorescent green *E. coli* gaining access to the xylem of cut leaf lettuce. (Magnification approx. 1,000x.)

A THESIS SUBMITTED FOR THE DEGREE OF

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The rise in demand for fresh fruits and vegetables has seen an increase in the risk of gastrointestinal disease, by pathogens such as *E. coli* O157:H7 and *Salmonella enterica,* since such produce is eaten uncooked. Although most produce is washed in chlorinated water, this processing may not be sufficient and can result in the formation of hazardous compounds.

Classical cell culture techniques as well as novel episcopic differential contrast and epifluorescence microscopy (EDIC/EF) combined with the BacLight^{TM} kit (to distinguish live and dead bacteria), the DAPI assay (to distinguish bacterial colonists and inorganic debris) and GFP-S*almonella* strains were combined for the first time to study the complex leaf surface (phylloplane). EDIC/EF microscopy was shown to be advantageous compared to other methodologies; as well as being able to visualize GFP-labelled *Salmonella* inoculated onto the phylloplane it was possible to observe the naturally residing microflora on this difficult matrix. The viable pathogens and microflora were shown to colonize by four strategies: they form clusters on the phylloplane; or single cells integrate into pre-existing aggregates of biofilm microcolonies; they become entrapped in niches such as stomata; or they actively swim into the stomata and become subsurface (confirmed using laser scanning confocal microscopy, LSCM). The clusters were sometimes surrounded by slime, suggesting the formation of biofilm on the phylloplane. The effects of treatments to the phylloplane were not directly comparable, due to large biological variations in each field of view; smaller treated sample areas should allow for qualitative and quantitative comparisons.

The Stomacher is at present used worldwide for the mechanical release of microorganisms from various matrices; here it was compared to the Pulsifier, which was shown to be more efficient in terms of cell recovery and causing less damage to the watercress phylloplane. Surface attachment was investigated by use of the Pulsifier release principle and refinements in its protocol were made. Pulsifier recovery techniques showed the inefficiencies of potential disinfectants in killing attached microorganisms, since they were not susceptible to attack until released into aqueous suspension. It is these 'protected' cells that then subsequently go on to produce foodborne illnesses. Further study showed the molecular signalling molecule nitric oxide (NO), to be an important physiological release agent, for enhanced recovery of coliforms, but not *Salmonella*, from the phylloplane.

Chemical methods of decontamination such as the use of ozone were shown to be efficient at reducing the numbers of viable cells, particularly when combined with pulsification mechanical release of cells into aqueous suspension, resulting in between 1- and 2-log reductions. However, this procedure is not ideal, due to chemical damage to the phylloplane and problems in maintaining constant ozone concentrations, both in the laboratory and at the factory. It was shown that chlorine levels could be reduced to 20 ppm compared to the industry standard of 90-120 ppm, this producing similar log reductions of between 1- and 2-log. The Pulsifier and NO were shown in combination to provide effective mechanical and physiological detachment strategies, releasing almost 4-log cells. It was found that 20 or 500 nM of NO, produced a 3-log dispersion of bacterial cells, including biofilm aggregates off the surface of watercress leaves. These studies demonstrate the importance of microbial physiology in the attachment of microorganisms on fresh produce phylloplanes and suggest that disinfection procedures are unnecessary for sanitation.

Publications and Presentations.

Published Papers:

1. Determination of calmodulin binding to metabotropic glutamate receptors with distinct protein-interaction methods. Biochemical Society Transactions. 2004 Nov; 32 (Pt 5):868-70. Lidwell, Dillon, Sihota, O' Conner, Pilkington.

2. Comparative study of surgical instruments from sterile service departments for the presence of residual Gram-negative endotoxin and proteinaceous deposits. Journal of Clinical Microbiology. 2006 Aug 23. Lipscomb IP, Sihota AK, Keevil CW.

3. Rapid method for the sensitive detection of protein contamination on surgical instruments. Journal of Hospital Infection. 2006 Feb;62(2):141-8. Lipscomb IP, Sihota AK, Botham M, Harris KL, Keevil CW.

4. Diathermy forceps and pencils: reservoirs for protein and prion contamination? Journal of Hospital Infection. 2006 Oct;64(2):193-4. Lipscomb IP, Sihota AK, Keevil CW.

5. QTLs for shelf life in lettuce co-locate with those for leaf biophysical properties but not with those for leaf developmental traits. Journal of Experimental Botany. 2007 March; 58 (6): 1433-49. Zhang FZ, Wagstaff C, Rae AM, Sihota AK, Keevil CW, Rothwell SD, Clarkson GJ, Michelmore RW, Truco MJ, Dixon MS, Taylor G.

6. Comparison between visible analysis and microscopy assessment of surgical instrument cleanliness from sterile service departments. Journal of Hospital Infection. 2008 January; 68 (1): 52-8. Lipscomb I, Sihota AK, Keevil CW.

Papers in Process to be Submitted:

1. Bacteria are highly efficient at colonizing furrows between epidermal cells in lettuce, watercress and spinach leaves, and thereby resist dislocation from the leaf surface during the washing process. Use of the Pulsifier to show this and dislocate cells in order for attack by disinfectants. Sihota AK, Keevil CW.

2. Efficacy of ozone, hypochlorite and pulsification disinfection strategies for *Salmonella, E. coli* and coliforms on salad leaves, determined using industry approved and novel detection methods.

3. SNP paper.

Powerpoint and Poster Presentations:

1. Enhanced recovery and molecular techniques for detecting viable pathogens on complex matrices. May 2004. Biochemistry research group seminar programme.

2. Development of a chlorine-free washing procedure 1. 28th February 2005. Vitacress Salads. Powerpoint presentation.

3. Development of a chlorine-free washing procedure 2. Vitacress Salads. Powerpoint presentation.

4. Safe decontamination strategies for coliforms and *E. coli* on salad leaves. Postgraduate symposium. June 2005. Poster presentation.

5. Salad lettuce decontamination. Enhanced recovery and molecular techniques for detecting viable pathogens on complex matrices. Vice chancellor, University of Southampton. Powerpoint presentation.

6. Efficiency of chlorine versus ozone decontamination of *E. coli* and Coliforms on baby leaf salad. Marks and Spencers presentation.

7. Survival of *Salmonella, E. coli* and coliforms on salad leaf phyllospheres and resistance to disinfection. Abstract for postgraduate symposium presentation. June 2006.

8. Efficacy of ozone, hypochlorite and pulsification disinfection strategies for *Salmonella, E. coli* and coliforms on salad leaves, determined using industry approved and novel detection methods. A. K. Gill, C. W. Keevil. American Society of Microbiology (ASM) 106th Annual General Meeting. Orange County Convention Center, Orlando, FL. Poster presentation.

9. Development of a chlorine-free washing procedure. Vitacress Salads, March 2007. Poster presentation.

10. The effect of Sodium Nitroprusside (SNP) on detachment of microbes off the watercress leaf matrix and susceptibility of these to disinfection. Separate presentations for Microgen Bioproducts, Vitacress Salads, Australia Research Group.

11. Development of a chlorine-free washing procedure and the effect of sodium nitroprusside (SNP) on detachment of microbes off the watercress leaf matrix and susceptibility of these to disinfection. Vitacress Salads, Waitrose and Plant Sciences Group.

Submission of Written Reports and Viva Examinations to date.

Enhanced Recovery and Molecular Techniques for Detecting Viable Pathogens on Complex Matrices:

1. 6 Month Stage, written report, 31st March 2004. Pass.

2. 10 Month Stage, written report and viva examination, 18th July 2005. Pass.

3. Transfer from MPhil to PhD Stage, written report and viva examination, 18th July 2006. Pass.

Awards

1. Prestigious ASM travel grant award for best poster abstract. Efficacy of ozone, hypochlorite and pulsification disinfection strategies for *Salmonella, E. coli* and coliforms on salad leaves determined using industry approved and novel detection methods. A. K. Gill, C. W. Keevil. American Society of Microbiology (ASM) 106th Annual General Meeting. Orange County Convention Center, Orlando, FL. Poster presentation. April 2006.

Further Projects that Initiate from this Study.

1. Development of molecular techniques for *in-situ* detection and tracking of pathogens on salad leaves. 2006 collaboration with Vitacress Salads and BBSRC.

2. KTP Project. Refinement of the mechanical mechanisms of the Pulsifier to potentially reduce the volume of water required for washing each kilogram of leaf. 2007 and enhance the recovery of microorganisms further. Collaboration with the School of Engineering Sciences, Research Institute for Industry and Vitacress Salads.

3. NO Project. Further investigation and refinement of the effect of SNP or other Nitric Oxide donors on aggregates of micro-organisms or biofilms.

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DECLARATION OF AUTHORSHIP

I, Arinder Kaur Sihota declare that the thesis entitled:

Enhanced Recovery and Molecular Techniques for Determining Bacterial Colonists and Viable Pathogens on the Complex Phylloplane Matrix

and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has been submitted for a degree or any other qualification at this University or any other institute, his has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

Signed: ……

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LIST OF ABBREVIATIONS

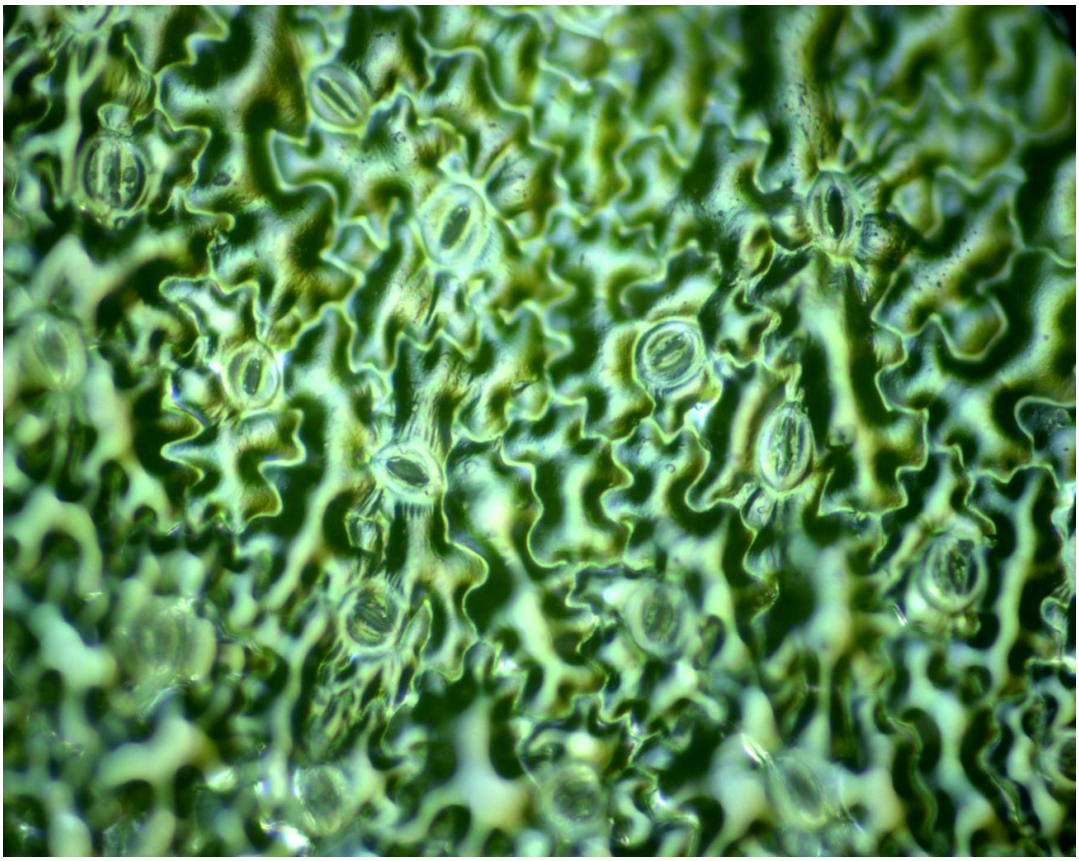
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CFU	Colony forming unit
DAPI	Diamidino-2-phenylindole
DNA	Deoxyribose nucleic acid
DPD	N,N-diethyl-p-phenylene-diamine
DPSS	Diode pumped solid state
E. coli	*Escherichia coli*
EDIC	Episcopic differential interference
EHEC	Enterohaemorrhagic E. coli
EF	Epifluorescence
EPS	Extracellular polysaccharide
FDA	Food and drug administration
GI	Gastrointestinal
GFP	Green fluorescent protein
HNOB	Haem nitric oxide binding domains
HOCl	Hypochlorous acid
HR	Hypersensitive response
LCD	Liquid crystal display
LSCM	Laser scanning confocal microscopy
MID-60	Microgen identification system software

MRSA	*Methicillin resistant Staphylococcus aureus*
NA	Nutrient agar
NO	Nitric oxide
NO+	Nitrosonium cation
NO-	Nitroxyl radical
NR	Nitrate reductase
NOS	Nitric oxide synthase
OCl-	Hypochlorite ions
OD	Optical density
P.	*Pseudomonas*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid
PPM	Parts per million
PR	Pathogenesis related
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
S.	*Salmonella*
SA	Salicylic acid
SAR	Systemic acquired resistance
SEM	Scanning electron microscopy

SOS	Oxidative stress
SNP	Sodium nitroprusside
Stx	Shiga toxin
TBX	Tryptone Bile-X-glucuronide agar
TSA	Tryptic soya agar
TSB	Tryptic soya broth
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VNC	Viable but nonculturable
VRBA	Violet Red Bile agar
VTEC	Verotoxin producing *E. coli*
3D	3-dimensional

CHAPTER ONE

INTRODUCTION



Watercress leaf upper surface, x100 Magnification, EDIC microscopy.

1.1 Overview

Due to numerous advertisement campaigns promoting food and health awareness to the public, there has been a large rise in the consumption and demand of fresh fruits and vegetables (Hampson *et al*., 2009). In particular, one advertisement campaign that has made a significant contribution advocates reducing the risk of cancer by eating five portions of fruit and vegetables a day (Kaufman *et al.,* 2000).

Foodborne illness resulting from the consumption of any food, particularly fresh foods, which are eaten raw, is dependent on a number of factors. The food produce must first be contaminated with a pathogen, such as *Escherichia coli* O157:H7 or *Salmonella*; the pathogen must then survive until the time of consumption, at levels that are sufficient to cause illness. The infective dose of most pathogens is very low, typically 50-5000 cells, so only minimal numbers of pathogens are required to cause illness.

The survival and/or growth of pathogens on fresh produce is influenced by numerous factors including the organism, the matrix, and the environmental conditions in the field and subsequent storage conditions post-harvest. In normal conditions, pathogens will survive on, but not grow on, the undamaged outer surface of fresh fruits or vegetables; this is due to the protective mechanisms of the plant's natural barriers (i.e. the waxy cuticle). In general, on the field, the physical environment of leaf surfaces is considered to be inhospitable for the growth and survival of bacteria since there is a lack of nutrients and free moisture, there are also temperature and humidity fluctuations, and there is the presence of UV light (Dickinson, 1986). However, if the environmental conditions are favourable, bacterial populations can flourish. For example, conditions such as the presence of free moisture on leaves, resulting from dew, rainfall or crop irrigation, may promote the growth and survival of bacterial populations (Blakeman 1981, Andrews 1992). Due to this risk, rapid detection methods for pathogens are required in the food industry; as well as an increased understanding of plant-bacteria and pathogen-epiphyte interactions. Current industry utilised disinfection strategies, such as that of the use of chlorine, only lead to typical reductions in pathogen load from leafy foods of between 1- and 2-log (Li *et al.*, 2001; Foley *et al.,* 2002; Koivunen and Heinonen-Tanski, 2005). Another problem with

the use of chlorine disinfection is that chlorine and its by-products have been shown to result in eye and respiratory irritation; additionally the by-products have been associated with causing cancer (Hery *et al.*, 1998; Komulainen, 2004). For this reason, alternative disinfection strategies must be sought, preferably chemical-free, but at least free of chlorine.

1.2 Characteristics and epidemiology of foodborne pathogens.

Numerous pathogens contribute to the outbreak of foodborne disease. This project focuses on microorganisms in particular that have led to many outbreaks worldwide and those that receive the most media attention, namely, *Salmonella enterica* and *Escherichia coli* O157:H7: these microorganisms are of major importance on raw food surfaces, such as that of salad leaves. Coliforms can also lead to foodborne disease outbreaks and strains of coliforms are now being found to also be antibiotic resistant, which could lead to wider problems in the future. Other pathogens that are also found on fresh produce include *C. jejuni, Listeria monocytogenes, Clostridium botulinum* and *Shigella* species (Francis *et al.*, 1999; Wadhwa *et al.*, 2002). Table 1 shows the reported fresh-produce related outbreaks of *Salmonella* and *E. coli* O157:H7 from 1996 onwards (adapted from Heaton and Jones, 2008). Figures 1 and 2 show the reported *E. coli* O157:H7 and *Salmonella* in England and Wales, between 1990 and 2008 (HPA, 2009).

Table 1: Reported fresh-produce outbreaks of *Salmonella* and *E. coli* O157:H7, 1997 onwards (adapted from Heaton and Jones, 2008).

Year	Pathogen	Vehicle	Reference
1997	*Salmonella* Enteritidis	Cauliflower	Anon (2005a)
1997	*S.* Enteritidis PT4	Pepper	Anon (2005a)
1997	*S.* Meleagridis	Sprouted seeds (alfalfa)	Taormina *et al.,* (1999)
1997	*S.* Infantis/*S.* Anatum	Sprouted speeds (alfalfa and mung bean)	Taormina *et al.,* (1999)
1997	*S.* Saphra	Cantaloupe	Sivaplasingham *et al.,* (2004)
1997-8	*S.* Senftenberg	Sprouted seeds (clover and alfalfa)	Taormina *et al.,* (1999)
1998	*S.* Havana/ *S.* Cubana/ *S.* Tennessee	Sprouted seeds (alfalfa)	Taormina *et al.,* (1999)
1998	*S.* Oranienburg	Cantaloupe	Anon (2001a,b)
1998-9	*S.* Baildon	Tomatoes	Anon (2001a,b)
1999	*S.* Muenchen	Unpasteurized apple juice	Anon (2001a,b)
1999	*S.* Thompson	Cilantro	Sivaplasingham *et al.,* (2004)
1999	*S. paratyphi* B var. java	Sprouted seed products	Stratton *et al.,* (2001)
2000	*S.* Enteritidis	Unpasteurized citrus juice	Anon (2001a,b)
2000	*S.* Poona	Cantaloupe	CDC (2002a)
2000	*S.* Typhimurium	Lettuce	Horby *et al.,* (2003)
2000	*S.* Typhimurium DT104	Lettuce	Anon (2005a)
2000	*S.* Typhimurium DT204B	Lettuce	Anon (2005a)
2000	*S.* Enteritidis 11b	Mung bean sprouts	Harb *et al.,* (2003)
2001	*S.* Kottbus	Sprouted seeds (alfalfa)	CDC (2002b)
2001	*S.* Newport	Mixed bag salad	Anon (2005a)
2001	*S.* Virchow	Salad items	Anon (2005a)
2001	*S.* Poona	Cantaloupe	CDC (2002a)
2001	*S.* Enteritidis	Mung bean sprouts	Honish and Nguyen (2001)
2002	*S.* Javiana	Tomatoes	CDC (2002c)
2002	*S.* Poona	Cantaloupe	CDC (2002a)
2003-4	*S.* Enteritidis	Almonds	CDC (2004)

2004	*S.* Newport	Lettuce	Gillespie (2004)
2004	*S.* Thompson	Rocket salad	Nygard *et al.,* (2004)
2004	*S.* Braenderup	Tomatoes	CDC (2005)
2004	*S.* Javiana	Tomatoes	Anon (2005a)
2005	*S.* Typhimurium DT104	Spanish lettuce	Takkinen *et al.,* (2005)
2005	*S.* Typhimurium DT104	Lettuce	Anon (2005a)
2005	*S.* Enteritidis	Bean sprouts	Anon (2005b)
2006	*S.* Newport	Tomatoes	Anon (2007)
2006	*S.* Typhimurium	Tomatoes	CDC (2006a)
2007	*S.* Senftenberg	Basil	Pezzoli *et al.,* (2007)
1997	*E. coli* O157:H7	Sprouted seeds (alfalfa)	Sivaplasingham *et al*., (2004)
1997	*E. coli* O157:H7	Salad	Anon (2005a)
1998	*E. coli* O157:H7	Salad	Anon (2001a,b)
1998	*E. coli* O157:H7	Fruit salad	Anon (2001a,b)
1998	*E. coli* O157:H7	Coleslaw	Anon (2001a,b)
1998	*E. coli* O157:H7	Sprouted seeds (clover/alfalfa)	Taormina *et al.,* (1999)
1998	*E. coli* O157:H7	Unpasteurized apple juice	Anon (2001a,b)
1998	*E. coli* O157:H7	Parsley	Sivaplasingham *et al*., (2004)
1999	*E. coli* O157:H7	Coriander (cilantro)	Campbell *et al.,* 2001
1999	*E. coli* O157:H7	Unpasteurized apple juice	Anon (2001a,b)
2003	*E. coli* O157:H7	Cucumber	Duffell *et al.,* (2003)
2003	*E. coli* O157:H7	Lettuce	Anon (2005a)
2005	*E. coli* O157:H7	Lettuce	Soderstrom *et al.,* (2005)
2006	*E. coli* O157:H7	Spinach	CDC (2006b)
2006	*E. coli* O157:H7	Lettuce	CDC (2006c)

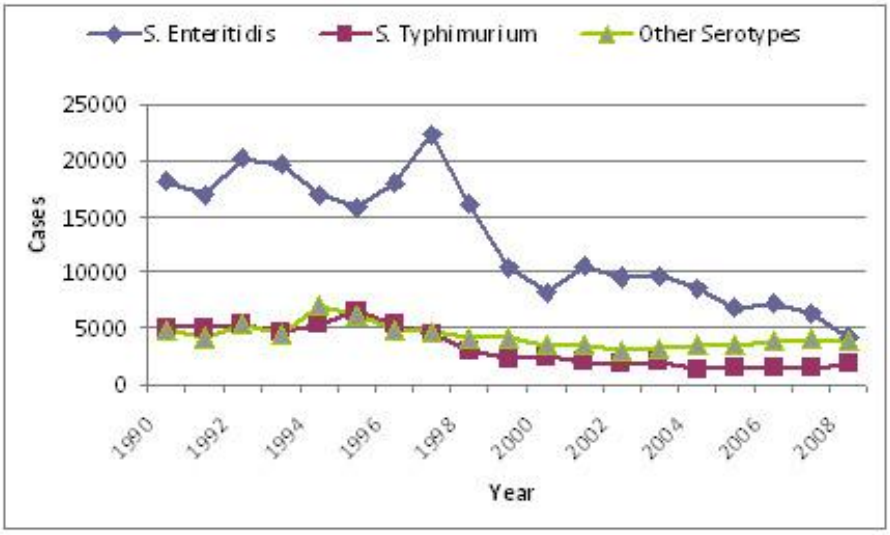


Figure 1: Cases of *Salmonella* in humans reported in England and Wales, 1990-2008. Faecal and lower gastrointestinal tract isolates reported to the Health Protection Agency Centre for Infections (HPA, 2009).

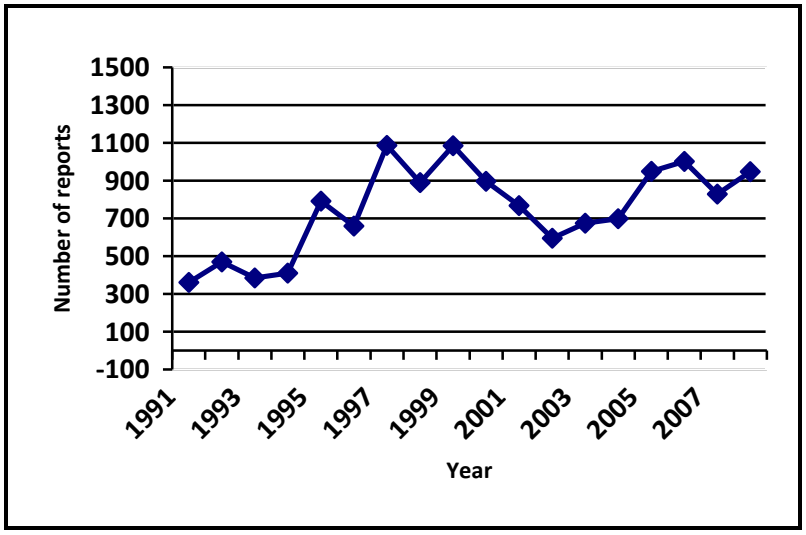


Figure 2: Total annual reports of *E. coli* O157:H7, from England and Wales between 1991 - 2008 (HPA, 2009).

Although laboratory reports for *E. coli* O157:H7 are lower than those for *Salmonella* spp., a significant drop in cases is not evident as it is for *Salmonella*. This drop in *Salmonella* cases is most likely due to a corresponding fall in the levels of *Salmonella* in eggs. Eggs are a raw animal product, and widespread vaccination of egg laying flocks against *Salmonella* Enteritidis, and improved flock hygiene measures have had a dramatic effect on the outbreak of foodborne disease due to eggs. Several vaccinations for chickens have been produced, to reduce the levels further (Salenvac, 2004). For *E. coli* O157:H7, an

upward trend in outbreaks is evident between 1991 and 2008. In the last four years a fairly steady number of reported cases, of approximately 900 per annum, are evident.

Numerous fresh produce related outbreaks are reported annually, for this reason it is important to investigate how these food matrices become contaminated with pathogens in the first instance.

1.2.1 Matrix contamination

The bacteria that are found in the leaf environment originate from soil, water, untreated manure, other plants, seeds, or they may be vectored by animals or insects. In general, the population of pathogens is lower on the leaf surface compared to the soil, since the soil has more stable conditions. Intra-protozoal growth of pathogens can also occur, such as the survival of *E. coli* O157:H7 in the soil parasite *Acanthamoeba polyphaga* (Barker *et al.*, 1999). Bacteria that are found around plants can be classified separately. Some microorganisms that are called 'casuals' do not establish a nutritional relationship with the living leaf; however, they may with senescent plant tissue. Casuals are generally found in higher concentrations closer to the soil around the roots. The second group of microorganisms are called 'residents or epiphytes', these establish a nutritional relationship with the plant, and can remain in large numbers over a long period of time.

The most common cause of bacterial contamination of fruit and vegetables has been attributed to the use of uncomposted or improperly composted animal manure which is used as a fertilizer. In the UK, over 90 million tonnes of animal waste are put to land annually (Heaton and Jones, 2008). *E. coli* O157:H7 and *Salmonella* are carried by livestock and are transmitted to these foods by faecal contamination, since these microorganisms reside in the intestinal tract of animals. Wang and Doyle showed in 1996 that *E. coli* survived in cattle faeces for 42 to 49 days at 37°C, for 49 to 56 days at 22°C and for 63 to 70 days at 5°C. A study by Chapman *et al.* in 1997 showed that the prevalence of *E. coli* O157:H7 was as high as 36.8% in cattle. Contamination is also likely to occur from irrigation water containing manure traces. Seventy-one percent of irrigation water in the UK is obtained from surface waters which receive treated sewage effluent

(Heaton and Jones, 2008). The third source of contamination is directly from the soil, the soil being either naturally contaminated originally, or it may be contaminated by fertilizers containing organic wastes. Pathogens within the soil can then go on to contaminate leaves by heavy rain or leaf splash.

Once pathogens have gained entry into a plant, via either wounds in its surface, or stomata (Figure 3), pathogenesis can progress intercellularly from the mesophyll through the parenchyma of the bundle sheath, and into the vascular system of a minor vein. Migration of pathogens then proceeds to the leaf blade.

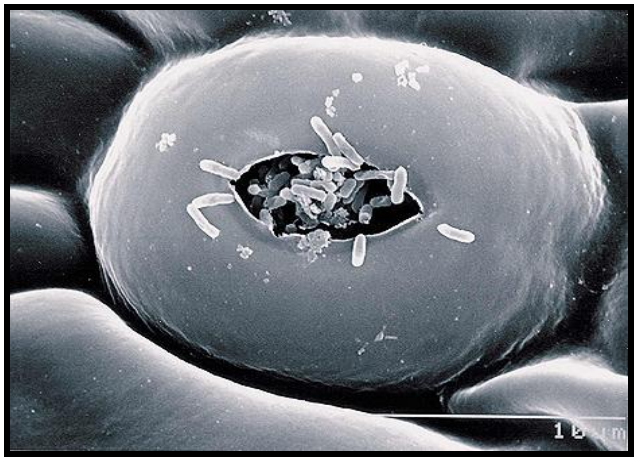


Figure 3: SEM of stomata on grapefruit leaf, *Xanthomonas* bacteria entering stomatal chamber (Gottwald *et al.,* 2002).

In the next three Sections, *Salmonella* spp., *E. coli* O157:H7 and coliforms will be characterized. These are human pathogens that can be found on plants and soil and can lead to infection through the consumption of fresh fruit and salads. They are the three main microorganisms of interest in this project.

1.2.2 Characteristics of *Salmonella*

Salmonella are a group of microorganisms that, in humans, can cause gastroenteritis, resulting from a foodborne infection or they can cause typhoid fever, resulting from bacterial invasion of the bloodstream. An asymptomatic carrier state may also occur, such as that known as Typhoid Mary, named after the most notorious carrier. *Salmonella* are

Enterobacteriaceae of the genus *Salmonella*, they are Gram-negative, rod-shaped bacilli that are flagellated and facultatively anaerobic; it has been estimated that 2000 serotypes can cause human disease. *Salmonella enterica* is a species of *Salmonella* and it has a number of serovars. The most commonly detected serovars with the greatest reported cases annually are *S.* Enteritidis and *S.* Typhimurium (Baumler *et al.,* 2000; Patrick *et al.,* 2004).

Young children, the elderly, and the immunocompromised are most at risk of having severe infections (Sivapalasingam *et al.,* 2004). It has been estimated that approximately 600 people die with acute salmonellosis each year in the USA (Mishu *et al.,* 1994). Between 1973 and 1997 *Salmonella* was isolated in 48 % of cases in the USA and in 41 % of cases between 1992 and 2000 in the UK (Heaton and Jones, 2008).

Salmonella are commensal microorganisms in the intestinal tracts of humans and other animals, including birds. Viable cells are therefore regularly shed in the faeces and have the potential to be deposited onto the surface of fresh food surfaces in investigation here; in migratory birds this could enable strains to cross continents. *Salmonella* species are usually transmitted to humans by eating foods that have been contaminated by faeces. The contaminated foods typically include poultry, beef, milk, eggs, vegetables and salads. Salads such as lettuce, spinach and watercress leaves are at particular risk since they are eaten raw, having been grown in open conditions in fertilized and irrigated soils (Thunberg *et al.,* 2002).

Pathogenic *Salmonellae* that have been ingested in food or via person-person spread, survive passage through the gastric barrier, and invade the mucosa of the small and large intestine and produce toxins (Chopra *et al.,* 1994). To be fully pathogenic, *Salmonellae* must possess a variety of virulence factors. These include the ability to invade cells, a complete lipopolysaccharide coat, the ability to replicate intracellularly, and to possibly elaborate toxins. After ingestion, the microorganisms colonize the ileum and the colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles (Finlay *et al.,* 1989).

The multistate outbreak of *Salmonella* Newport led to vast media attention to the problems of foodborne illnesses due to microorganisms. Fifteen people were left hospitalised and seventy-eight others were affected by the pathogen, two died. Contaminated fruit, possible mangoes were identified as a likely source, although health officials have revealed that the exact cause of the illness may never be known (Sivapalasinam *et al.,* 2003).

In February 2009, a *Salmonella* Saintpaul outbreak linked to raw alfalfa sprouts resulted in two hundred and thirty-four cases across fourteen states. There were twenty-five hospitalisations but no deaths. The FDA issued a warning nationwide. The alfalfa sprouts were produce from several farms, however, the seeds used to grow these sprouts were from one seed company that obtained the seeds originally from one grower; thus, strongly suggesting that the seeds were contaminated (CDC, 2009).

More recently, the multistate outbreak of *Salmonella* Typhimurium has received vast media attention. The first reported case was in March 2009, there was a total case count of seven hundred and fourteen in forty-six states. The outbreak was linked to peanut butter crackers and a multistate recall notice was issued (CDC, 2009).

1.2.3 Characteristics of *Escherichia coli*

Over 700 antigenic types or serotypes of *Escherichia coli (E. coli)* are recognised based on the presence of O, H, and K antigens. Serotyping is important in distinguishing the small number of strains that actually cause disease, such as *E. coli* O157:H7 or *E. coli* O121:H19 (Clarke, 2001). *E. coli* O157:H7 is an emerging cause of foodborne illness. Between the months of August and September 2006, one hundred and ninety-nine people fell ill with *E. coli* O157:H7 in an outbreak sourcing from California; one hundred and two hospitalizations resulted and three deaths due to the consumption of spinach (CDC, 2006). As few as ten *E. coli* O157:H7 bacteria can lead to disease. The GI tract of most warm-blooded animals and humans is colonized by *E. coli* within hours or a few days after birth; the bacterium is ingested in foods or water or obtained directly from other individuals handling the infant (Robins-Browne *et al.,* 2002).

E. coli is the best known of the large bacterial family, Enterobacteriaceae, or the enteric bacteria which are facultatively anaerobic, Gram-negative rods that live in the intestinal tracts of animals in health and disease. *E. coli* is a consistent inhabitant of the human intestinal tract, although it makes up a very small proportion of the total bacterial content; it is the predominant facultative organism in the human GI tract. The fact that *E. coli* is regularly present in human and animal intestines has led to the use of *E. coli* as an indicator of faecal pollution and water contamination where it is present in the environment (Stewart *et al.,* 2001). Medically, the Enterobacteriaceae are the most important bacteria. A number of genera within this family are human intestinal pathogens, such as *Salmonella* or *Shigella;* several others normally colonise the human GI tract, such as *Escherichia enterobacter*, but these bacteria may also be associated with human disease (Robins-Browne *et al.,* 2002; Solomon *et al.,* 2002). Cattle have natural reservoirs of *E. coli* O157:H7, and Fratamico *et al.* in 2002 showed prevalence rates of 1.8 to 28%. Outside of the host most enteric pathogens survive well, such as in faeces, hence the risk posed by non-composted animal manure, and therefore, contaminated irrigation water. Houseflies have also been observed to serve as a vector of these microorganisms since they can carry the pathogen in their intestines.

E. coli O157:H7 produces at least one powerful toxin and can cause severe illness. This strain of *E. coli* carries trait O157:H7-associated virulence factors or genes including *stx1* that encodes shiga toxin (stx) 1; *eae* for gamma-intimin that is essential for cell attachment; *ehxA* for enterohaemolysin and a +93 *uidA (gusA)* base mutation that is unique to the O157:H7 serotype and abolishes glucuronidase activity (Monday *et al.,* 2001). These strains are often referred to as either Verotoxin-producing *E. coli* (VTEC) or Shiga-like toxin producing *E. coli* (STEC).

E. coli can grow in both the absence or in the presence of oxygen. Under anaerobic conditions it grows by fermentation, producing mixed acids and gas as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilise NO_3^-, NO_2^-, or fumarate as terminal electron acceptors for respiratory electron transport processes. This characteristic of *E. coli* facilitates adaptation of this bacterium to its

intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats (Clarke, 2001). *E. coli* has been shown in numerous experiments to respond to environmental signals such as chemicals, pH, temperature, and osmolarity in very remarkable ways considering it is a single-celled organism. It has been shown that *E. coli* can sense the presence or absence of chemicals and gases in the environment and can swim towards or away from them in a chemotactic manner. *E. coli* can stop swimming and produce fimbriae that will specifically attach it to a cell or surface receptor. In response to temperature and osmolarity, the bacterium can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or exude inhibitory substances. Due to these complex mechanisms for regulation of metabolism, *E. coli* can survey the chemical contents of its environment in advance before it synthesizes any enzymes that are necessary to use these compounds. This method is very energy efficient since the bacterium does not excessively produce enzymes for degradation of carbon sources unless they are available, the second advantage of this mechanism is that it does not produce enzymes for the synthesis of metabolites if they are already available as nutrients in the environment (Ray, 1996; Clarke, 2001).

An outbreak of *E. coli* O157:H7 in 1995 in which seventy people were infected was associated with lettuce. The lettuce plants were grown downhill from a cattle pasture. Scientists concluded that the contamination may have been due to contaminated water being used to irrigate the fields in Montana by a company located on California's Salinas Valley (Wang *et al.*, 1996).

The *E. coli* O157:H7 outbreak in Minnesota in September 2005, which infected residents that had eaten pre-packaged Dole lettuce from four different food stores, shows just how important it is to disinfect salad leaves effectively before they reach the consumer level. Two residents were hospitalised, and the cases ranged from residents between the ages of 3 to 68 years (CDC, 2006).

A more recent outbreak is a multistate outbreak of *E. coli* O157:H7, associated with beef. Twenty-three people were infected from nine states. Two patients developed haemolytic uremic syndrome. Most ill persons reported that they had consumed undercooked ground

beef. A multistate recall of the beef packages was issued and consumers were urged to check their refrigerators and freezers (CDC, 2009).

Godstone animal farm in Surrey was recently linked to ninety-three outbreaks of *E. coli* O157:H7, in 2009. The HPA received information about the first case on 27 August 2009. The HPA Outbreak Control Team concluded that the outbreak was of animal origin, ruling out water, food, or catering as potential sources. Some of the children affected were left seriously ill and needed dialysis; all ninety-three were discharged from hospital within a month after contracting the pathogen (HPA, 2009).

1.2.4 Characteristics of Coliforms

The coliform bacteria group consists of several genera of bacteria belonging to the family Enterobacteriaceae. These are rod-shaped, Gram-negative organisms that ferment lactose with the production of gas when incubated at 35ºC and are commonly found in plants, soil and animals. Members of this group include *E. coli*, *Citrobacter*, *Acinetobacter*, *Serratia*, *Enterobacter* and *Klebsiella* species. Total coliform bacteria counts are sometimes used to test for water contamination by faeces, however, these organisms are less precise as faecal contamination indicators because many can also live and reproduce in soil and water, without having a human host. All of these strains except for *Klebsiella* spp. produce flagella (McFeters *et al.,* 1982,). The coliform counts produced in this present study can be used to indicate bacteria that are originating from the soil and have had time to adapt to the prevailing environmental conditions.

Coliforms have been found in various hospital-acquired infections. *Klebsiella, Enterobacter,* and *Serratia* species are frequent causes of bacteraemia at some medical centres and are also frequently involved in infections associated with the respiratory tract, and procedures using contaminated inhalation therapy equipment. *Klebsiella* and *Serratia* species commonly cause infections following intravenous and urinary catheterization and infections complicating burns. The role of *Citrobacter* species in human disease is not as great as that of the other coliforms. *Citrobacter freundii* and *C. diversus* (*C. koseri*) have been isolated predominantly as superinfecting agents from urinary and respiratory tract

infections. *Citrobacter* septicaemia may occur in patients with multiple predisposing factors; *Citrobacter* species also cause meningitis and pulmonary infections in neonates and young children. Strains of coliforms are emerging to be antibiotic resistant, and therefore, it is all the more important to remove these microorganisms from the surface of fresh foods before they are consumed (Selvaratnam *et al.,* 2004).

Some strains of coliforms have pili (fimbriae). Pili are associated with adhesive properties and in some cases are related with virulence. The process of disease production by coliforms is, in many cases, poorly understood. The bacteria may be acquired indirectly via various vehicles, or by direct contact. A variety of vehicles have been implicated in the spread of nosocomial pathogens. For example, *Klebsiella, Enterobacter*, and *Serratia* species have all been recovered in large numbers from hospital food, particularly salads, with the hospital kitchen being a primary source (Coyne *et al.,* 1995). This present study looks at the numbers of coliforms being recovered from the leaf surface in order to indicate those bacteria originating from the soil and contaminating salad leaves.

1.3 The matrix microenvironment and survival in the phyllosphere.

The phyllosphere is the term given to the bacterial environment that is created by a leaf surface and the phylloplane is used to describe the leaf surface. The phyllosphere is a harsh environment resulting in wide fluctuations in populations of microorganisms. Several factors are involved in the process. The diversity of the natural leaf microbiology is yet to be clarified and there are very few published studies in this area. Hirano and Upper (2000), found that bacterial populations in the phyllosphere totalled on average 7-log colony forming units (CFU) per cm^3 of leaf surface. However, subsequent studies by Karamanoli *et al.* (2005), showed that overall bacterial numbers varied greatly between plant species and they concluded that this may be due, in part, to species-specific plant phenolic compounds having antimicrobial properties. Gram-negative rod bacteria, mainly of the genera *Pseudomonas* and *Erwinia* are generally found to constitute approximately 90% of the natural bacterial communities of fresh produce (Francis *et al.,* 1999). The *Erwinia* species are important necrotrophic plant pathogens that are responsible for a variety of soft rots and tissue necroses, particularly on vegetable bulbs and tubers. The growth-promoting

colonists, the pink-pigmented facultative methylotrophs or PPFMs are the least abundant colonists (Madhaiyan *et al.*, 2005).

Plant surfaces are hostile environments for most pathogens because of the prevailing rapid and repeated fluctuations in temperature, water availability and solar radiation. The upper epidermis of leaves is covered with a waxy, waterproof cuticle, which reduces water loss from the leaf and prevents the penetration of pathogens (Figure 4). The lower epidermis of the leaf contains thousands of pores, called stomata. Each stoma is surrounded by two guard cells. Guard cells are elongated in shape and contain chloroplasts (Figure 4). They regulate the opening and closing of the stomata and therefore, control the exchange of gases between the leaf and the surrounding atmosphere (Nawrath, 2006). The individual plant cell walls are semi-rigid and provide cell shape, structural support and cement adjacent cell walls. They also play an important role in plant-pathogen interactions, in that they act as a barrier to pathogen invasion and spread within infected tissue (Figure 4). The plant cell wall is composed of complex carbohydrate polymers, the most abundant being cellulose and pectin (Carpita and McCann, 2000). Some pathogens have the ability to degrade these polymers into polysaccharides and can use these as a carbon source.

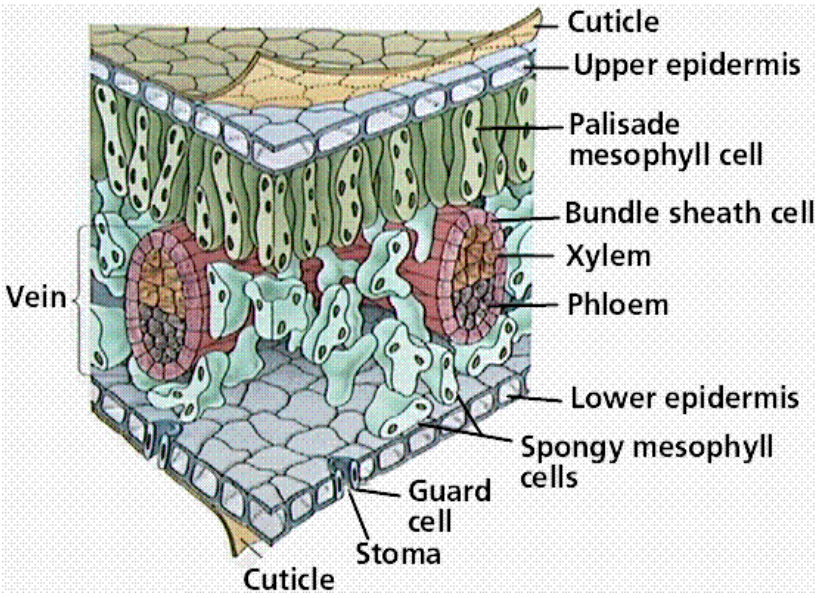


Figure 4: Cross-section of leaf. The waxy cuticle prevents water loss and protects the leaf from pathogen entry. From http://www.cartage.org.lb/en/themes/sciences/botanicalsciences/plantsstructure/plantsstructure/leafstru.gif

Unlike commensals, pathogenic bacteria alternate between free living and host-associated states. This results in different demands and stresses on the bacterium due to alterations in the environment between each state, because of this pathogens have evolved highly sophisticated mechanisms for sensing external conditions. Pathogens respond by altering the pattern of gene expression with the activation of a set of genes whose products assist in survival, particularly by the induction of virulence factors (Yura *et al*., 1993). Stress conditions and different environmental stimuli result in the induction of complex processes, both at the level of transcription and translation, which control the regulatory systems.

The temperature of the phylloplane fluctuates hourly during the day and the night, since the atmosphere does not provide the same insulation properties as the soil; these changes can be as great as 35°C between the summer and winter, and during a 24 hour period (Gniwotta *et al*., 2005). In pathogenic microorganisms, temperature stress results in the induction of virulence genes and also the expression of a set of heat shock proteins (HSP). The induction of HSPs, known as the Heat Shock Response, occurs primarily at the level of transcription. In *E. coli*, the product of the rpoH gene, a σ-factor (subunit of RNA polymerase), is required for the transcription of heat shock genes (Yura *et al.,* 1993). σ 32 binds to the core RNA polymerase and recognises the heat shock promoters which differ significantly from regular promoter regions and the length of the space between these regions. At high temperatures, σ 32 is present at a higher level in the cell, which results in a greater association with RNA polymerase and therefore, a greater transcription of the heat shock genes (Yura *et al.,* 1993)

The surface of a leaf contains very little in terms of food for the pathogen, unless the waxy cuticle has been damaged, in which case a leakage of amino acids, carbohydrates and organic acids is likely to occur. In many pathogens, an increase in osmolarity is associated with the expression of virulence factors. Osmolarity is an important factor that enables a pathogen to distinguish between the external (environmental waters) and host (human body for infection) associated environments; the intestinal lumen having a higher osmolarity than the aqueous environment.

Since the cuticle is hydrophobic, it is expected that pathogenic microorganisms use hydrophobic interactions to attach to the surface. Pathogens can locate between plant cells in the extracellular space, however, this area is relatively low in nutrients and plant defence responses induced due to microbial attack is often targeted to this area (Hammond-Kosack and Jones, 1996). Therefore, in order to colonize the plant surface successfully, pathogens need to tolerate, evade, or suppress the antimicrobial defences of the matrix and also render the environment suitable for further growth, by eliciting release of nutrients and water from the host.

The upper surface of the leaf is also exposed to infrared and UV radiation, these lead to heating and bactericidal effects, respectively. For this reason, it may be expected for phylloplane bacteria to colonize the underside of leaf surfaces instead; or those microbes that are able to survive UV radiation may be efficient in UV-induced DNA damage repair (Jacobs and Sundin, 2001; Heaton and Jones, 2008). The fluorescent *pseudomonads* are able to tolerate UV radiation from the sun and they are species that are commonly found on the upper surface of plant leaves. In *Pseudomonas syringae*, DNA repair is induced after the expression of the gene *rulAB* and *E. coli* and *S. enterica* possess homologues of *rulAB*, enabling the ability to withstand UV radiation (Brandl, 2006; Heaton and Jones, 2008). However, it is to be noted that these are not the only species found on the surface of leaves. Less tolerant species are also found to be present and are not completely eliminated on exposure; this suggests that cross protection can come from factors other than a single bacterium's defences (Francis *et al.*, 1999).

On the phylloplane the relative humidity is greatly variable; this is a disadvantage to most microorganisms which require free water for replication. However, some pathogens have been shown to continue to multiply even during times of low humidity. Bacterial extracellular polysaccharides (EPS), such as alginate, are hydrophilic and absorptive; they may be important in holding water in close proximity to the cell even if free water is lacking on the surface of the leaf. As stated previously, *Pseudomonas* species are the most abundant on the leaf surface, and they comprise plant-pathogenic bacteria and neutral colonists. *P. fluorescens* is not harmful to plants; however, *Pseudomonas syringae* is one

of the most virulent bacterial plant pathogens, which most likely accounts for its dominance in the plant phyllosphere (Dulla *et al*., 2005). In *P. syringae*, expression of some genes that are involved in alginate production is optimal at 32°C, and this may correspond with the formation of the alginate capsule. The fact that the ideal replication temperature for this organism is 30°C, may suggest that the production of alginate is triggered by adverse conditions, such as infrared heat (Penaloza-Vazquez *et al.,* 1997). Solomon *et al.,* in 2003, also reported that the application of water directly to the surface of plant leaves (as is done while lettuce, watercress or spinach are growing – spray irrigation) is linked to the contamination of crops. It was suggested that repeated exposure of water to the leaves increased the levels of *E. coli* O157:H7 on plants. *Xanthomonas campestris* is also a virulent plant pathogen that is able to attain high numbers on leaves (Jaques *et al.*, 2005); it secretes large quantities of exopolymeric material which is commonly used as a food thickner (xanthan gum). The presence of virulent plant pathogens on the phylloplane may increase the likelihood for the growth and penetration of enteropathogens. This is due to damage of the waxy cuticle of the plant by the plant pathogen, resulting in the leakage of nutrients and water. The co-inoculation of *S. Poona* with the virulent plant pathogens *Cladosporium cladosporoides* or *Penicillium expansum* on the wound sites of cantaloupe has been shown to result in increased penetration of *Salmonella* into the internal tissue, due to tissue breakdown by the fungi (Heaton and Jones, 2008; Richards and Beauchat, 2005).

The anatomy of the leaf also affects pathogen attachment. A leaf that is more folded will protect pathogens from environmental stresses such as UV radiation. The leaf thickness will also have an affect; leaves that are thicker in volume have greater insulatory properties than thinner ones. Areas of the waxy cuticle that are thicker, are less likely to leak nutrients, therefore, bacteria do not colonize those areas as readily. Also, at junctions of epidermal cells, depressions in the cuticle are normally present, allowing for higher populations of bacteria. The crevices and grooves that are formed between the leaf blades may also provide locations for greater pathogen colonization. Even before growth of lettuce, alfalfa seeds that are wrinkled (i.e. have greater grooves and crevices), have been shown to harbour more aerobic bacteria than seeds that are smooth (Charkowski, 2001).

Plants have evolved numerous basal defence mechanisms to protect themselves against microbial attack (Heath, 2000). They constitutively produce non-specific antimicrobial compounds that act as biochemical barriers to colonization (Dixon, 2001). In additional to this basal defence, plants activate a complex series of responses to microbial attack. These responses include a rapid oxidative burst, accumulation of increased levels of endogenous signalling molecules (such as salicylic acid, jasmonic acid and ethylene), induction of defence genes (pathogenesis related or *PR* genes), the production of lytic enzymes which are antimicrobial, and the reinforcement of plant cell walls surrounding the site of infection (Hammond-Kosack and Jones, 1996; Glazebrook *et al.,* 1997; Felix *et al.,* 1999; Gomez-Gomez and Boller, 2002). The expression of these defences limits the spread of the pathogen and therefore, the severity of any disease on the leaf surface. For this reason, it is widely believed that a successful plant pathogen must be able to evade or inhibit induction of these defences for colonization. A successful plant pathogen is distinguished from a non-pathogenic organism by its ability to tolerate the host tissues defences, evade them, avoid them, or suppress the activation of these (Alfano and Collmer, 1996; Felix *et al.,* 1999; Jin *et al.,* 2003). To facilitate colonization of plant tissue, as stated above, pathogens also elicit release of water from plant cells and can interfere with the integrity of the plant cell wall that surrounds the initial site of infection. Pathogens have evolved virulence strategies to modulate the host physiology, they release low molecular weight phytotoxins into the extracellular space (Bender *et al.,* 1999), protein virulence factors directly into the cytosol via a specialized secretion system (Galan and Collmer, 1999; Jin *et al.,* 2003), degradative enzymes targeting the cell wall, and extracellular polysaccharides (EPS). Some plant pathogens have also been shown to produce plant hormones or analogues of hormones to modulate the host hormone physiology. Pathogens express genes to allow them to adapt to the hosts defence responses, such as the production of proteins and enzymes (such as glutathione S-transferase, superoxide dismutase and catalase) to counteract the oxidative stress and also enzymes to detoxify antimicrobial compounds (Boch *et al.,* 2002; Salanoubat *et al.,* 2002; Buell *et al.,* 2003). A successful plant pathogen is able to suppress or tolerate the host defence system until colonization has occurred, and once high levels of growth have resumed it deploys a further virulence strategy, the

production of the symptoms of the disease or infection on the leaf. The pathogen uses this to spread to uninfected tissue and neighbouring plants (Agrios, 1997).

Common strategies for bacterial fitness in association with eukaryotic hosts and plants are emerging for animal and plant pathogens. A study by Barak *et al.* in 2005, showed that *S. enterica* genes that are important for virulence in animals are also required for attachment and colonization of plants. Curli and other gene products regulated by RpoS were shown to be important for *S. enterica* attachment to plant tissue - similar to that shown for eukaryotes (Barak *et al.,* 2005). RpoS is an important adaptation regulator; it controls a large regulon and plays a critical role in survival against stresses, such as UV-exposure, low pH, prolonged starvation, thermal stresses and oxidative stress, some of which may occur on the phylloplane. RpoS is a sigma factor that directs RNA polymerase to recognize specific genes required for survival. Studies have found that the occurrence of mutations in the *rpoS* gene is vital to the central role that RpoS plays in survival (Chen *et al.,* 2004). For example, growth of *E. coli* on weak acids (i.e. succinate and acetate) strongly selects for mutations in *rpoS* for enhanced survival. The weak acid concentration (i.e. of acetate) is relatively high in the human colon where *E. coli* colonizes, so *E. coli* may face a similar selective pressure within the host environment. Selection for loss and gain of RpoS function may be an important adaptive mechanism to ensure that *E. coli* survives in complex natural environments (Chen *et al.,* 2004) such as the phylloplane. Components of the type III secretion system (TTSS), which enable the delivery of pathogenicity proteins to the host cell, have been identified in both animal and plant pathogens, although the proteins that are secreted are different (Heaton and Jones, 2008; Huek, 1998). Epiphytic colonization of the phyllosphere is mediated by the TTSS in *P. syringae* pv. *syringae.* The TTSS in *S. enterica* has been shown to be expressed and assembled in plant roots, since mutations reduce the ability of *S. enterica* to colonize plant roots in vitro (Brandl, 2006).

A variety of stress conditions can result in a co-ordinated or overlap in induction of stress proteins, this has been well characterised for microorganisms including *Escherichia coli* and *Bacillus subtilis* (Yura *et al.,* 1993; Hecker *et al.,* 1996). This overlap in protective mechanisms has also been shown for *Lactococcus lactis*. Carbohydrate starved stationary

phase cells of *L. lactis* have been shown to exhibit increased resistance to acid, heat, ethanol and osmotic and oxidative stress (Hartke *et al.,* 1994). UV-irradiated cells of the same strain of *L. lactis* resulted in enhanced survival against acid, ethanol, heat and hydrogen peroxide (Hartke *et al.,* 1995). Microorganisms on the phylloplane may be similarly exposed to UV irradiation which might confer enhanced stress survival. An overlap between acid and starvation responses is well documented in the literature, and many experiments have determined the responses to various nutrients by *E. coli*, S*almonella* Typhimurium, *B. subtilis* and *L. lactis* (Nystrom, 1993; Spector and Foster, 1993; Hartke *et al.,* 1994, 1995, 1996; Bernhardt *et al.,* 1997). Svensater *et al.* (2000) showed this response for *Streptococcus mutans*: the starvation response in this microorganism resulted in acid protection and this effect was significant in the dental plaque environment. The ingestion of dietary carbohydrate can result in a rapid reduction in pH (to values close to pH 4), depending on the age of the plaque. Thus, it has been speculated that as the plaque pH and carbohydrate concentrations decrease, the cells receive at least two signals to induce the acid response. One signal triggered by acid, and the other by the concentration of carbohydrate. The significance of multiple stress resistance is that the combined effect generates a level of tolerance (e.g. acid tolerance) greater than that achieved by either signal alone (Svensater *et al.,* 2000). This multiple stress adaptation may be important in salad leaf processing since microorganisms may have survived UV and desiccation stress in the field and are subsequently exposed to a weak acid rinse which has been advocated by several food companies to wash the leaves. The literature suggests that there is an overlap in the stress responses that result in microbial resistance to sanitizers and microbial resistance to antibiotics that are used therapeutically. Studies have suggested that bacteria may become resistant to antibiotics through exposure to pine oil disinfectants. Moken *et al.* (1997), showed that pine oil resistant *E. coli* mutants were also more resistant to the antibiotics tetracycline, ampicillin and chloramphenicol. It was found that the resistant mutants over expressed a gene that triggered an increase in the general antimicrobial efflux pump (Moken *et al.,* 1997; Mangalappalli-Illathu *et al.,* 2008).

Penetration into the leaf is vital if pathogens are to survive chlorination or any other exterior bactericidal factors. Pathogens are more readily able to penetrate into the leaf

through stomata. As stated, humid gases and some nutrients escape to the leaf surface via these apertures. Variations in oxygen concentration results in the expression of adherence and invasion factors in several pathogens. High concentrations of oxygen generally repress, whereas, low concentrations of oxygen induce invasiveness. A second method by which pathogens are able to penetrate into the leaf, is through cuts in the leaves, such as those produced by food industries in order to produce the increasingly popular pre-cut salads. *E. coli* O157:H7 has been shown to penetrate into the leaf, becoming entrapped 20 to 100 µm below the surface cuticle (Garg *et al.,* 1990; Seo *et al.,* 1999). Pathogens in irrigation water can also be internalised by being taken up by the root systems. Recently, a study by Kroupitski *et al.* in 2009, showed that the internalization of *Salmonella enterica* into lettuce leaves is induced by light and it involves chemotaxis and penetration through open stomata. Some pathogens have other means for gaining nutrients for survival. Necrotrophic pathogens cause cell death in the host which results in the leakage of nutrients for their survival and biotrophic pathogens produce analogues of plant hormones which induce nutrient leakage without causing cell death (Brandl *et al*., 2001; Marco *et al*., 2005). Once pathogens have penetrated the leaf surface they begin rapid reproduction. Bacterial cells produce large amounts of exo-enzymes, these break down the semi-permeable properties of the cell wall, leading to the leakage of food material within the cell walls, which can be used by the pathogen (Seo *et al.,* 1999; Takeuchi *et al.,* 2000).

Other survival strategies have also been found to be utilised by microorganisms, such as the formation of spores under conditions of limiting nitrogen, carbon or phosphorus. In general, the spore structure can be visualised due to a rounding up of the cell and a decrease in cell volume. Studies have found that the spore state is very resistant to desiccation, temperature changes, pesticides, antibiotics and dyes. The water content of spores is very low, and very low to no metabolism occurs, as has been shown for marine bacteria (Jones and Rhodes-Roberts, 1980; Jones and Rhodes-Roberts, 1981). When optimal conditions resume, germination can occur. Once the spore has been activated, it generates ATP, synthesizes RNA, and assembles amino acids into proteins. Other survival states include the formation of vegetative cells, which use up their energy reserves slowly, due to a lower metabolic rate. Also, the existence of a state of dormancy, which can be

caused by stresses such as starvation and results in a decreased size and activity of the bacteria - once the stress is removed, normal development resumes (Roszakt and Colwell, 1987).

The first step in survival on the phylloplane requires attachment of microorganisms, this confers advantages due to protection from environmental stresses and this also provides the opportunity for gene exchange.

1.3.1 Mechanisms of bacterial attachment.

Research to date suggests that it is likely that most of the pathogens that have been discussed in the previous sections attach to salad and leaf surfaces using mechanisms similar to those of naturally occurring bacteria that reside on these surfaces. As discussed above, the attachment of pathogens to humans has also been shown to resemble their attachment to leaves (Barak *et al.*, 2005).

The first stage of bacterial attachment in many species involves the use of small proteins that exist on the surface of some bacterial cells (such as those of *Rhizobium* spp.). These are non-structural and in the case for *Rhizobium* spp. it is a bacterial Ca^{2+} binding protein (called rhicadhesin) that attaches the bacteria to root hairs. Bacterial strains with knockouts of rhicadhesin have been shown to be inhibited from attaching to plant roots cells (Smit *et al.*, 1989).

In some bacteria the next stage in attachment requires the bacteria to synthesize cellulose fibrils that bind the cells to the host surface more tightly, these cellulose fibrils (unlike the proteins involved in the first stage) aid in the formation of bacterial aggregates. In *Salmonella* and *E. coli* these fibrils have been shown to be formed *in vitro*, however, they are yet to be shown in the adhesion to actual plant cells (Zogaj *et al.*, 2001). A naturally occurring colonizer of plants is *Pseudomonas fluorescens*, which has been shown to require cellulose polymer formation to attach to the plant phyllosphere and rhizosphere (Gal *et al.*, 2003).

Lipopolysaccharides and exopolysaccharides (described later on in this study) are commonly found on Gram-negative bacteria. Studies performed by Solomon *et al.* in 2006, showed that *E. coli* O157:H7 that was grown in media that produced more lipopolysaccharide (media that was more hydrophilic, such as tryptic soy broth), attached to surfaces of lettuce better than those grown in other media (such as nutrient broth). Solomon *et al.* concluded that this may suggest that these polysaccharides may be involved in the direct attachment of *E. coli* O157:H7 to plant surfaces. These act as receptors for lectins on plant surfaces (Solomon *et al.,* 2006). Bacterial leaf spot and headrot in lettuce is caused by *Xanthomonas campestris* pv. *Vitians*, a Gram-negative plant-associated bacterium. The lipopolysaccharide of this microorganism is suspected to be an important molecule for adhesion to and infection of lettuce leaf (Molinaro *et al.,* 2002).

Research also shows that bacteria produce lectins, which mediate their interaction with carbohydrates that are present on the surfaces of plants. Studies have shown that *E. coli* O157:H7 and *Listeria monocytogenes* preferentially attach to cut edges of lettuce leaves). This could be related to the fact that at cut edges carbohydrates leak from the damaged plant cells; thus the lectins on the surfaces of these pathogens maybe interacting directly with specific carbohydrates in these areas (Takeuchi *et al.,* 2000).

Pathogenic bacteria have also been shown to produce pili (fimbriae) and flagella (structural adhesions) that play a role in their attachment to host cells and possibly also in their virulence. The same has been shown for the interaction of plant-associated bacteria with plant surfaces (Barak *et al.,* 2005). Pili are proteinaceous hair-like appendages that are seen on Gram-negative bacteria. *Pseudomonas* spp. have been shown to attach to the stomata of bean leaves via pili; knockout studies that contained minus-pili mutants were shown to be more easily washed from leaves than wild type strains (Hirano *et al.,* 1996). Although flagella are most commonly thought of in association with the motility of bacteria, this is not only the case. Mutational studies have shown that the bacterial flagella can also play a role in attachment of pathogens to plant surfaces. Immunoelectron microscopy, using antibodies specific to the flagella, has been used to show *S. enterica* serovar Thompson anchored to leaf surface cells via its flagella (Brandl *et al.,* 2002).

If potentially pathogenic bacteria are to be removed from fresh food surfaces, it is essential to understand the mechanisms by which this attachment occurs in the first place. Factors which enhance stronger attachment need to then be investigated and their involvement reduced. It needs to be investigated if bacteria are strongly attached to the matrix in the first place, or if there are other factors which enable foodborne disease outbreaks to occur, despite the use of disinfection procedures such as 90 ppm hypochlorite. Bacteria attached to plant surfaces are phenotypically different from their free-living counterparts. Genes associated with the physical attachment mechanism are upregulated and the close-proximity of high numbers of bacteria can induce co-ordinated responses by means of dispersible signal molecules known as autoinducers, this is known as quorum sensing (Waters and Bassler, 2005; Antunes and Ferreira, 2009). This close proximity further allows for the opportunity for horizontal gene exchange between bacteria via the transfer of plasmid DNA (Christensen *et al.*, 1998, Bailey *et al.*, 2002). Attachment has been shown to occur in two stages, the reversible and transient association stage and then the irreversible adhesion; these are phenotypically distinct from one another as well as from the planktonic existence, the transition between the two requires significant changes in gene expression (Hinsa *et al.*, 2003). Adhesion to surfaces is aided by the secretion of polysaccharide material which forms a slime layer around the bacterial cell; each bacterial strain produces only one type of capsular polysaccharide (Leigh and Coplin, 1992). However, where species co-exist in close proximity, the polysaccharides are capable of interacting with one another to form a heterogeneous matrix. The great differences in attachment strength by different bacteria may be explained by the composition of the exopolysaccharide secretion.

1.3.2 Biofilm structure and environment.

Bacteria on surfaces can frequently form biofilms; these are highly complex but ordered three-dimensional structures of multi-layered micro-colonies, bound together by secreted extracellular polymer material and interspersed with water channels (Donlan, 2002). The polymer material is composed mainly of polysaccharide but also proteins, glycoproteins and material trapped from the surrounding environment (Figure 5) and is what facilitates the formation of these complex three-dimensional structures. Without this polymer

material, the bacteria could be expressed nothing more than an aggregate of cells. Staining by use of fluorescent conjugated lectins can be used to view the exopolysaccharide matrix directly (Neu *et al.*, 2001). Biofilm formation has specific phenotypic requirements which may be species dependent. In the case of *P. aeruginosa* biofilm formation, there is the requirement for type IV pili production on the cell surface (O'Toole and Kolter, 1998). The bacteria within a biofilm communicate with each other about the physiological, developmental, and morphological state, via quorum sensing, as discussed above. Several signalling systems have been described, the main type being N-acyl-homoserine-lactose molecule mediated cell-cell signalling in the Gram-negative bacteria. These molecules pass freely across the cell membrane in and out of cells. Peptide-based signalling molecules are employed by Gram-positive organisms for quorum sensing, these are extracellular and bind to receptors on the cell surface, Another type involves auto-inducer-2 (AI-2), which is directed by the LuxS protein, and has been implicated in interspecies communication and biofilm formation by both Gram-negative and Gram-positive organisms (Welch *et al.*, 2005; De Keersmaecker *et al*., 2006). AI-2 signalling has been investigated in numerous enteric pathogens, affecting flagellar motility and the ability to form biofilms (Xavier *et al*., 2007).

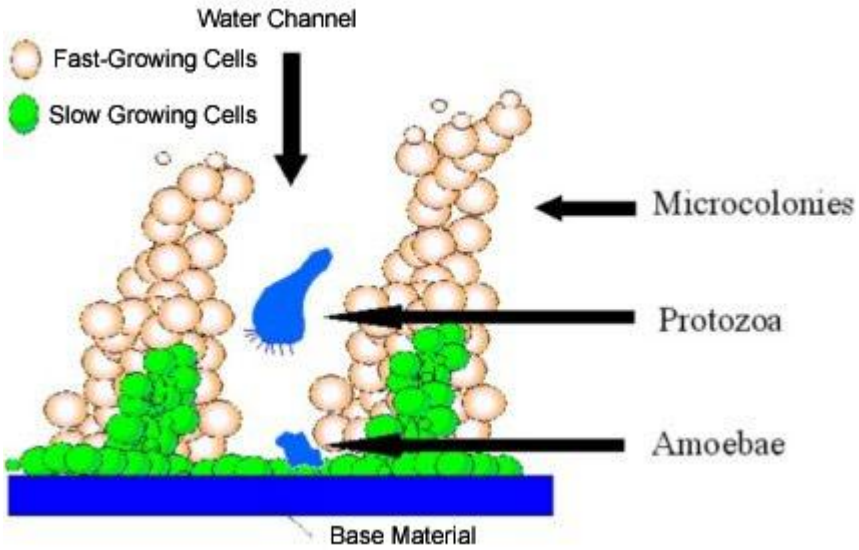


Figure 5: A mixed species ordered biofilm showing heterogeneous three-dimensional structures extending from the surface (Keevil, 2003).

Biofilms are thought to be the stable point in the biological cycle for many bacterial species. Biofilms protect cells within them from stresses, such as naturally occurring UV irradiation on leaves and also from artificial chemical disinfection (Simoes *et al.*, 2005). Established biofilms have been shown to resist antimicrobial agents at concentrations many times that needed to kill genetically identical planktonic cells (Lewis, 2001). This is therefore, of great importance in fresh produce industries and medicine.

Environmental biofilms, such as those in study here, are mostly heterogeneous, containing both anaerobic and aerobic species. The initial steps to form these structures are in response to environmental cues including local nutrient availability, osmotic stress, toxic molecules, and temperature. The literature reports that most microorganisms in biofilms live in mixed populations (O'Toole *et al.*, 2000). In 1997, Jones and Bradshaw demonstrated mixed biofilm interactions using *Klebsiella pneumoniae* and *Salmonella* Enteritidis. They found that the level of attachment of *S.* Enteritidis was higher in a binary biofilm than in a single species biofilm, suggesting a synergistic interaction between the two. They also noticed that there was a larger population of *S.* Enteritidis in the binary biofilm compared to the single species biofilm which may have been due to enhanced growth in the presence of *K. pneumoniae*. *K. pneumoniae* is a facultative anaerobe; when oxygen supply becomes limited, nitrogen fixation by *K. pneumoniae* in a mixed biofilm may be augmented, therefore increasing the overall metabolic activity of the biofilm. The introduction of *S.* Enteritidis to an existing biofilm of *K. pneumoniae* was shown to increase the proportion of metabolically active cells in the biofilm from 40 % to 77 %. As discussed by Jones and Bradshaw, previous studies have demonstrated that *K. pneumonia* grows up to five times faster and is more active in the deeper layers of a biofilm when grown with *P. aeruginosa* compared to monoculture (LeChevallier *et al.*, 1987; Jones and Bradshaw, 1997). This therefore suggests that mixed biofilms are more active than single species biofilms.

Studies have shown that the bacteria within a biofilm are held together very tightly and although solitary cells do often detach off, the major loss of biofilm biomass occurs when large sections of the biofilm break off, this mass does not disintegrate and usually follows

mechanical shear stress (Stoodley *et al.,* 2001). Conversely, Barraud *et al.,* (2006), have more recently shown a fundamental physiological process of cell mobilization and detachment, which is not dependent on shear stress. Overall, biofilm biomass is determined by a balance between attachment, growth and detachment processes. Studies have investigated biofilm detachment, and processes that appear to be important in this process include nutrient levels (Hunt *et al.*, 2003), fluid shear stress, abrasion or erosion (Picioreanu *et al.,* 2001), the detachment processes allow cells to spread and colonize new sites. The presence of biofilms on the phylloplane suggests how such a highly hostile surface supports such a diversity and abundance of microorganisms. Possible integration of potential pathogens into these pre-existing biofilms confers protection from the disinfectant processes that are in use today to decontaminate leaf surfaces. The reasoning behind biofilms having increased resistance to antibacterials, is thought by some, to be because of the physical barrier hypothesis (slime), preventing the penetration of disinfectants into the full depth of the biofilm. The second mechanism is thought to be due to the enzymatic obstacle hypothesis, which states that biofilm bacteria exhibit a different phenotypic response to adherence, compared to their planktonic counterparts (Gorman *et al.,* 2001). Studies by LeChevallier *et al.,* in 1988, showed that attached *E. coli* is 2,400 times more resistant to chlorine than unattached *E. coli*, and biofilms grown on granular activated carbon particles, showed more than a 3,000 fold chlorine resistance (LeChevallier *et al.*, 1988a,b). Research has shown that in potable water, coliforms that are attached to surfaces are able to withstand at least 12 ppm free residual chlorine. Many of the viable bacteria in chlorinated potable water have been shown to be attached to surfaces (LeChevallier *et al.*, 1988a,b).

Studies have shown that planktonic cells in the stationary phase show physiological resemblance to cells within a biofilm, due to both being situated in environments of high cell density and low nutrient availability (Fux *et al.*, 2004). Many genes related to cell division, cell wall synthesis, nucleic acid replication and translation are expressed at lower levels in biofilms, which may also suggest that the majority of the biofilm population are in the stationary phase. Furthermore, slow growing planktonic bacteria (or those in the stationary phase) and biofilm bacteria are both notoriously tolerant to antibiotics, compared

to logarithmic phase planktonic cells (Spoering and Lewis, 2001). However, more recently, studies have shown that biofilms may be more similar to planktonic cells in exponential phase compared to those in stationary phase (Mikkelsen *et al.*, 2007). The medium used for biofilm growth can be explained for the discrepancies between these studies. Hentzer *et al.* (2005), used a minimal medium, and found biofilms to be more similar to planktonic cells in stationary phase; whereas Mikkelsen *et al.* (2007) used undiluted rich medium (Mikkelsen *et al.*, 2009).

The biofilm mode of growth encompasses surface-associated microbial communities and the extracellular matrix in which these cells are embedded. Biofilm formation is a huge problem in industrial, environmental and medical settings. Biofilms act as reservoirs for pathogens in drinking water systems; MRSA biofilms have been shown to be highly antibiotic resistant and pathogens such as *Pseudomonas aeruginosa* cause persistent infections in the lungs of cystic fibrosis patients which are associated with the emergence of antibiotic-resistant subpopulations of bacteria. Furthermore, the formation of biofilms on the phylloplane may be the prime cause of foodborne outbreaks associated with fresh produce such as salads.

1.4 Bacterial detection methods applicable within the food industry

The sterility of the environment within which food production and processing occurs is very important in determining the safety and quality of the final food product. Two major reasons lie behind the theory for maintaining food industrial areas as sterile as possible, the most important being that food containing pathogens above a certain level, depending on the potency of the microorganism, is likely to cause illness and possible death; secondly, microorganisms lead to food spoilage and are likely to reduce shelf life, which is costly to food manufacturers. The problem is that many food surfaces can be the ideal environment for microbial growth, and in addition, food contact areas in industries may contain high levels of organic material which can lead to the formation of a conditioning film onto which microorganisms can become attached.

The last few years have seen many improvements in the methods used for the detection of pathogenic microorganisms in the food industry. Modifications have been made in sample preparation, plating techniques, and counting viable pathogens, while new identification test kits have been produced (James and Keevil, 1999). In particular, it is now recognised that faecally transmitted bacteria may become stressed, and sublethally damaged when exposed to the environment, such as soil, water and perhaps plant surfaces. New resuscitation methods have been developed for any stressed pathogens such as for *C. jejuni*, *Salmonella enterica*, *E. coli* O157:H7 and *Listeria monocytogenes*, in faecal wastes, and these may be applicable to pathogens in the phyllosphere (Horan *et al.*, 2004).

Other than the conventional microbiological methods, many different techniques are increasingly being used. Methods such as the ATP bioluminescence technique are currently being used to measure the efficiency of cleaning surfaces and utensils. Immunoassays and nucleic-acid based methods are being employed for the rapid detection and identification of microorganisms (Fratamico, 2001). The polymerase chain reaction (PCR) and other amplification techniques are used to increase the sensitivity of these assays (Kadivar and Stapleton, 2003). Each of these methods are useful for the detection of bacteria to avoid and reduce foodborne disease, however, the protocols themselves carry many disadvantages and improvements must be sought, particularly in techniques for separating microorganisms from food matrix and for concentrating the bacteria before detection (such as improvements in the mechanisms of the Pulsifier – see later). A problem with ATP bioluminescence is that it does not distinguish different bacterial species; and the disadvantage of using PCR-based methods is the large costs involved and low reproducibility of results between laboratories (de Boer and Beumer, 1999). Alternative methods must meet many requirements including sensitivity, specificity, accuracy, validation, speed and automation (Moore and Griffith, 2002). Within the food industry timing is very important. If pathogens are present in raw or finished foods, then this needs to be discovered as early as possible in order for remedial action to be implemented before control of a product or process is lost.

1.4.1 Sample preparation

Sample preparation is the first task involved in the detection and identification of pathogenic organisms and is an important initial stage of concentrating bacteria. Two main instruments are generally used for this purpose, namely the Stomacher or the Pulsifier.

1.4.2 The Stomacher

The Stomacher was designed by Anthony A. Sharp and has been in use internationally for the preparation of food samples for microbiological analysis since 1972 (Figure 6). It has been estimated that more than 40,000 Stomachers are in use worldwide (Fung, 2002). At the time of its introduction, the Stomacher's main advantage was to serve as an alternative to blending food samples for use in research and food industries.

Figure 6: The Stomacher. Mechanical forces crush the matrix, leading to pathogen release. From www.iuk.edu/ACADEMICS/ artsci/nims/equipment.htm

The Stomacher works by use of mechanical forces. The sample to be analysed is massaged between two steel paddles, which reciprocate out of phase at approximately 300 rpm with amplitude of approximately 4 cm: this causes the sample to be crushed against a solid door plate, driving the liquid from one side of the bag to the other (Sharpe *et al.*, 2000). This leads to the release of microorganisms from the surface of food matrices by violent shearing forces. Depending on the volume of the sample to be analysed, the Stomacher is available in three different sizes. The Stomacher 3500 is ideal for samples that are 300 to

3000 ml, the Stomacher 400 for samples of 40 to 400 ml and the Stomacher 80 can handle sample volumes of 8 to 80 ml.

Several health science groups have studied the effectiveness of the Stomacher compared to the gold standard Waring blender. In 1978, Andrews *et al.* studied six hundred and seventy-one food matrix samples; these represented thirty food categories (including fruits, vegetables, meats, pasta, nuts, spices and desserts). They compared the microbial recoveries produced by the Stomacher 400, the Stomacher 3500 and the Waring blender. The group initially soaked the food samples, so that there would not be a risk of sharp edges tearing Stomacher bags, they then processed the samples for two minutes. The authors concluded from their results that the relative efficiency of the Stomacher 400, the Stomacher 3500 and the Waring blender depended on the actual food matrix being investigated. Different food groups were suited to either one of the types of microbial removal and concentrating mechanism (Andrews et al., 1978).

Although the Stomacher does not always produce a higher viable plate count than the Waring blender, the Stomacher has major advantages over the blender. If the Stomacher is used there is no need to re-sterilize the instrument between samples, this is since the sample is placed into a sterile plastic Stomacher bag, and is contained inside, and so the sample does not come into direct contact with the instrument. The Stomacher bags are disposable, so there are no excess washing stages involved. Therefore, in a set period of time a large number of samples can be processed more efficiently. There is no danger to the scientist using the instrument, particularly if the food matrices are being inoculated with an infectious microorganism, since no heat or aerosols are generated. The sample is also contained within an isolated bag, therefore time course studies can be performed easily (Fung, 2002).

1.4.3 The Pulsifier

In the late 1990s Anthony A. Sharp designed a new instrument for the preparation of food samples for microbiological analysis, the Pulsifier (Figure 7). Unlike the Stomacher, the Pulsifier utilises vibrational waves to drive the microbes on the matrix into suspension. In

place of the paddles that are present in the Stomacher, the Pulsifier has a steel oval ring that essentially shakes off the pathogens. Operation occurs at a frequency of 3,000 rpm with amplitude of approximately 20 mm; this leads to the formation of shock waves and intense stirring that drive the microbes into suspension. Since no mechanical forces are involved the sample matrix remains predominantly intact (Sharpe, 2003). The latest model is PUL100E that operates at 220-250 volts with 0.6 amps of current (Microgen Bioproducts Limited).

Figure 7: The Pulsifier. Vibrational waves drive the microbes into suspension. From www.teagasc.ie/../paper08.htm

The Pulsifier has many advantages over the Waring blender, and also over the Stomacher. The Pulsifier produces less sound disturbance, and it is also lighter and smaller, and therefore easier to shift and transport. Samples placed in the Pulsifier, unlike the Stomacher, do not need to be double-bagged and as the most important factor of improvement, it does not damage the sample, instead leaving it intact and the diluent much clearer. Other features that make the Pulsifier more user-friendly is that it possesses a transparent door enabling the progress of the sample to be viewed, and a specific bag clamping mechanism in the door that completely seals the bag and prevents the formation of dangerous microbial aerosols. If the instrument requires cleaning then the door can be completely lifted out by use of a "freedom from lever" action (Kang, 2001). The second generation Pulsifier has now been produced. This version is more automated, with an LCD screen and the option of at least ten different program settings that can be stored for the processing of various matrices; it is also much quieter and user-friendly.

In 1998, Fung *et al.* compared the first generation Pulsifier to the Stomacher, for the preparation of ninety-six different food samples for microbiological analysis. The samples were serially diluted, plated on agar media and counted for aerobic colony forming units. Fung *et al.* concluded from the experiments that pulsified samples always contained a lot less debris; it was also reported that total aerobic counts obtained from the use of the Pulsifier for most samples may be expected to equal or slightly exceed those by stomaching (Fung *et al.*, 1998).

Due to the substantial decrease in food matrix debris, the Pulsifier is recommended as the initial step in sample preparation for general analysis as well as PCR amplification, ATP bioluminescence tests, DNA/RNA hybridization, flow cytometry, and enzymatic assays, which can easily be affected by tissue extracts. To date, there have been no studies investigating the effect of pulsification on inoculated pathogens, such as *Salmonella* Thompson; there are also no studies that have investigated the *in-situ* effects of pulsification on the spatial distribution of microorganisms on the phylloplane. These investigations would begin to elucidate the working mechanisms of the Pulsifier and how it leads to the cell recovery effects.

1.4.4 Microscopy methods for *in situ* analysis of surface and sub-surface micro-organisms.

In 1957 Marvin Minsky discovered the confocal principle, however, it was not until 1979 that the Dutch physicist Brakenhoff applied the principal and built the first confocal microscope. The confocal microscope is similar to the light-microscope, however, it is able to do three-dimensional imaging of biological specimens, it is better than the electron microscope in that the samples do not have to be sliced and dehydrated, and therefore none of the physiological information is lost and the resolution is good (White, 1987, Takeuchi *et al.*, 2001, Pamp *et al.*, 2009).

In laser scanning confocal microscopy (LSCM) a laser light beam is expanded to make maximal use of the optics in the objective. The lasers excite fluorophores in fluorescent

dyes and stains that have been applied to the sample. In general, a confocal microscope that is set up correctly will always give a better image than can be obtained with a standard epifluorescence microscope. All this improvement essentially comes from the rejection of out-of-focus interference (White *et al.*, 1987). Amongst other studies, pathogen attachment and detachment from food matrices can be observed *in situ* using LSCM.

When the specimen to be observed is transparent, differential interference contrast microscopy (DIC) is excellent for rendering contrast. DIC microscopy is a beam-shearing interference system in which the reference beam is sheared by a minuscule amount. This produces a monochromatic shadow-cast image that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimen. A newer technique is episcopic differential interference contrast (EDIC) microscopy, which provides a real time analysis of curved/flat matrices without the need for cover slips or oil and is ideal for opaque matrices (Keevil, 2003). EDIC transmits light off a sample rather than through the surface and differences in the plane of the sample alter the path of the returning light, producing areas of contrast. EDIC can be coupled with epifluorescence microscopy (EDIC/EF), and used to visualise 3-D structures of matrices, physiological niches and localization of specific pathogens in combination with fluorescent dyes and stains, see Figure 8. The EDIC/EF microscope can be used to follow events occurring on the surface of matrices, such as drinking water pipes (Wilks and Keevil, 2007) and surgical instruments (Lipscomb *et al*., 2007), however, it has never before been used for the study of the phyllosphere, this study will investigate its use for this purpose.

Figure 8: The EDIC microscope.

1.4.5 Use of molecular techniques to track pathogens

Traditionally, bacterial pathogens have been detected indirectly by use of immunological probes, such as by the use of fluorescent antibodies. Scientists have also attempted to directly coat microorganisms with fluorochromes to study real time interactions with both plant and animal host cells. Other methods used to investigate the adherence of pathogens to matrices include electron microscopy, radiolabelling of bacteria, Giemsa staining and acridine orange-crystal violet staining (Valdivia *et al.,* 1996). Direct fluorescent staining of the natural microflora of leaves is in its early stages; most studies have detached the microflora from the phylloplane onto filters and stained indirectly. However, these approaches are of limited value since it is difficult to image the results obtained and compare the leaves *in-situ*. The methods are destructive, time-consuming, and subject to photo-bleaching since these tagging mechanisms are extrinsic and difficult to perform. More recently, green fluorescent proteins are attracting huge interest; they are the first method for creating strong visible fluorescence by purely biological means, they are an intrinsic tag that can be used to detect bacterial cells that are present both intracellularly and extracellularly.

The green fluorescent protein (GFP) was discovered in the mid-1970s by Shimomura *et al.* but it was not until molecular biologists managed to clone GFP did scientists begin to exploit its enormous potential as a biological marker. GFP has already been used as a molecular tracking tool as a reporter of gene expression, tracer of cell lineage and as fusion tags to monitor protein localization within living cells. The fluorescent marker has potential use in the study of bacterial pathogenicity, already GFP is allowing quantitative measurements to be taken of bacterial association with mammalian cells. Localisation experiments involving whole organisms such as rats by use of GFP have also proven very successful (Bumann *et al.,* 2002).

Among the many uses of GFP-labelled bacteria are as controls in the microbiological testing of matrices within the food industry. At present, unmarked, positive control strains are used to ensure that the media and methods have worked as required in isolating the target bacteria. However, in food testing, cross contamination of samples with control strains does occur sometimes, therefore, GFP-marked control strains would be particularly useful, since they would be easily distinguishable in case of cross-contamination (Chalfie *et al.,* 1994).

Another potentially important use of GFP-marked bacteria is in the detection of strains that are viable but non culturable (VNC). Bacteria may enter this state, due to environmental stresses imposed within the food industry; this is where a pathogen remains infectious but can no longer be cultured by conventional methods. Due to this, it could go undetected; however, strains that are marked with GFP will be detectable no matter what state they are in, since bacteria that express the plasmid stably would constitutively fluoresce (Cho and Kim, 1999).

1.4.6 The green fluorescent protein.

GFP is a 26.9kDa protein that is produced by the jellyfish, *Aequorea victoria* which fluoresces in the lower green portion of the visible spectrum. Wild type GFP has two excitation peaks, a major one at 395 nm (UV range) and a minor one at 475 nm (blue); and has an emission peak at 509 nm (Margolin *et al.,* 2000).

The 3-D crystal structure of recombinant wild-type green fluorescent protein has been solved to a resolution of 1.9 angstroms. The protein has the beta-can structural motif, and is made up of a single chain of 238 amino acids. The residues form an 11 stranded anti-parallel beta-barrel wrapped around a co-axial central helix; short segments of the helices close the top and bottom of the beta-can structure. The cylindrical barrel has been found to measure 42 angstroms in length and 24 angstroms in diameter, protecting and scaffolding the central helix and the p-hydroxylbenzylideneimidazolidinone chromophore that is enclosed. It is this fluorophore that is responsible for the fluorescence of GFP (Figure 9, Tsien *et al.,* 1998).

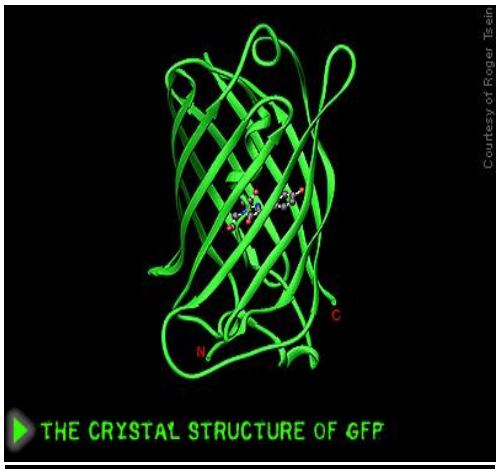


Figure 9: The crystal structure of GFP, a beta-can. From www.its.caltech.edu/ ~sciwrite/journal03/pan.html

The role of GFP is to serve as an energy-transfer acceptor; the protein receives energy from a Ca^{2+} activated photoprotein. After stimulation, *A. victoria* emits a green light. GFP acts as a secondary fluorescent protein; it receives energy from aequorin (blue chemiluminescence) and transduces this into green fluorescent light. Upon protein folding, the fluorescent p-hydroxylbenzylideneimidazolidinone chromophore is formed spontaneously, without any cofactors or accessory proteins, from three main amino acids, Ser65, Tyr66 and Gly67. The first step is a nucleophile attack of the amino group of Gly67 onto the carbonyl group of Ser65 resulting in a pentamer. Elimination of water then results in the formation of the imidazole ring. In the final step the C-C bond within the hydroxybenzyl side chain of Tyr66 is oxidised with atmospheric oxygen forming a large

delocalised pi-system, this step is the rate limiting step in the reaction (Cubitt *et al.,* 1995; Tsien *et al.,* 1998).

Once stable strains expressing GFP of the pathogen of interest have been produced, they are virtually ready to be used in both extracellular and intracellular detection studies. The strain of interest can be inoculated onto matrices and the behaviour of the pathogen followed by use of either a UV lamp or for more detailed investigation the microscope. To date there are few studies examining GFP-labelled bacteria on leaf surfaces, however, Brandl and Mandrell (2002) used LSCM to provide the first conclusive evidence that GFP-labelled *Salmonella* are capable of penetrating the leaf surface in the sub-stomatal areas of cilantro leaves. More recently, as discussed earlier, Kroupitski *et al.* have shown the internalization of *Salmonella* enterica into lettuce leaves using LSCM, furthermore, they have shown that the internalization is induced by light and involves chemotaxis and penetration through open stomata (Kroupitski *et al.,* 2009). One of the aims of this present study is to show this further by the use of *Salmonella* GFP and watercress leaves.

1.5 Chemical disinfection methods.

Adams *et al.* in 1989 reported that washing lettuce leaves with tap water alone only reduces indigenous microflora by approximately 1-log, this is significant, however, it is not sufficient to ensure microbiological safety of fresh cut salads that are eaten raw (Nguyen-the and Carlin, 1994; Sapers, 2001). The water used to wash the produce can become a source of contamination, therefore chemical disinfectants are also required for this reason (Beuchat and Ryu, 1997). Studies have shown that the addition of a chemical disinfectant to the wash water reduces the microbial load (Beuchat and Ryu, 1997). The most frequently used sanitizer at present is chlorinated water. However, using chlorinated water is not as effective as required, since reductions are less than 2-log on salads, and there are also other risks of using chlorine such as the formation of chlorinated bi-products, which on exposure can lead to cancer (Komulainen *et al.,* 2004). Therefore, alternative disinfection treatments must be sought, such as the potential use of ozonated water.

1.5.1 Chlorine.

The most widely used disinfectant in the fresh fruit and vegetable industry is chlorine, the most commonly used free chlorine being hypochlorite. The recommended concentration at which chlorine has been shown to disinfect food surfaces is between 50 to 200 ppm (at pH below 8.0 with a contact time 1 to 2 minutes). This is very high, particularly when compared to the 5 ppm hypochlorite concentration used by the water industry. A higher concentration is used in the food industry due to the more complex nature of the food matrices compared to water. Chorine is a very reactive oxidising halogen that combines with any likely substrates that can be oxidised to form compounds such as trihalomethanes. The bactericidal activity of chlorine depends on the pH, temperature, organic matter content and contact time.

When temperature is increased and humid conditions are maintained there is an exponential growth of bacteria, for this reason cooler water temperatures are more effective at sanitizing processing equipment and surfaces of fresh foods. These conditions are best for chlorine disinfection too, since maximum solubility of chlorine in water occurs at approximately 4°C (Beuchat, 2000). The pH of chlorinated water is also important in the reduction of bacteria. The following equation shows the reaction that occurs when chlorine is added to water; it is the hypochlorous acid that is the primary compound responsible for the antimicrobial properties of chlorine.

$$\mathbf{Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-}$$

Sodium hypochlorite is used instead of chlorine gas, by most fresh produce industries,

$$\mathbf{NaOCl + H_2O \rightarrow HOCl + NaOH}$$

The hypochlorous acid (HOCl) can then dissociate to form hypochlorite (OCl^-) and hydrogen ions, which is dependent on pH. At pH less than 5 the proportion of undissociated HOCl is the greatest, if the pH drops below 4 then the proportion of chlorine gas increases (which can be potentially hazardous). HOCl has greater bactericidal effects

than OCl^- therefore, if the solution has a pH range between 6 and 7.5 then this is best (Sapers, 2003).

$$\mathbf{HOCl \leftrightarrow H^+ + OCl^-}$$

A 1.3 to 1.7-log CFU/g reduction was observed for *Listeria monocytogenes* on shredded lettuce and cabbage after treatment with 200 ppm chlorine for 10 minutes at 4°C and 22°C respectively (Zhang and Faber, 1996).

Although it is not yet entirely clear how chlorine kills bacterial cells, it is thought that it attacks the bacterial cell wall, altering it physically, chemically and bio-chemically, thereby, terminating the cell's vital functions and killing the microorganism (Gil *et al.*, 2009). It is thought that a possible sequence of events during chlorination is as follows. The initial disruption of the cell wall barrier by the reaction of chlorine on the cell surface, vital cellular contents in the bacteria are then released, followed by termination of membrane-associated functions and finally termination of cellular functions within the cell. Studies have shown that chlorine has deleterious effects on bacterial DNA, including the formation of chlorinated derivatives of nucleotide bases (Shih *et al.*, 1976; Dennis *et al.*, 1979; Dukan *et al.*, 1996). Chlorine has also been shown to disrupt oxidative phosphorylation and other membrane-associated activity (Camper *et al.*, 1979). It is thought that due to the highly oxidative nature of chlorine the cellular activity of proteins is destroyed (Bloomfield *et al.*, 1996). McKenna and Davies have also described the inhibition of bacterial growth by hypochlorous acid (McKenna *et al.*, 1988). As discussed previously, although chlorine is an effective disinfectant, its main problem is that it leads to the formation of trihalomethanes which have been shown to have irritation effects and may also lead to cancer, for this reason and also since foodborne disease outbreaks are still occurring, this study investigates alternative methods of disinfection, such as the use of ozone.

1.5.2 Ozone

When oxygen molecules are exposed to a high voltage electrical discharge, ozone is produced. The oxidising power of ozone can get to 3,000 times faster than that for chlorine. Even small concentrations of ozone (0.01 to 0.04 ppm) can be detected by humans by smell (Guzel-Seydim *et al.*, 2003).

Ozone was permitted by the Food and Drug Administration for the treatment of drinking water in 1982; it was also allowed to be used as a disinfectant in bottled water (FDA, 1982). In 2001 ozone was approved to be used as an antimicrobial agent for the treatment of raw and minimally processed fruits and vegetables (FDA, 2001).

Due to the instability of ozone, it has to be generated on site. The half-life of ozone in distilled water at a temperature of 20°C is approximately 20 to 30 minutes (Khadre *et al.*, 2001). As is the case when using chlorine as a disinfectant, ozone's half-life is decreased when the organic content of the solution is high, this is because the ozone readily oxidises the organic molecules. The most effective way to produce ozone commercially is through the use of pulse injected corona discharge.

The mechanisms of bacterial destruction by ozone need to be further elucidated. It is known that the cell envelope of bacteria is made up of polysaccharides and proteins, and it is also known that in Gram-negative organisms fatty acid alkyl chains and helical lipoproteins are present. Disinfection by ozone is thought to occur through the rupture of the cell wall (Macauley *et al.*, 2006). When the effectiveness of ozone as a disinfectant was measured, there was little or no disinfection up to a certain dosage. At higher levels the sanitizing effect increased greatly. For complete disinfection a surplus of ozone must be maintained in the solution to assure that all microorganisms have been contacted. The bacteria are sealed by a relatively solid cell membrane and their vital processes are controlled by complex enzymatic systems. Ozone interferes with the metabolism of bacteria cells, most likely through inhibiting and blocking the operation of the enzymatic control system. A sufficient amount of ozone breaks through the cell membrane, and this

leads to the destruction of the bacteria, possibly by oxidising thiol groups in enzymes and proteins (Macauley *et al.*, 2006).

The significant advantage of ozone use as a disinfectant over hypochlorite use is that ozone quickly decomposes to oxygen, whereas chlorine has been shown to produce other compounds that have been found to be associated with causing cancer. Ozone is also a more powerful oxidant than chlorine; therefore, it should also reduce the numbers of microorganisms more effectively than hypochlorite. The practicalities of ozone production and effectiveness must be elucidated in this study in order to determine of this disinfectant could potentially be used in replace of chlorine.

1.5.3 Bacterial resistance to disinfectants.

Resistance to disinfectants in Gram-negative bacteria, such as those being investigated in this study, is generally an intrinsic or natural property of the organism and such strains are generally more resistant to disinfectants. It has already been discussed previously how for a disinfectant molecule to reach its target site the outer layers of a cell must be crossed; these outer layers may act as a permeability barrier, leading to reduced uptake of these disinfectant molecules (Russel *et al.,* 1994). The bacteria may also constitutively synthesize enzymes that can degrade the disinfectant molecules (Bloomfield *et al.,* 1994). These mechanisms are chromosomally controlled. The outer membrane of Gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types of antimicrobial agents (Russell *et al.,* 1988; Ayres *et al.,* 1993). Peptidoglycan, which is present in higher quantities in Gram-negative bacteria compared to Gram-positive bacteria, may play a role in this resistance. Porins restrict molecules that are greater than 600-800 Da in size, however, disinfectant molecules are relatively small, and so they may readily pass through the membrane. The cytoplasmic or inner membrane may provide one mechanism of intrinsic resistance. The cytoplasmic membrane is composed of lipoprotein and could prevent the passive diffusion of hydrophilic molecules.

Microorganisms also undergo physiological changes when they encounter a new environment. As discussed previously, the association of bacteria with solid biotic or

abiotic surfaces leads to the formation of a biofilm, when there is sufficient time interval between bacterial colonisation onto the surface of the food and the time when the food is washed with the disinfectant. Biofilms are important for several reasons, mainly biocorrosion, reduced water quality and foci for contamination of hygienic products (Anderson *et al.,* 1989; Anderson *et al.,* 1990). Bacteria in different parts of a biofilm experience different environments and therefore this affects their physiological properties. For example, within the depths of a biofilm, nutrient availability is likely to reduce growth rates, therefore affecting susceptibility to disinfectants. The reduced sensitivity of bacteria within biofilms to disinfectants can be explained in different ways. There may be reduced access of a disinfectant to the cells within a biofilm, the microenvironment may be altered, degradative enzymes may be produced, and there may also be genetic exchange between cells in a biofilm. Many aerobic microorganisms develop intrinsic defence systems that confer tolerance to peroxide stress. The oxidative-stress or SOS response has been well studied in *E. coli* and *Salmonella* and includes the production of neutralising enzymes (including catalases, peroxidases, and glutathione reductase) to prevent cellular damage and also to repair DNA lesions (including exonuclease III) (Demple *et al.,* 1991; Demple *et al.,* 1994; Storz *et al.,* 1994). In both *E. coli* and *Salmonella* increased tolerance can be obtained by pre-treatment with a subinhibitory dose of hydrogen peroxide (Demple *et al.,* 1983; Winquist *et al.,* 1984). Studies have shown that pre-treatment induces a series of proteins, some of which are under the positive control of a sensor/regulator protein (OxyR), including glutathione reductase and catalase (Storz *et al.,* 1994) and other non-essential proteins that accumulate to protect the cell (Mukhopadhyay, 1997). Cross-resistance to heat, ethanol, and hypochlorite has also been reported in studies (Musser *et al.,* 1995; Chesney *et al.,* 1996; Dukan *et al.,* 1996). As discussed previously, studies have shown that due to cross-resistance, constant use of disinfectants may also increase the development of resistance to antibiotics used therapeutically (Moken *et al.,* 1997; Mangalappalli-Illathu *et al.,* 2008).

The location of microorganisms on produce surfaces may affect their inactivation by disinfectants. Bacteria attach to pores, stomata, broken or cut areas or natural indentations of salad leaves, therefore also allowing internalisation to occur, which protects the bacteria

against sanitising agents. Studies have shown that the ineffectiveness of many sanitising agents on strawberries is to do with the surface roughness of the fruit, the indentations allowing the bacteria to attach more firmly and form biofilms (Yu *et al.*, 2001). If the salad is cut then the surface area for bacterial attachment is increased further, therefore, greater internalisation and subsequent protection from detergents can occur. Sap contents that are released when leaf surfaces are cut also increase the organic load in the disinfectant solution, thereby reducing the effectiveness of the sanitising agent (Rodgers *et al.*, 2004).

Studies have also shown that the surface structure of lettuce can protect *E. coli* O157:H7 cells from disinfection by chlorine (Ukuku and Sapers, 2001). If this is the case then the potential use of pulsification must be investigated, in order to remove as many cells from niches on the matrix surface as possible, and hopefully thereby enabling the microorganisms to be more susceptible to subsequent disinfection.

1.5.4 The physiological signalling molecule, nitric oxide, for pathogen detachment and enhanced disinfection susceptibility.

Nitric oxide (NO) is a free radical that can either gain or lose an electron to energetically more favourable structures (the nitrosonium cation, NO^+ or the nitroxyl radical, NO^-). Up until the late 1980s nitric oxide was considered to be a toxic gas, and it was not until the early 1990s that evidence began to emerge that NO was an integral part of physiological processes in animals (Mayer *et al.*, 1997). By the late 1990s NO was also identified as an important messenger in plant defence signalling against microbial pathogens (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Animal defence against microbial pathogens is also similar to that of plants; one of the earliest changes that are detected following pathogen attack in both is a rapid increase in reactive oxygen species (ROS). NO is an intermediate of the anaerobic respiratory pathway known as denitrification and is a by-product of anaerobic nitrite respiration in the enteric bacteria. Further studies then revealed NO to play a role in the regulation of normal plant physiological processes, including stomatal closure, growth and development (Neill *et al.*, 2002; Guo *et al.*, 2003; Pagnussant, 2003). The fact that NO leads to stomatal closure

(Desikan *et al.,* 2002; Garcia-Mata and Lamattina, 2002), would, however, be an added advantage in terms of disinfection processes since it should reduce the internalization of potential pathogens. NO functions include modulation of hormonal, defence and wound healing processes (rapid restoration of damaged tissue after application of Sodium nitroprusside, SNP) in potato leaflets, Paris *et al.*, 2007), as well as regulation of cell death, and a factor delaying leaf senescence. Recently, Tatiana *et al.* in 2007 showed that the expression of a NO degrading enzyme induced a senescence programme in *Arabidopsis.*

In animals, NO is generated primarily by cytosolic enzymes called nitric oxide synthases (NOS). In plants, equivalent plant enzymes are yet to be discovered using antibodies against mammalian NOS(s), although NOS-like activity has been reported widely. Recently, however, two groups of NOS-like enzymes have been identified in plants, a pathogen-inducible NOS (Chandok *et al.,* 2003) and a hormone activated NOS (Guo *et al.*, 2003). The first enzyme that was found for NO synthesis was nitrate reductase (NR), which has an important role in nitrogen assimilation and produces NO when nitrite (substrate for NR) can be accumulated (Yamasaki, 2000). NR has been recognized as a candidate for NO production during plant-pathogen interactions (Yamamoto *et al.,* 2003). Several studies have shown that after challenge with an avirulent pathogen or elicitor, NO accumulates in resistant plants and establishes a direct correlation between disease resistance responses against biotrophic pathogens and NO.

NO has been shown to promote germination, low levels promote leaf extension and root growth (Lesham and Haramaty, 1996). A reduction in NO levels was shown to be associated with fruit maturation and flower senescence by Beligni and Lamattina in 2001, showing that NO has an involvement in these physiological processes too. All of these roles of NO as a developmental regulator would be beneficial to fruit and vegetables. Furthermore, NO has been shown to act as a key signal in plant resistance to incompatible pathogens by triggering resistance associated hypersensitive cell death, or the hypersensitive response (HR). The HR is characterized by the formation of necrotic lesions at the site of infection of a pathogen, and these lesions function to restrict pathogen

spread and infection, therefore leading to plant disease resistance (Lamb and Dixon, 1997). After pathogen recognition, a complex signal transduction system has been shown to trigger defence responses based on accumulation of ROS and NO (Levine *et al.,* 1994; Keller *et al.,* 1998; Delledonne *et al.,* 1998). Delledonne *et al.* detected a peak of NO in soybean and *Arabidopsis* suspension-cultured cells after approximately six hours of challenge with an avirulent pathogen. The simultaneous increase of NO and ROS activate a hypersensitive cell death in soybean and tobacco cell suspensions; the independent increase of only one of these components ceasing the induction of cell death (Delledonne *et al.,* 1998). The kinetics of accumulation of NO and progression of the HR suggest that NO is involved in cell-to-cell spreading of HR rather than triggering cell death (Zhang *et al.,* 2003). The cytotoxic effects of NO and ROS derive from the diffusion-limited reaction of NO with O_2^- to form the peroxynitrite anion $ONOO^-$ which then subsequently interacts with cellular components (Koppenol *et al.,* 1992). The exact method by which NO cooperates with H_2O_2 to elicit hypersensitive cell death is still the object of extensive studies.

Plants react to pathogen invasion by eliciting a HR at the site of infection and by establishing systemic acquired resistance (SAR), which is defined as a long-lasting systemic immunity that protects the entire plant from subsequent invasion of a broad range of pathogens (Ryals *et al.,* 1996). Establishment of SAR is associated with the systemic expression of defence genes encoding pathogenesis-related proteins, whose physiological functions have not been clarified, although most possess anti-microbial activity (Van der Biezen and Jones, 1998). Salicylic acid (SA) plays a critical role during incompatible plant-pathogen interactions in both local and systemic resistance, resulting in the stimulation of the initial oxidative burst and leading to defence gene expression (Shirasu *et al.,* 1997). Durner *et al.,* in 1998 showed that NO treatment induced SA accumulation in tobacco, and therefore, these result indicated that NO plays an important role in the induction of signalling pathways leading to the establishment of SAR.

Exposure of plants to low levels of NO improved the response of plants to various stresses, Garcia-Mata and Lamattina in 2002 found that pre-treatment of wheat-seedlings to low levels of a NO donor enhanced tolerance to drought, NO was also shown to have an

antioxidant role, thereby protecting tomato plants from damage (Beligni and Lamattina, 2001). Beligni *et al.* showed in 2002 that in barley cells, the ROS-dependent giberellin-induced programmed cell death in the presence of NO was delayed. NO production has been recorded in response to high temperature, osmotic and UV-B stresses (Beligni and Lamattinia, 2001; Gould *et al.*, 2003).

Numerous studies have shown that bacteria within biofilms undergo coordinated dispersal events, in which attached biofilm cells convert to free-swimming planktonic bacteria (Sauer *et al.*, 2004). This process is thought to benefit bacteria cells, since it allows for the single free-swimming cells to travel to a new matrix area and colonise new habitats.

Two main types of stress have been shown to play important roles in biofilm dispersal and lysis, Webb *et al.*, in 2003 showed that this was due to the activation of a prophage and the generation of nitrosative or oxidative stress inside *P. aeruginosa* biofilms. Nitrosative stress is due to the production and damage produced by reactive nitrogen intermediates (RNI) including amongst others, nitric oxide (NO), peroxynitrite (ONOO), formed after reaction of NO with the superoxide free radical, $O2^-$), nitrous acid (HNO_2). Oxidative stress is due to exposure to reactive oxygen intermediates, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl ion (OH^-).

RNIs are small and potentially highly reactive molecules that can be continuously produced in the organisms as by-products of anaerobic respiratory metabolism. When the production of ROI and/or RNI overwhelms the capacity of the cell to remove these, DNA, protein and lipid damage (polyunsaturated fatty acids) may occur (Barraud *et al.*, 2006). The resulting products of this oxidation are, lipid hydroperoxides (when polyunsaturated fatty acids react with NO in aqueous phosphate buffer at pH 7.4) or lipid nitrates (when polyunsaturated fatty acids react with NO in the presence of cyclohexane), carbonylated proteins, and DNA with oxidised bases e.g., 7,8-dihydro-8-oxoguanine (Ischia, 2005; Covarrubias *et al.*, 2008). In proteins, one of the main targets for nitrosation are tyrosine residues, the reaction involves one electron oxidation of the phenol ring to give a phenoxyl radical, which

couples with NO_2 to form 3-nitrotyrosine (Figure 20, Ischia, 2005). This results in loss of structural integrity, enzyme inactivation, receptor modification and enhanced proteolysis.

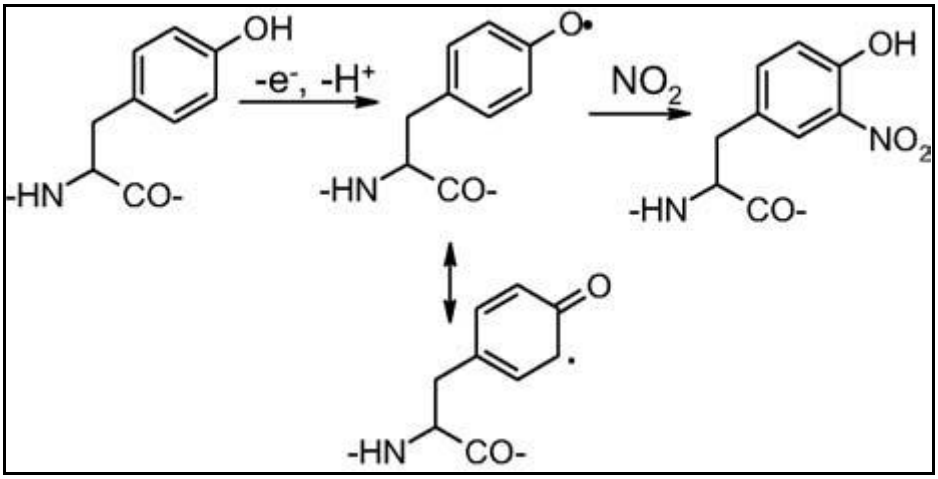


Figure 10: Tyrosine nitration in proteins (From Ischia, 2005).

The main speciality of mammalian phagocytes (including leukocytes and activated macrophages) is their high output of ROI. High output production of RNI is also attainable by many mammalian cells in response to appropriate inflammatory stimuli. Macrophages can produce superoxide and nitric oxide in almost equal amounts, and therefore, they can produce high levels of the combination of these, peroxynitrite (OONO-). Any dietary RNI are utilised in mammals as antimicrobial agents in gastric juice (a main component of the innate immune system of the epithelium). In mammals, phagocytes sacrifice themselves and any volume of uninfected host tissue to prevent microbial metastasis, resulting in the formation of an abscess (Xian and Zweier, 1997). Similarly, plants have the hypersensitivity reaction, although this does not involve phagocytes, it depends on the production of ROI and RNI and results in the formation of an abscess (Bolwell, 1999). In addition to their damaging properties, ROIs are also involved in signalling and regulatory pathways in prokaryotic and eukaryotic organisms (Nathan, 2003; Thannickal, 2003). In eukaryotic organisms NO and other RNI's derived from arginine have been shown to be essential in many physiological signalling roles, including in neurotransmission (Garthwaite 1991), the regulation of blood flow and pressure (Palmer *et al.,* 1987; Kilbourn *et al.,* 1993), in cell apoptosis, differentiation and proliferation (Moncada *et al.,* 1998).

Numerous studies in *E. coli* have shown that RNI activate global regulatory networks such as the SOS response (Lobysheva *et al.,* 1999) or the genetic response to oxidative stress controlled by OxyR and SoxRS (Demple, 1991).

High concentrations of NO were recently shown to effectively kill *P. aeruginosa* in its mucoid form in both planktonic and biofilm cultures, when it was added back in sterile supernatants from CF transplant patients and in the mouse model of chronic CF airway infection (Yoon *et al.,* 2006). Conversely, Barraud *et al.* in 2006 found that NO, the main precursor of ONOO- in vivo (Beckman *et al.,* 1990), was able to induce biofilm dispersal at concentrations in the nanomolar range that were non-toxic to wild-type *P. aeruginosa.* It was proposed that this was due to NO inducing biofilm mode cells to revert to the planktonic phenotype. Barraud *et al.* (2006) demonstrated that a *P. aeruginosa* mutant that lacked the only enzyme that is capable of generating metabolic nitric oxide through anaerobic respiration (nitrite reductase) did not lead to dispersal whereas enhanced dispersal was evident in a *P. aeruginosa* NO reductase mutant when exposed to low nanomolar concentrations of nitric oxide. A mechanism by which bacteria could detach from the biofilm phenotype was suggested by the *P. aeruginosa* microarray studies which previously revealed that upon exposure to NO, genes involved in adherence were down-regulated (Firoved *et al.,* 2004). Furthermore, this showed that bacteria that were exposed to low levels of NO were more effectively removed from surfaces using combined antimicrobial treatments (hydrogen peroxide, sodium dodecyl sulphate and tobramycin) compared to control antibiotic treated biofilms.

Barraud *et al*. (2009) have recently suggested a role for NO and cyclic-di-GMP signalling at nM concentrations in triggering the biofilm dispersal event. This event is induced by NO, as dispersal requires phosphodiesterase (PDE) activity and addition of NO stimulates PDE and induces the concomitant decrease in intracellular cyclic-di-GMP levels in *P. aeruginosa* (Barraud *et al*., 2009). Furthermore, gene expression studies indicated global responses to low, non-toxic levels of NO in *P. aeruginosa* biofilms, including upregulation of genes involved in motility and energy metabolism and down regulation of

adhesins and virulence factors. There is currently no literature investigating if NO has this effect on the release of coliforms in the phyllosphere.

Studies have shown that NO has a deleterious effect on the growth of wild type cultures of *E. coli*, in the presence and the absence of oxygen; it has been shown that NO can be toxic (Gardner & Gardner, 2002). The main mechanisms of the antimicrobial action of NO include interference with cell division and energy production at concentrations between 10-100µm (Lobysheva *et al.*, 1999), this is via inhibition of DNA synthesis and electron transport proteins due to the nitrosative deamination of DNA and the nitrosylation of protein SH groups (Natan, 1992; Keefer *et al.*, 1996).

Furthermore, the study of the effect of nitric oxide on the dispersal of coliforms and its potential use in food processing or the field environment is supported by the fact that recently nitric oxide has been found to inhibit shiga-toxin (Stx) synthesis by enterohaemorrhagic *E. coli* (Vareille *et al*., 2007). Shiga-toxin is the virulence factor of enterohaemorrhagic *Escherichia coli* (EHEC) and results in the development of haemolytic syndromes. In 2007 Vareille *et al.*, demonstrated that chemical or cellular sources of NO inhibit *stx* mRNA expression and Stx synthesis, without altering EHEC viability. This inhibitory effect is suggested to occur through the NO-mediated sensitization of EHEC; this was shown to be the case because mutation of the NO sensor nitrite-sensitive repressor results in loss of NO inhibiting activity on *stx* expression. If NO also has a positive effect on the dispersal of coliform bacteria from the phyllosphere matrix then there is potentially more reason to use NO in food processing. Numerous studies have shown that NO potentiates the antimicrobial and cytotoxic activities of ROI, could the same be applicable when NO donors are combined with antimicrobials such as hypochlorite which are also suggested to assert their bactericidal effects by oxidation.

1.6 Aims and objectives

The overall aim of the study has been to investigate enhanced recovery and molecular techniques for detection of viable pathogens and heterotrophic bacteria on the salad

phylloplane matrix as a means of understanding their persistence as attached biofilms and potential threat to human health.

The first objective of this study was to establish the characteristics of the matrix surface and presence of an autochthonous biofilm microflora, using novel light microscopy techniques. The literature indicates that due to the complex structure of the leaf surface very little is known about the endogenous microbes or coliforms on leaves. The natural microbial colonization patterns on the phylloplane were established in this Section, comparing watercress, spinach and lettuce. Initial attempts were made to develop a technique for the *in-situ* quantification of microorganisms on the phylloplane.

The next objective of this study was to determine the efficiency of the Pulsifier to detach foodborne pathogens from difficult matrices such as watercress leaves. At present the current industry adopted method is to use the Stomacher which uses mechanical waves to extract bacteria from matrices into solution. It was necessary to establish if the Pulsifier was more efficient than the Stomacher in cell recovery from the watercress phylloplane and if so, what the optimum working conditions of the Pulsifier were, so that the same could be carried out throughout this study for optimised sample preparation. This allowed the establishment of the characteristics of the microflora and pathogens such as *Salmonella*, on the phylloplane, since it could be determined how strongly/loosely attached these cells were depending on length of treatment required for detachment.

The third objective of this study was to determine the effect of chlorine, ozone and pulsification treatments on the numbers of coliforms and *E. coli* extracted from the surface of young and aged watercress leaves, using conventional cell culture methods and also adapted for detecting sublethally damaged, slow-growing cells.

The final objective was to investigate the potential ability of exogenous nitric oxide to disperse coliforms from the surface of watercress leaves. This was to determine whether this potent signalling molecule emulated the same dispersive activity as for *P. aeruginosa*

biofilms, and whether conversion of coliforms from the biofilm to planktonic phenotype rendered them more susceptible to disinfection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Leaf material

Unless otherwise specified, fresh baby salad leaves that were provided by Vitacress Salads were used in this study. The main leaves of interest were watercress, lettuce and spinach. One to two day old salad samples from Portugal were used throughout this study, unless otherwise specified. In one study (where stated) a comparison of the microbiology of the watercress phylloplane from Portugal and the USA were compared. The salads that arrived from Vitacress Salads for experimentation had been through a simple water wash, as was required by industrial collaborators, and also to ensure that as much organic matter and soil etc. were removed from the samples prior to investigation. Salad leaf samples were transported in cool boxes until arrival at the laboratory, where they were stored at 4°C until use.

In Chapter Three the lettuce, spinach and watercress phylloplanes were studied. From Chapter Four onwards, the watercress phylloplane was the main salad type used throughout this study, as required by the industrial collaborators.

2.2 Sterilization

Culture media, glassware, distilled water (to prepare ultra pure water) and heat-resistant plastics were sterilised by autoclaving at 121ºC for the recommended times, as suggested by the manufacturer.

2.3 Culture media

Both solid (agar) and liquid (broth) media were used for bacterial subculture. The media types used in this project are listed in Table 2. Media was dissolved in one litre of distilled water and sterilised by autoclaving for 15 minutes (if required). Heat-liable supplements, for example antibiotics were added aseptically to cooled media (55ºC).

Table 2: Culture media used throughout this project, with abbreviations and supplier.

Culture Media	Abbreviation	Bacterial Detection	Supplier
Tryptic Soya Broth	TSB	*E. coli, Salmonella*	Oxoid, UK
Tryptic Soya Agar	TSA	*E. coli, Salmonella*	Oxoid, UK
Nutrient Agar	NA	*E. coli, Salmonella*	Oxoid, UK
Rambach Agar		*Salmonella*	VWR, UK
Tryptone Bile-X-Glucuronide Agar	TBX	*E. coli*	Oxoid, UK
Violet Red Bile Agar	VRBA	Coliforms	Oxoid, UK
R2A Agar	R2A	Total heterotrophs	Oxoid, UK

2.4 Bacterial strains

Total heterotrophs, coliforms and *E. coli* that were naturally residing on the phylloplane were studied. In addition, GFP-labelled *Salmonella* Thompson was used for inoculation studies, details for which are in Table 3, below. This was grown in Tryptic Soya Broth with 50 µg/ml of the antibiotic kanamycin. The *Salmonella* Thompson strain RM2311 was kindly provided by R. Mandrell from the United States Department of Agriculture. This strain contains the plasmid pWM1007, which incorporates the gene that encodes the GFP protein. The plasmid also contains genes that are required for replication and for kanamycin resistance. The kanamycin resistance (*kan*) gene acts as a selectable marker for expression of the GFP (Brandl and Mandrell, 2002).

Table 3: The characteristics and source of the bacterial strain used in this study.

Bacterial Strain	Characteristics	Reference
Salmonella enterica serovar Thompson RM2311	RM1987 (pWM1007)	Brandl and Mandrell, 2002).

2.5 Storage of Viable Organisms

2.5.1 Preservation of *Salmonella* Thompson using the Protect bead protocol.

From a young (18-24 hours), solid culture (either stab or slant) the colonies that were to be preserved were picked off using a sterile loop, making a thick suspension in the cryopreservation fluid (glycerol). The tube was capped and inverted six times, then left to stand for at least 30 seconds. The glycerol was then pipetted off until there was just enough left to cover the top of the beads. The glycerol/inoculum mixture was disposed of safely into an infectious agents bin. The vial was capped and the pathogen details recorded. This was repeated five times to ensure sufficient levels were available for future use. The vials were then stored at -70°C. In order to make removal of the beads easier in future, the vial was placed horizontally in the freezer for the first few minutes.

2.6 Growth Curves

Growth curves of *Salmonella* Thompson (GFP) were carried out as follows. Isolates were cultured in TSB for 16 hours at 37ºC with shaking, and subsequently diluted in TSB to 10^8 CFU/ml. A 100µl aliquot of diluted culture was then inoculated into 100 ml of pre-warmed TSB to give a density of approximately 10^5 CFU/ml. Cultures were incubated at 37ºC with shaking and the CFU/ml was determined by plating ten-fold serial dilutions onto TSA plates at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 hours.

2.6.1 Growth patterns of *Salmonella* Thompson.

Tryptic Soy Broth was prepared for *Salmonella* Thompson. Four universal tubes were labelled and 5 ml of broth was added into all four flamed universal tubes (three repeat samples and one control). A vial of the relevant pathogen was taken out of the freezer and a bead was removed using a sterile inoculating loop and dropped into the broth of the three replicate universal tubes (not into the control).

In order to obtain optical density readings, 100 µl volumes of each universal tube sample (as above) were pipetted into a microtitre plate, this representing time 0; the universals were then placed into the 37°C incubator. The microtitre plate containing samples at time 0 was placed into the OD reader (Tecan SunRise microtitre plate reader) at wavelength 620

nm, and the results were recorded onto a spreadsheet, allowing for the control broth (background) OD to be taken into account. This enabled growth curves to be produced for *Salmonella* Thompson.

In order to determine the effect of initial broth temperature on the growth of *Salmonella*, the optical density for this organism was recorded with a starting temperature of either 15°C or 37°C. It was established that the starting concentration of any broth should be approximately 37°C for efficient growth.

2.7 Preparation of sanitizing solutions.

2.7.1 Bore-hole water or PBS treatment

A bore-hole water or PBS treatment consisted of placing flat on the bench-top, 25 g of salad leaf sample in a Stomacher bag containing 225 ml of either, bore-hole water for 2 minutes (when investigating the effect of salad disinfection treatments in Chapters Five and Six) or PBS for 30 seconds (when investigating the effect of cell recovery procedures for laboratory use in Chapter Four).

Phylloplane samples were treated as described in Section 2.8

2.7.2 Hand-wash

A hand-wash treatment consisted of physically rubbing 25 g of salad leaf sample in a Stomacher bag containing 225 ml of either, borehole water for 2 minutes (when investigating the effect of salad disinfection treatments in Chapters Five or Six) or PBS for 30 seconds (when investigating the effect of cell recovery procedures for laboratory use in Chapter Four)

2.7.3 Hypochlorite

Hypochlorite solutions were prepared as specified by the guidelines. One Haztab tablet was added to 1 litre of distilled water to prepare the stock solution. Appropriate dilutions were made to obtain working solutions of 20 ppm, 50 ppm and 90 ppm. Fresh stock hypochlorite solutions were prepared weekly. Hypochlorite concentrations were recorded

pre- and post-treatment using the DPD assay, utilising DPD no. 1 tablets (VWR, International).

The DPD no. 1 tablet reacts with chlorine resulting in the production of the red coloured 'Wurster dye' which is the stable primary oxidation product. The chlorine concentration can be subsequently measured semi-qualitatively by a visual comparison of the colour of the measurement solution with the colour windows of a colour disk.

Phylloplane samples were treated as described in Section 2.8

2.7.4 Ozone

Corona discharge ozonators, as supplied by Steer Engineering were used; initially one that used air as its main supply, followed by an ozonator that used medical oxygen. The ozonator was set up and 1 litre of 4°C borehole water was added to the attached storage container. Concentrations of ozone incorporated into the borehole water were diluted to obtain final concentrations of 0.2 ppm. Ozone solutions were prepared immediately prior to use. Ozone concentrations were recorded pre- and post-treatment using the DPD assay, utilising no. 4 tablets (VWR, International). A third Corona discharge ozonator supplied by Ozone Industries was also used in this study. Unless otherwise specified the ozonator was allowed to run for 2 hours prior to use of the ozonated water.

As described for chlorine, ozone reacts with DPD no. 4 tablets to form a red-violet dye. The ozone concentration can be subsequently measured semi-qualitatively by a visual comparison of the colour of the measurement solution with the colour windows of a colour disk. Ozone concentrations were measured in triplicate at each time point.

Phylloplane samples were treated as described in Section 2.8

2.7.5 Sodium nitroprusside

A stock solution of SNP (NO donor) was prepared by dissolving 0.298 g SNP in 1 litre distilled water, resulting in a solution of concentration 1 mM. Dilutions of this were

subsequently prepared using distilled water, resulting in 500 nM, 20 nM and 5 mM SNP treatment solutions. All solutions were prepared immediately before use.

Phylloplane samples were treated as described in Section 2.8.

2.8 Preparation and treatment of phylloplane sample.

In order to ensure that the same conditions were utilised in all experiments throughout this study (unless otherwise specified), and to imitate those conditions that are used in the food industry, the following steps were adhered to for each treatment. These were established by visiting a local food factory and recording the decontamination conditions.

Treatment solution (225 ml) and the phylloplane matrix (25 g) to be tested were added to a Stomacher bag (Figure 11). The treatment bag was placed in an ice-bath at 4ºC, and treatment was allowed for either 2 minutes or 30 seconds, with a gentle agitation to adequately mix the contents.

When investigating disinfection for salad industries, a 2 minute incubation time was used (as described above) and for the control treatments borehole water was utilized. However, when investigating cell recovery by use of the Pulsifier (Pul100E, Microgen Bioproducts Ltd, Camberley) or the Stomacher, PBS and an incubation time of 30 seconds were utilized.

In most investigations, unless otherwise stated, pre- and post- stomached or pulsified samples were obtained (as specified). Borehole water or PBS samples, 30 second hand-washed samples and post-treatment samples were analysed.

After treatment (with bore-hole water or specific disinfection treatment), the suspension was quickly decanted into a measuring cylinder labelled ‘pre-pulsification’. The pre-pulsification samples determine numbers of viable cells (whichever are being tested for) after treatment. Immediately, 225 ml of PBS was added to the same Stomacher bag containing the same 25 g sample of watercress and this was mixed well. This was then

pulsified for 30 seconds to release adherent cells, and the PBS was immediately decanted into another measuring cylinder labelled as 'post-pulsification'. This was to determine if there were any viable cells (whichever were being tested for) present now, or if the treatment had attacked those cells that were attached to the leaf surface and killed them too.

The sanitizing solutions, as described in Section 2.7 were utilised during the treatment times. When required, all of the steps above were repeated in order to compare the effect of using the Stomacher for cell recovery instead of the Pulsifier. In this case, the Stomacher was therefore used to release the cells, in order for the quantification of viable numbers remaining after treatment.

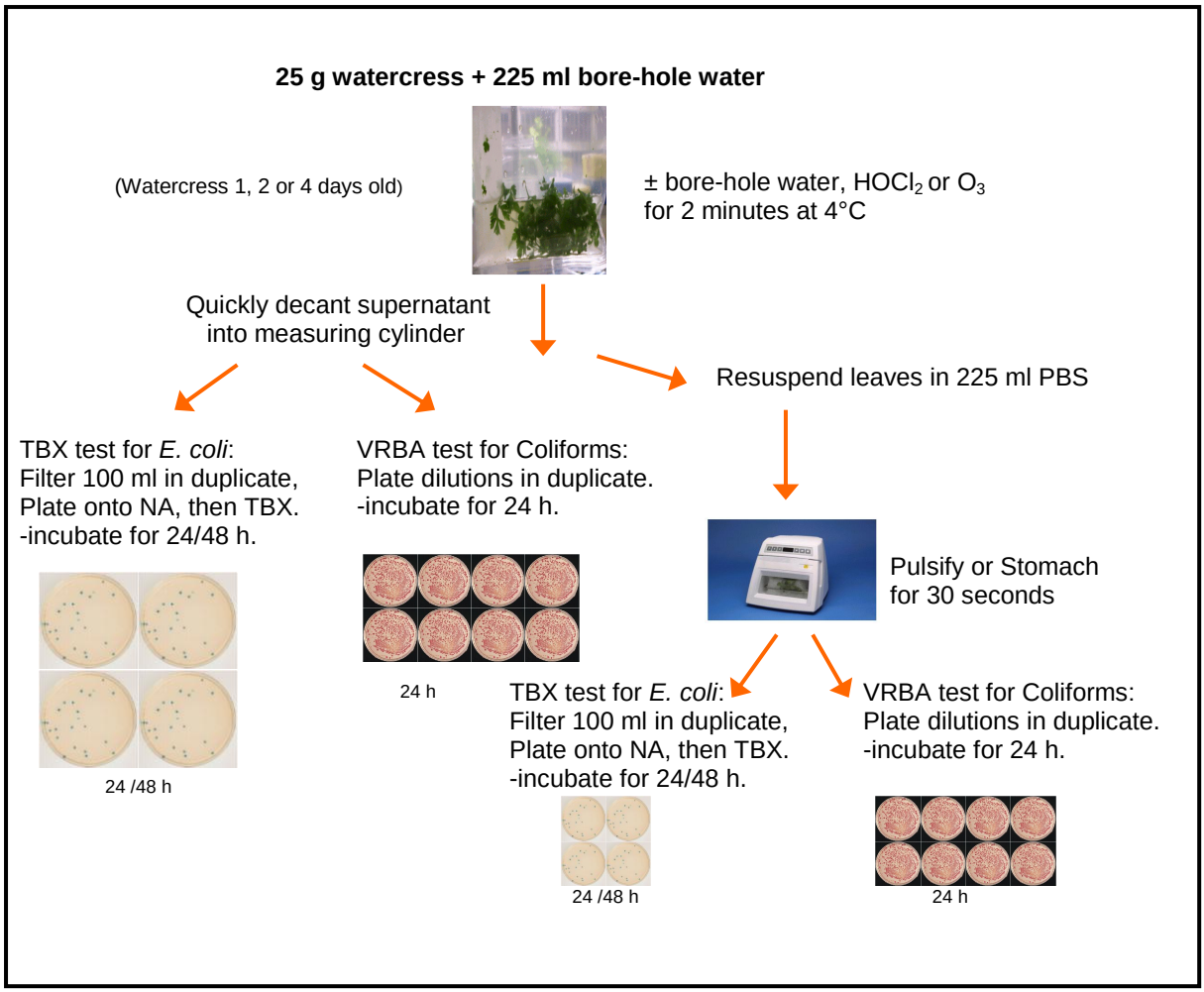


Figure 11. Watercress was treated for 2 minutes at 4ºC, followed by using TBX agar and VRBA to enumerate viable cells, both before and after pulsification.

2.9 *In situ* visualisation of the phylloplane.

2.9.1 Microscopic validation

2.9.1.1 Use of EDIC/EF microscopy

This study used the EDIC microscope for the first time to view the phyllosphere and its microenvironment; previously this technique has been used to visualize metal, tissues and plastics (Walker and Keevil, 1994; Keevil, 2001; Keevil, 2003).

Conventional differential interference contrast (DIC) microscopy works by splitting the source light into two polarized beams (from below the microscope) before it passes through the sample to be observed; the paths that the waves travel down are altered according to the thickness, size and refractive index of the sample. The final pseudo 3-dimensional image is visualized due to the variations in the sample resulting in interference between the two beams.

In EDIC microscopy, the prism that splits the light source is placed above the stage and light refracted from the sample is used rather than light that is transmitted through the sample from the base of the microscope. This makes EDIC microscopy ideal for observing opaque surfaces.

EDIC microscopy combined with epifluorescence (EF) microscopy was utilized for comparing and observing bright field images (EDIC) and fluorescent images of stained samples (EF). The relevant filter sets were used when investigating fluorescent microorganisms, as described for each protocol.

The EDIC microscope was supported by Best Scientific, Swindon with a high power metal halide illumination system and an image analysis software package (see Section 2.9.3).

2.9.1.2 Use of LSCM - visualization and image analysis with LSCM

In LSCM the specimen is irradiated in a point wise manner. The physical interaction between the laser light and the specimen fluorescence is measured point by point. To obtain information about a whole specimen, the laser beam is scanned across the specimen.

With a confocal LSM it is possible to exclusively image a thin optical section out of a thick specimen, by only collecting in focus light through a pin hole aperture. The thickness is referred to as the Z plane. In this way, a number of slices can be sectioned through a specimen and recorded at different planes; the specimen is moved along the optical (Z) axis in controlled increments. This results in a 3D data set, providing information about the spatial structure of an object.

Watercress phylloplane sample disks were analyzed using a Leica TCS SP2 LSCM equipped with a Leica DMR upright stand (Leica Microsystems Heidelberg GmbH, Germany), z-galvo stage and Leica LCS software package. Laser excitation of the samples was performed with an Argon laser (488 nm excitation wavelength) and a Melles Girot diode pumped solid state (DPSS) laser (561nm excitation wavelength), respectively. The position and bandwidth of the detection ranges for the emitted light were freely adjustable and set appropriately. Images were acquired at a bit depth of 8 bits.

Fluorescence of the GFP cells was detected with 488 nm excitation wavelength and an emission detection range of 500 to 540 nm. For the GFP channel the pinhole diameter was set to 114.53 µm (2.0 airy units). Autofluorescence of the chlorophyll of the leaves was detected with 561 nm excitation and within an emission range of 590 to 700 nm. To monitor autofluorescence the pinhole diameter was set to 20.0 µm (1.0 airy units). GFP fluorescence and autofluorescence were imaged in sequential scan mode at a scan speed of 400 Hz. Stacks of 40 images (z-stacks) were acquired at a step size of 488 nm along the z-axis. For each channel and in each focus plane 8 images were acquired and averaged. Due to time constraints, it was not possible to produce three independent repeats of this experiment; therefore, statistical analysis of the data obtained was not possible.

2.9.2 The preparation of leaf samples for microscopic viewing.

In general, four leaves were randomly collected from a particular study using a pair of forceps; subsequently, these leaves were cut into disks with a diameter of 1 cm, using a cork borer. Both the abaxial and adaxial surfaces were examined where stated and triplicate disks were obtained from each leaf. Samples were placed onto labelled glass

slides and observed using either x10, x50 or x100 objective lenses. Images were then taken as explained below for qualitative analysis (Section 2.9.3).

If it was possible, quantitative analysis was attempted for each sample disk to determine total counts *in situ*. As such, numbers of bacteria were recorded in 10 random fields of view for each sample disk using the eyepiece grid. A graticule slide could then be used to enumerate the area viewed on the grid so that counts could be converted to bacterial numbers per unit area of the phylloplane.

In addition, the 1 cm diameter watercress disks were directly inoculated with *Salmonella* Thompson by use of pipetting techniques in which the leaf surface was fully covered with approximately 10^8 cells. The watercress leaf samples (as specified) were allowed to dry in a laminar flow hood for 20 minutes; prior to drying, a cover slip was placed on the samples and it was fixed in place using nail-polish. The samples were then transferred to the confocal microscope.

For the experiments that were not directly investigating the use of EDIC/EF microscopy, 25 g of watercress or spinach leaves (as specified) were inoculated with *Salmonella* (as described in Section 2.11). Ten leaves were then randomly selected and disks were cut, as above; the leaves were subsequently dried and fixed prior to the use of EDIC/EF microscopy for analysis.

2.9.3 Image analysis

ImagePro plus software was used to capture the images viewed by the EDIC/EF microscope. Following image capture, measuring bars were added by use of ImagePro plus.

When using LSCM, images were acquired at a bit depth of 8 bits. For each detector channel, gain and offset of the detector were set in a way that made full use of the available range of grey levels. Image acquisition was performed using a HCX PL APO 63.0x 1.40 oil objective, a pixilation of 512 x 512 pixels, and a zoom factor of 1.88-times. After

fluorescence detection, stacks of 40 images (z-stacks) were acquired at a step size of 488 nm along the z-axis. For each channel and in each focus plane, 8 images were acquired and averaged. Further processing of the z-stacks with the Leica LCS software involved calculation of the projection along the z-axis (combined image of all image planes) or, alternatively, optical sectioning of the z-stack in three orthogonal planes.

2.9.4 Molecular methods for viewing the phyllosphere *in situ*

2.9.4.1 Use of the BacLight™ kit to determine viability

The BacLight™ (Molecular Probes) live/dead assay was evaluated for use on the phylloplane to confirm viability of attached bacteria. Solution A or SYTO 9 (5 µl) was added to a microfuge tube, subsequently, an equal volume (5 µl) of solution B or propidium iodide was added to the same microfuge tube, these were then mixed thoroughly. The dye mixture (3 µl) was then added to 1 ml of distilled water and thoroughly mixed. This was then added to salad leaf samples in a Petri dish and incubated at room temperature in the dark for 20 minutes. After the incubation time, any excess dye was allowed to drip from the leaf. The leaf samples were allowed to air dry on labelled glass slides in the dark, prior to visualization using the EDIC/EF microscopy as described above. The FITC filter set was used to visualize the SYTO 9 stained bacteria, and the TRITC filter to visualize those stained by propidium iodide.

The nucleic acid stain, SYTO 9, fluoresces green upon intercalation with dsDNA, ssDNA and RNA, and is able to penetrate bacteria with intact and damaged cell membranes, thereby labelling all bacteria in a population. Propidium iodide fluoresces red upon intercalation with dsDNA, ssDNA and RNA, however, it is only able to penetrate those bacteria that have damaged cell The combined use of these stains results in live bacteria in a population being labelled green (SYTO 9), and dead bacteria being labelled red. Quantitative analysis was attempted as described in Section 2.9.2.

2.9.4.2 Nucleic acid staining by use of diamidino-2-phenylindole (DAPI)

DAPI is a nucleic acid stain that stains dsDNA. The ability of DAPI to stain naturally residing bacteria on the watercress phylloplane was investigated. Leaf discs were covered

with 10 µg/ml of DAPI solution and the samples were subsequently stored in the dark for 20 minutes and then washed in sterile distilled water. The water on the surface of the samples was allowed to dry for 5 minutes in the dark, after which the leaves were viewed under the EDIC/EF microscope using the DAPI filter set. As described in Section 2.9.2, quantitative enumeration was attempted using the eyepiece grid.

2.10 Cell culture techniques to determine viable cells from the phylloplane.

Current industry adopted mechanisms for detecting viable numbers of recovered epiphytes and/or inoculated pathogens were utilised. Details of the manufacturers of the media used throughout this study can be seen in Table 2.

2.10.1 Total heterotrophs

R2A agar plates were prepared and the spread plate technique was utilized for enumeration of total viable heterotrophs recovered from the phylloplane (Figure 12). R2A is a low nutrient medium and combined with a lower incubation temperature (22°C) and longer incubation times (4 to 5 days), it may stimulate the growth of any possible stressed, sublethally damaged bacteria.

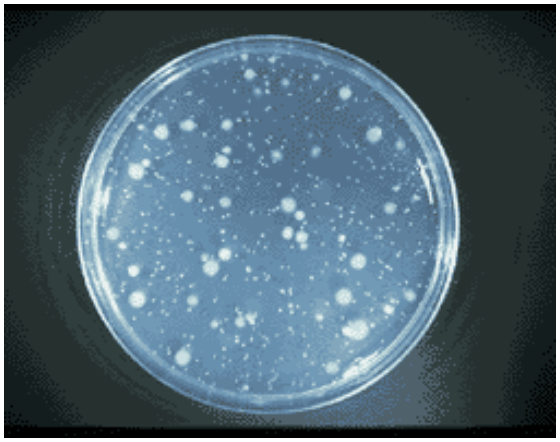


Figure 12: Total recovered heterotrophic bacteria growing on a Rambach agar plate.

2.10.2 Coliforms

VRBA was utilised for the enumeration of viable recovered levels of coliforms from the phylloplane (Figure 13). VRBA is a selective medium for the detection and enumeration of

coliform organisms in water, food and dairy products. An overlay methodology was used to improve the specificity of the medium. Samples containing the bacteria were appropriately diluted and pipetted in triplicate into labelled Petri dishes. To these samples 15 ml of VRBA that had previously been tempered to 45°C in a water bath was added. The Petri dishes were rotated in a clockwise and anticlockwise manner to ensure that the sample and agar were thoroughly mixed together; this was then allowed to solidify. Once this first layer of agar had set, a second layer of 5 ml VRBA was then pipetted over the original layer to cover it, and it was then also allowed to solidify. All of the plates were incubated for 24 hours at 37°C.

After incubation, all typical colonies that were pink-purplish red, had a diameter of +0.5 mm and were with or without halos were counted and the results recorded.

Figure 13: Coliforms growing on a VRBA plate.

2.10.3 *E. coli*

TBX was advocated for use in the water industry; and furthermore complies with the ISO/DIS standard 16649 for enumeration of *E. coli* in food and animal foodstuffs (Anon, 2001). TBX agar plates (Figure 14) were utilized as well as nutrient agar plates. After direct plating, no *E. coli* growth was evident; in order to ensure that this was in fact the case, the method was refined to make it more sensitive. A lower temperature pre-incubation stage was also incorporated to allow for any possible stressed, sublethally damaged cells to be resuscitated. After initial studies, results showed that due to the low

natural levels of *E. coli* residing on the phylloplane, a filter mechanism was necessary for concentration of the cells.

Using filter equipment, a volume of 100 ml sample solution was filtered through cellulose nitrate membranes. The cellulose nitrate membranes were subsequently placed onto nutrient agar and incubated for 4 hours at 37°C to resuscitate any possible stressed, sublethally damaged cells. The membranes were then transferred to TBX agar at 44°C for 24 hours to determine the viable numbers of *E. coli* surviving treatment. Triplicates of each sample were used and at least six samples were taken.

After incubation any blue colonies formed on the TBX agar plates were counted and recorded. At the start of the ozone experiments, the plates were re-incubated for another 24 hours, so as to detect any possible sublethally damaged cells (48 hours). TBX agar is a selective medium for the enumeration of *E. coli* in food products.

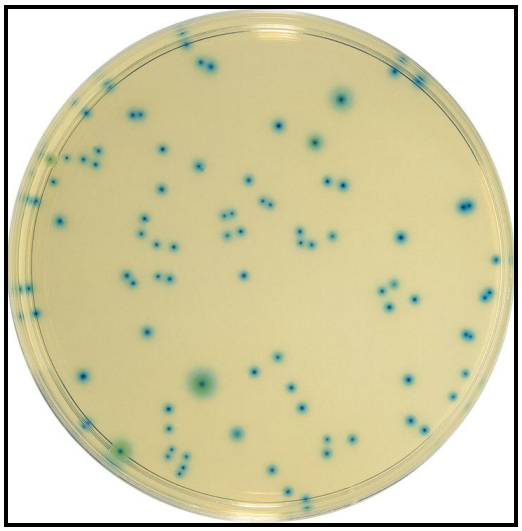


Figure 14: *E. coli* colonies growing on a TBX agar plate.

2.10.4 *Salmonella*

Rambach agar plates were prepared and spread plate techniques were utilized for enumeration of viable *Salmonella* from the phylloplane (Figure 15). Plates were incubated at 37°C for 24 hours.

Rambach agar is a species-specific positive chromogenic medium for the detection and selective isolation of *Salmonella* from complex environmental samples (Rambach, 1990). The *Salmonella* can be rapidly and reliably detected as red colonies.

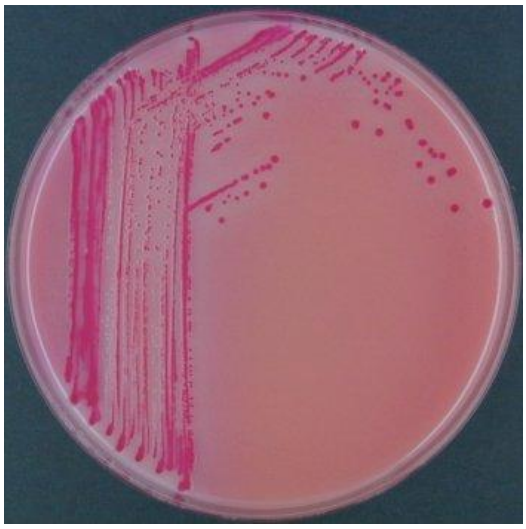


Figure 15: *Salmonella* colonies growing on a Rambach agar plate.

2.11 Use of GFP-labelled *Salmonella* Thompson to detect the viable pathogens on the watercress phylloplane.

The use of the EDIC/EF microscope was investigated for the first time in this study in combination with GFP-labelled *Salmonella,* to track the locations of bacteria on the watercress phylloplane.

GFP-labelled *Salmonella* Thompson was inoculated into TSB (10 ml) and allowed to grow at 37°C for 12 hours. Subsequently, appropriate volumes were then added to 225 ml of PBS in a Stomacher bag, to give an approximate final concentration of 10^{10} colony forming units (CFU) per ml. Following this, 25 g of watercress leaves were weighed out and added to the GFP-labelled *Salmonella* suspensions.

The Stomacher bags containing the leaf samples and the GFP-labelled inoculum were then left at room temperature overnight to allow for attachment. These were then ready to be used in subsequent experiments.

After the contact time, 225 ml of treatment water was added to the watercress that had been inoculated with the *Salmonella* Thompson and the appropriate treatment required was followed, as described in Section 2.8. After treatment, methods as described in Sections 2.9 and 2.10 were followed in order to obtain results for microscopic evaluation and for cell culture.

2.12 Enhanced recovery of coliforms by use of the Stomacher or the Pulsifier.

PBS (225 ml) was added to the watercress sample (25 g) in the Stomacher bag and this was stomached for 10 seconds. This was repeated, however, the stomaching duration was changed to 20, 30, 40, 50 or 60 seconds. The whole experiment was repeated three times in order to obtain an average. The same procedure was followed for the Pulsifier. Photographs were taken of the leaf samples at the optimal microbial release times for PBS-washed, stomached and pulsified samples.

The coliforms that were recovered were detected using VRBA, see Section 2.10.2.

2.13 Effect of air or no air in a treatment bag on coliforms extracted from the watercress phylloplane by the Pulsifier.

Twelve 25 g watercress samples were placed into separate Stomacher bags. One of the samples had 225 ml of PBS added; all of the air was removed out of that Pulsifier bag. This sample was pulsified for 30 seconds and the sample was subsequently serially diluted using PBS and then plated into VRBA plates. A second sample was filled with air prior to pulsification; this sample was once again serially diluted and then plated into VRBA, as described in Section 2.10.2. This was independently repeated 6 times for each treatment.

2.14 Quantification of leaf damage

Variations in the quality of the phylloplane (pre- and post- stomaching or pulsifying and with or without air) were validated by investigating quantitative effects on the suspension pre- and post-treatment. Optical density recordings were made in triplicate for each sample. Measurements were recorded as optical density at 620 nm, using a Tecan SunRise microtitre plate reader. The higher the value of the OD of a sample related to a greater

damage to the phylloplane, this was due to an excessive release of chlorophyll and debris. Photographs were taken of the suspensions after treatment in order for qualitative descriptions to be made.

2.15 The effect of shelf life on coliform or *E. coli* recovery.

Watercress that was 1, 2 and 4 days old was used in three independent experiments to determine the effect of shelf-life on the cells recovered after bore-hole water, hypochlorite and ozone treatment. Sample preparation was followed as in Section 2.8, followed by the appropriate tests for coliforms and *E. coli* (Sections 2.10.2 and 2.10.3).

2.16 Determination of the sterility of borehole water

Throughout this study, borehole water was obtained from borehole number two (Vitacress Salads Ltd). Before experiments were initiated, the sterility of the borehole water was tested. The *E. coli* and coliform detection steps were followed, as in Sections 2.10.2 and 2.10.3. The tests were repeated six times for accuracy.

2.17 Determination of background levels of *Salmonella* on the watercress phylloplane

A 100 ml aliquot of each of the samples (pre- and post-stomached) was filtered through the filter equipment in duplicate, using cellulose nitrate membranes. This filtration method was initially used since it was unclear as to how many *Salmonella* may be present in the samples; a 10 ml aliquot of each of the pre- and post-stomached samples was also filtered in duplicate using cellulose nitrate membranes (a 10 ml sample would be sufficient for low numbers of *Salmonella,* however, a 100 ml sample would be needed for greater concentration of the *Salmonella* if the numbers were very low). Samples were plated on agar media in triplicate.

The cellulose nitrate membrane filters were then transferred to Rambach agar plates and stored in the dark at 37ºC for 24 hours. After 24 hours the plates were removed from the incubator and the colonies counted (See Section 2.10.4).

2.18 Effect of ozone or hypochlorite treatment on the watercress phylloplane inoculated with *Salmonella* Thompson; use of revised protocol to reduce any quenching effect.

Salmonella Thompson was inoculated into samples as described in Section 2.11. The steps as in Section 2.8 were followed, however, every 30 seconds during the 2 minute treatment time, the treatment solution was refreshed (decanted quickly into a measuring cylinder, labelled, and then replaced with fresh treatment solution). After 2 minutes, the treatment solution was quickly decanted into another measuring cylinder (labelled as 'pre-pulsified'). To the same watercress sample, 225 ml of PBS was added; this was then pulsified for 30 seconds. After 30 seconds, the 225 ml of PBS was quickly decanted into another measuring cylinder (labelled as 'post-pulsified'). This protocol was followed in order to try to reduce any quenching effect caused by the organic content in the treatment bags.

2.19 Effect of 0.1 ppm ozone on numbers of coliforms naturally present on watercress leaves, using an industrial scale model at Vitacress Salads.

Vitacress Salads cleared line one of their salad washing line and allowed an ozonated water trial to be incorporated into that wash line. The aim was to retain the first wash (which removes organic debris), and to replace the subsequent 90 ppm chlorine wash with a 0.1 ppm ozone wash (see Figures 16, 17 and 18). Treatment time was allowed for 2 minutes at 4°C. Five 25 g samples of watercress were collected and bagged just before the ozone wash, 225 ml PBS was added to each of these Stomacher bags and then they were labelled and sealed. Five 25 g samples of watercress were then collected and bagged just after the 2 minute ozone wash, 225 ml PBS was added to each of these stomacher bags (to dilute any of the ozone); the bags were then labelled and sealed. These 10 samples were then brought back to the laboratory.

Each of the samples were in turn: pulsified for 30 seconds, serially diluted, and samples was taken from the Stomacher bags and plated into VRBA to determine coliform numbers, as described in Section 2.10.2.

Figure 16: Line one of several wash lines available for use at Vitacress Salads.

Figure 17: Watercress being washed at 4ºC, in a borehole water wash, to remove organic content and soil etc.

Figure 18: A large industrial scale ozonator at Vitacress Salads, being tested for use.

2.20 Numbers of coliforms recovered from the watercress phylloplane with two separate pulsification steps.

The aim of this protocol was to investigate the potential use of pulsification as a means for mechanical disinfection of the phylloplane without the use of any chemicals (rather than solely to recover bacteria for sample preparation for diagnostic studies).

Samples were prepared as described in Section 2.8. The samples was pulsified for 30 seconds, after which a sample of this treatment water was removed and plated out in duplicate into VRBA (see Section 2.10.2), this was labelled as "A". This PBS water was quickly decanted and replaced with 225 ml fresh PBS. The sample was then pulsified again, to find out how many cells were "missed" in the first pulsification step. This was independently repeated six times.

2.21 Effect of a 30 second pulsification step being incorporated into treatment prior to 50 ppm hypochlorite treatment on coliform numbers.

Samples were prepared as described in Section 2.8. The samples were pulsified for 30 seconds, after which an aliquot of this treatment water was removed and plated out into VRBA (see Section 2.10.2) this was labelled as "A". Hypochlorite solution was added to this treatment bag in order to obtain a final concentration of 50 ppm; the treatment sample was then quickly placed in an ice-bath at 4ºC for 2 minutes. After 2 minutes, a further sample was removed from this and plated into VRBA; this was labelled as sample "B". The hypochlorite treatment water was quickly decanted and replaced with 225 ml PBS, the sample was then pulsified again for 30 seconds and the sample plated out into VRBA, this was labelled as "C". This was repeated independently for six samples. The experiment was then repeated for another six samples, however, this time no hypochlorite solution was added at stage "B", this was in order to investigate the effect of pulsification as a means for mechanical disinfection combined with chemical disinfection (hypochlorite) via enhanced recovery (rather than only for use in improving detection).

2.22 Numbers of coliforms recovered from the watercress phylloplane after treatment with varying SNP concentrations followed by hypochlorite disinfection or pulsification.

Watercress (25 g) was added to 25 different Pulsifier bags. Subsequently, either 225 ml of 4°C bore-hole water, 20 nM SNP, 500 nM SNP or 5 mM SNP was added to the Pulsifier bags, each treatment being replicated four times. The treatment bags were then incubated at room temperature for 12 hours. After the twelve hour treatment time, a sample was removed from each treatment bag and serial dilutions were performed, these were plated into VRBA (see Section 2.10.2) in triplicate and were labelled as 'A', this showed the number of cells recovered post-treatment but pre-pulsification. Hypochlorite stock solution was then added to the treatment bag to produce 50 ppm hypochlorite treatment, and treatment was allowed for 2 minutes. After chlorine treatment, a sample of the treatment water was once again serially diluted and plated out into VRBA in triplicate, this was labelled as 'B' and showed the cells surviving chlorine treatment. Finally, the hypochlorite treatment water was quickly decanted off and replaced with 225 ml of PBS. The watercress was pulsified for 30 seconds and samples removed, serially diluted and plated out into VRBA in triplicate. This was labelled as 'C' and showed the number of cells that were protected from hypochlorite treatment, and the number of cells that were not removed from the leaf during the initial treatment (stage A). This indicated which concentration of SNP had the greatest effect on coliform detachment from watercress leaves. The VRBA plates were incubated at 37°C for 24 hours (Section 2.10.2).

The above protocol was repeated for:

Four borehole water samples; four 20 nM SNP samples and four 500 nM SNP samples. Two of each of the bore-hole water, 20 nM SNP and 500 nM SNP samples were treated with 50 ppm hypochlorite solution. The remainder (two bore-hole water, two 20 nM SNP and two 500 nM SNP) samples were not treated with hypochlorite and served as the controls in terms of chlorine disinfection. All twelve treatment samples were pulsified.

The flow chart, Figure 19, shows an outline of this protocol for further clarification.

2.23 The effect of 12 hour, 30 minute and 10 minute incubation times on coliforms recovered from the watercress phylloplane, with 20 nM and 500 nM SNP treatment.

Watercress (25 g) was added to a Pulsifier bag, followed by 225 ml of borehole water. The watercress was gently rubbed for 10 seconds, and then a sample was removed from this and plated out onto VRBA. This was labelled as A* and showed the number of coliforms present initially, i.e. at time zero.

Watercress (25 g) was then added, to twelve different Pulsifier bags. Subsequently, the different treatments were carried out by adding either 225 ml of 4°C bore-hole water, 20 nM SNP, 500 nM SNP or 5 mm SNP in replicates of four to the Pulsifier bags. A sample of each of the 16 treatments was plated in triplicate into VRBA as above (see Section 2.10.2). This was to determine the number of viable coliforms present before treatment, i.e. at time 0, A*. The treatment bags were then incubated at room temperature for either 10 minutes, 30 minutes or 12 hours. After the treatment time, a sample was removed from each treatment bag and serial dilutions were performed, these were plated into VRBA in triplicate, and were labelled as 'A', this showed the number of cells recovered post-treatment. Hypochlorite stock solution was then added to the treatment bag to produce 50 ppm hypochlorite treatment, treatment was allowed for 2 minutes. After chlorine treatment, a sample of the treatment water was once again serially diluted and plated out into VRBA in triplicate, this was labelled as 'B' and showed the cells surviving chlorine treatment. Finally, the hypochlorite treatment water was quickly decanted off and replaced with 225 ml of PBS. The watercress was pulsified for 30 seconds and samples removed, serially diluted and plated out into VRBA in triplicate. This was labelled as 'C' and showed the number of cells that were protected from hypochlorite treatment, and the number of cells that were not removed from the leaf during the initial treatment (stage A). The VRBA plates were incubated at 37°C for 24 hours as described in Section 2.10.2.

The above protocol was repeated for:

Four borehole water samples, four 20 nM SNP samples, and four 500 nM SNP samples. Two of each of the borehole water, 20 nM SNP and 500 nM SNP samples were treated with 50 ppm hypochlorite solution. The remainder (two bore-hole water, two 20 nM SNP

and two 500 nM SNP) samples were not treated with hypochlorite and served as the controls in terms of chlorine disinfection. All twelve treatment samples were pulsified.

The flow chart, Figure 20, shows an outline of this protocol for further clarification.

2.24 The effect of bore-hole water, 20 nM and 500 nM SNP treatments on the shelf-life of watercress and spinach leaves.

Samples (25 g) of watercress and spinach were treated with 225 ml of bore-hole water, 20 nM SNP or 500 nM SNP for 30 minutes in triplicate. The samples of watercress and spinach were then analysed for sensory attributes. They were stored for 0 to 13 days at 4°C, and shelf-life was determined through a visual assessment everyday. On each day the sample was placed on a sterile white plate (22 cm diameter) before being presented to randomly selected panellists. The bore-hole water sample, together with samples treated with 20 or 500 nM SNP were coded with random three digit numbers and simultaneously presented to each panellist on each day of analysis. The untrained panel consisted of three undergraduate or PhD students in different disciplines. They visually evaluated produce for appearance, colour, bruising, water-logging of the leaf tissue, leaf blackening, tearing, slime production, in order to determine the overall quality. Sensory attributes were rated by assigning scores, a score of 1 being in a bad condition and that of 3 being in a good condition. All evaluations were carried out within 1 hour of removal of samples from storage.

2.25 The use of a biochemical kit for the identification of the types of coliforms recovered from watercress leaves with different treatment conditions of SNP.

The purpose of this test was to identify the coliforms that were being recovered from the watercress leaves after treatment with different concentrations and incubation times of SNP. After treatment, as described in Section 2.10.2, the samples were plated into VRBA. Subsequently, after incubation, colonies that were identified as coliforms, but had physical differences, were removed using an inoculating loop and emulsified in sterile 0.85% saline.

The Microgen GN-ID *enterobacteriaceae* identification assay was used. The GN-ID assay comprises microwell strip, GN A. Each strip contains 12 standardized biochemical

substrates for the identification of Enterobacteriaceae. A saline suspension of the sample to be identified was initially used to reconstitute the dehydrated substrates in each well. If the organism metabolizes the individual substrates a colour change occurred during incubation or after the addition of other reagents. This was recorded and interpreted into a profile number.

Microgen Identification System Software (MID-60) was used to interpret the profile number obtained from the GN A strip, this allowed for the identification of the test microorganism.

2.26 Statistical analysis

All experiments had at least three independent repeats unless otherwise stated; this was in order to obtain sufficient data for statistical analysis. Analysis was performed using the SigmaStat 3.5 software package.

1	2	3	4	5	6	7	8
							
25g:225ml WX : H2O [BORE-HOLE WATER]	25g:225ml WX : H2O [BORE HOLE WATER]	25g:225ml WX : SNP [SNP 20nM]	25g:225ml WX : SNP [SNP 500nM]	25g:225ml WX : SNP [SNP 5mM]	25g:225ml WX : SNP [SNP 20nM]	25g:225ml WX : SNP [SNP 500nM]	25g:225ml WX : SNP [SNP 5mM]
X2	X2	X2	X2	X2	X2	X2	X2

OVERNIGHT 12 HRS RTP

[A] PLATE SAMPLE INTO VRBA (24 HR INCUBATION 37°C)

ADD 50ppm $HOCl_2$ TO THE TREATMENT WATER
2 MINUTES 4°C

[B] PLATE SAMPLE INTO VRBA (24 HR INCUBATION 37°C)

DECANT TREATMENT WATER, REPLACE WITH 225ml PBS, PULSIFY 30 SECONDS

[C] PLATE SAMPLE ONTO VRBA (24 HR INCUBATION 37°C]

Figure 19: SNP protocol One.

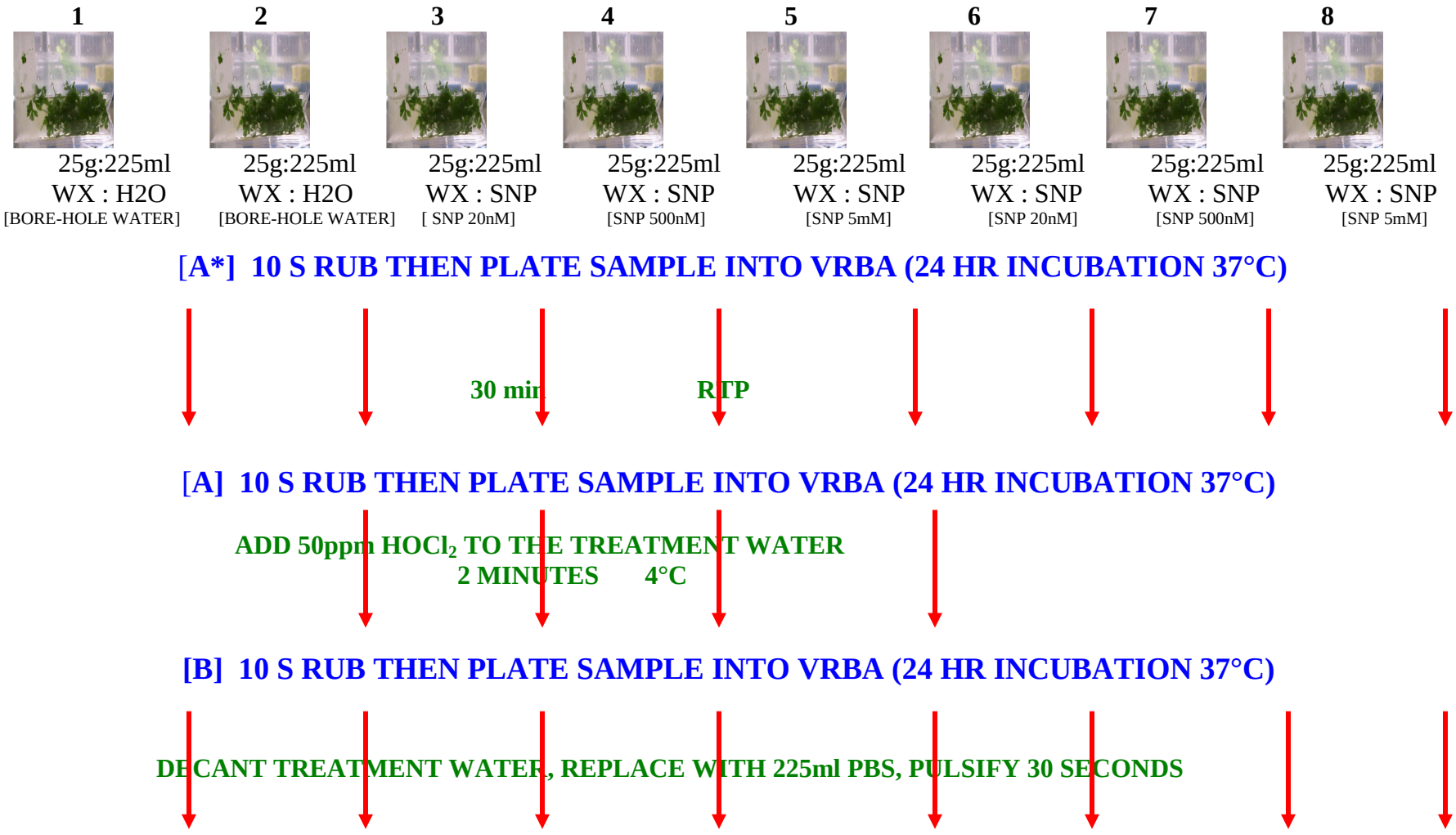
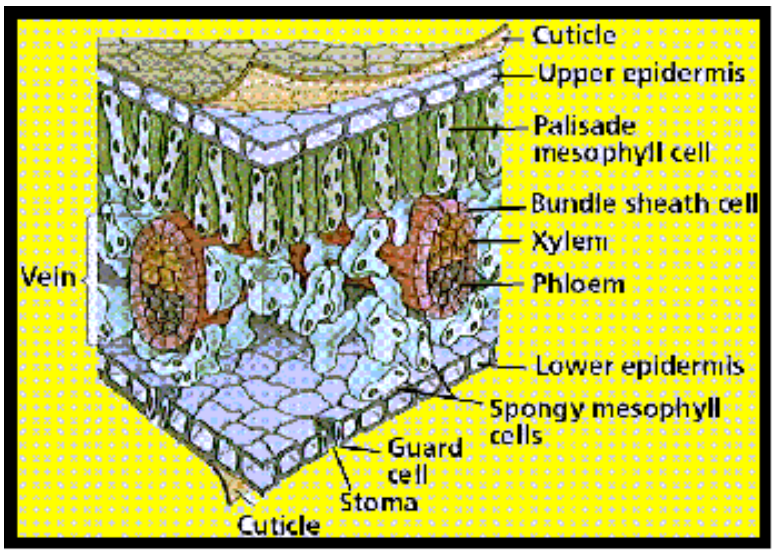


Figure 104 : SNP protocol Two.

CHAPTER THREE

USE OF THE NOVEL EDIC/EF MICROSCOPE FOR ENHANCED EXAMINATION OF THE PHYLLOPLANE AND TRACKING ZOONOTIC PATHOGENS



3.1 Introduction

The EDIC microscope has been shown to be more advantageous than other types of microscopy due to its rapid and non-destructive nature on even complex, opaque convoluted surfaces (Keevil, 2003), and this suggested that its use may also provide valuable insight into phyllosphere structure and physiology. The literature indicates that due to the complex structure of the leaf surface little is known about the location and structural integrity of endogenous microbes, coliforms or zoonotic pathogens on leaves. The *in situ* detection of microorganisms on the phylloplane is also less advanced since it is difficult to analyse leaves due to the autofluorescent properties of their photosynthetic pigments (Lang *et al.,* 1991). Techniques that are currently being used worldwide may not be entirely accurate, since these microscopy methods require some degree of sample preparation which could alter the dynamics of the living leaf. The natural microflora colonization patterns across the lettuce, spinach and watercress leaf surfaces was established and compared in this section using EDIC microscopy. At present, there are few studies that have demonstrated the presence of biofilm on the phylloplane; therefore, one of the aims of this study was to attempt to directly show biofilm *in situ* using EDIC microscopy. The commonly used stains, BacLight^{TM} (for differentiating live/dead bacteria) and DAPI (for detecting total microbial cells) were also evaluated for use on the watercress and spinach phylloplanes. These stains were also tested for use in the possible quantitative analysis of the leaf matrix microflora.

One of the most important zoonotic pathogens that have been shown to be the cause of gastroenteritis due to the consumption of fresh produce is *Salmonella*. There have been few studies to date describing how *Salmonella* spp. interact with the phylloplane to prolong persistence. Several studies have utilized LSCM to track GFP-labelled strains on the leaf surface (Brandl and Mandrell, 2002; Lapidot *et al*, 2006). However, the studies are difficult to perform with convoluted leaves and they do not readily differentiate endogenous microbial cells or biofilm to investigate pathogen interactions. These studies also do not directly show the localisation of pathogens in relation to all of the topographical features on the leaf surface. Consequently, *Salmonella* Thompson that had been genetically modified with green fluorescent protein was inoculated onto the surface of the watercress phylloplane and the EDIC/EF microscope was used to establish the colonization patterns of this pathogen. LSCM was also used in this section of work for

comparison with EDIC/EF microscopy and to investigate if any GFP-labelled *Salmonella* Thompson cells became internalized after a minimal incubation period.

The pattern of microflora interaction on the leaf surface may have an effect on the attachment of new cells to the matrix and/or the detachment of cells pre- or post-disinfection. Therefore, the effects of leaf washing on the microbial distribution, biofilms and inoculated pathogens was also investigated.

3.2 Methods

Please refer to Chapter Two.

3.3 Results

3.3.1 Evaluation of EDIC microscopy for the study of the lettuce phylloplane matrix.

Prior to tracking the fate of inoculated pathogens on the phylloplane, it was essential to characterize the structure of the phylloplane matrix and bacterial colonization using EDIC microscopy. The EDIC microscope was used to scan the lettuce leaf surface, locate stomata, and identify areas of the leaf with greater risk of bacterial contamination. The leaf matrix was observed directly without prior treatment, cover slips or specimen fixation.

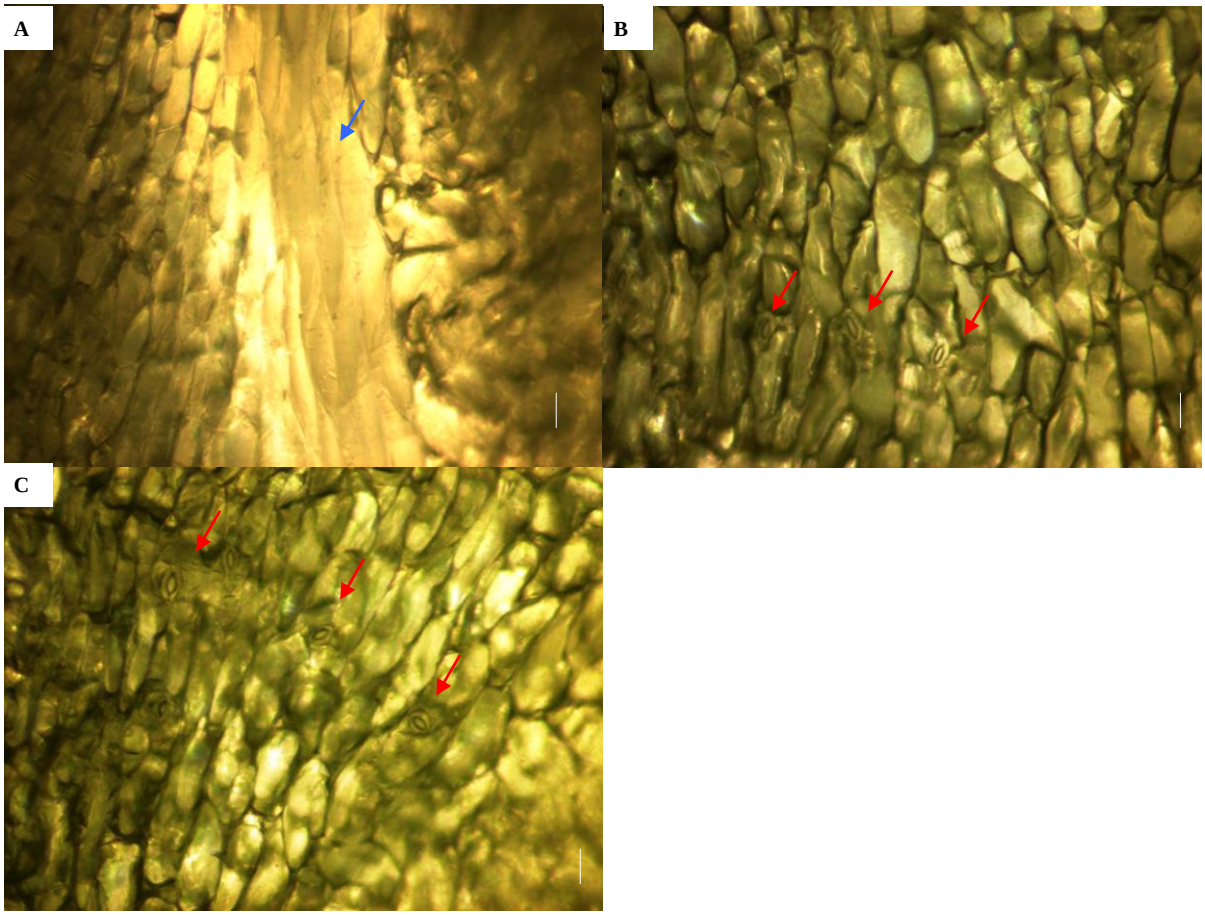


Figure 21. EDIC micrographs of lettuce leaves: (A) Xylem and phloem (blue arrow), (B) Outside of lettuce; epidermal cells and stomata (red arrows) are visible, (C) Inside of lettuce; epidermal cells and stomata (red arrows) are visible. Bars depict 10 µm.

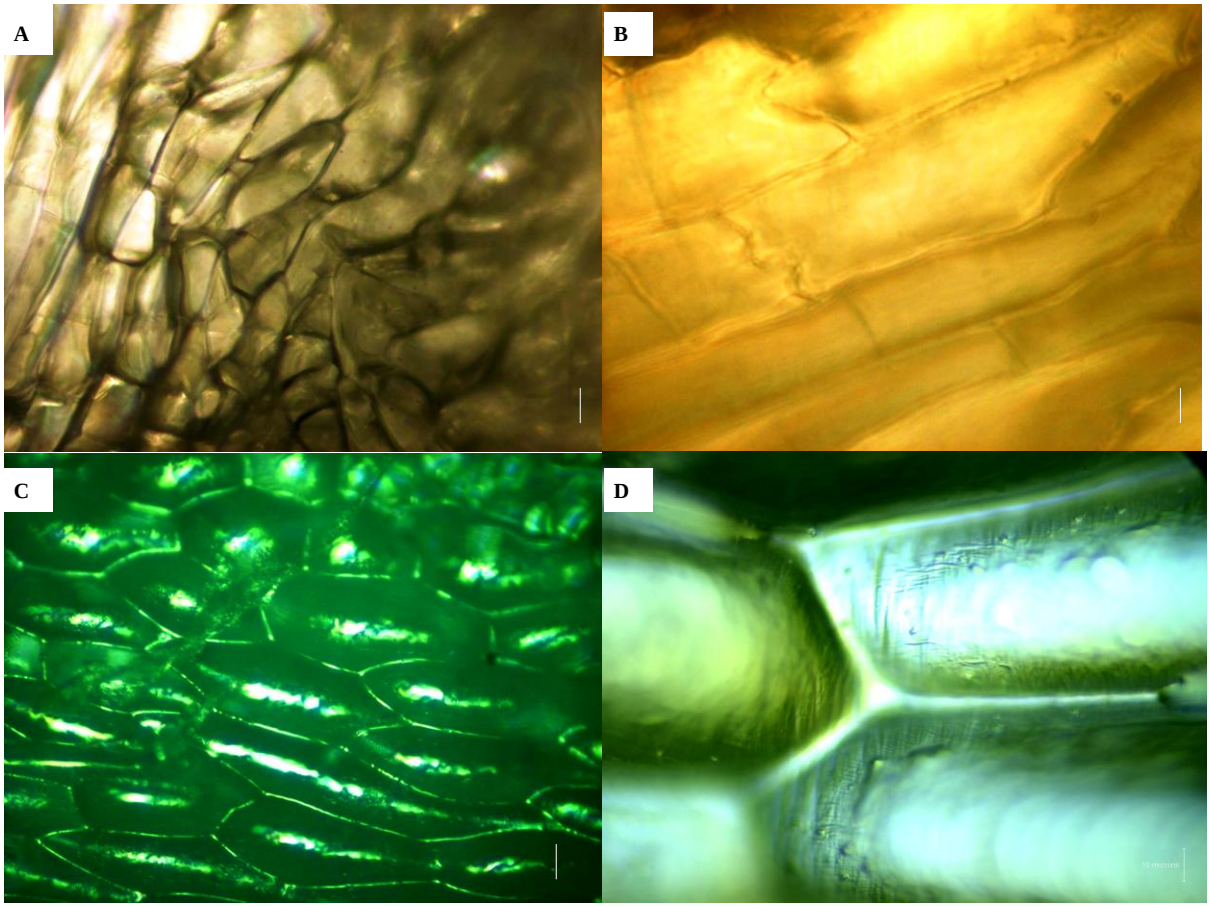


Figure 22. EDIC micrographs of lettuce leaves: (A) Stalk end of leaf, inside of lettuce; epidermal cells but no stomata are visible, (B) Mid-leaf epidermal cells, (C) Stalk end of leaf, outside of lettuce; epidermal cells, but no stomata are visible, (D) White stalk area, outside of leaf; showing areas of contamination or surface debris and larger epidermal cells. Bars depict 10 µm.

Figure 21 (A) shows that the xylem and phloem (blue arrow) of lettuce leaves can be easily resolved using EDIC microscopy and these regions offer large areas of grooves (approximately 30 – 40 µm in diameter) where endogenous microflora or pathogens might locate.

As can be seen in Figures 21 (B) and (C), epidermal cells of the main lettuce leaves (both inside and outside surfaces) are packed very closely. There is little intercellular space, and stomata (red arrows) can also be seen on the inside and on the outside of lettuce leaves.

Figures 22 (A), (B), (C) and (D) show that stomata are not present on the inner or outer surfaces of the white stalk of lettuce. Only epidermal cells can be seen at x100 and x500 magnification. The white stalk cells are larger, and therefore, there are fewer grooves for colonization compared to other regions. From these microscopy pictures the structural

environment that pathogens face if they were to be on the lettuce leaf surface can now be clearly seen. From these images, it is apparent that, lettuce leaves contain minimal debris and/or minimal areas of contamination; it is likely that contamination is confined to the edges of the epidermal cells.

3.3.2 EDIC microscopy to assess the structure and microenvironment of the spinach leaf matrix, before and after washing.

In this Section, the structure and microenvironment of the spinach matrix was investigated, using EDIC microscopy. These spinach leaves were then compared to the lettuce leaves examined in the previous section.

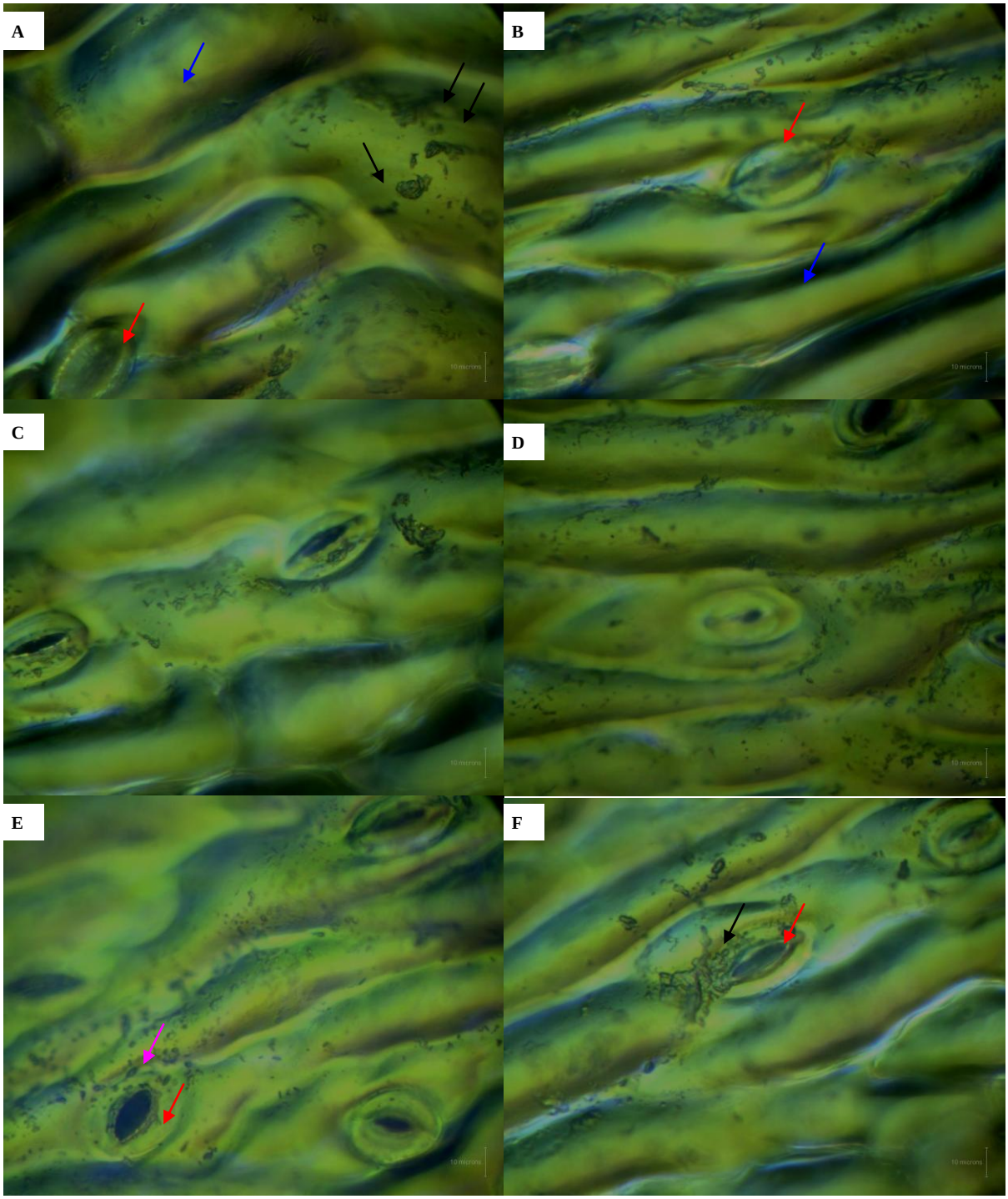


Figure 23. EDIC micrographs of spinach leaves: (A) Epidermal cells (blue arrows), stomata (red arrows), and 3D contamination (black arrows) can be seen; (B) Mid-leaf, showing epidermal cells (blue arrow) and stomata (red arrow); (C) Unwashed leaf, (D) Washed spinach leaf sample; it is apparent that on average no significant difference is seen between washed and un-washed phylloplanes; (E) Surface contamination varies, high numbers of single bacteria (pink arrow) can be seen close to the stomata (red arrow) (F) A large aggregate of bacteria (black arrow) is present close to the stomata (red arrow). Bars depict 10 µm.

It is apparent from the high quality images obtained in Figure 23, that spinach leaves have a much greater degree of surface contamination than lettuce, and the epidermal cells are

more irregular in shape (blue arrows). Stomata (red arrows) can be seen to be present on both the under-side and upper-side of the leaf surface, as seen with lettuce leaves.

In Figures 23 (A) and (B) it is apparent that individual bacterial bacilli cells are present (appropriate size and shape of microbial cells) as well as large 3D clusters (black arrows); in general, this distribution is across epidermal cells and cell margins, there being no apparent preference for attachment of these on spinach. The debris that is present on the leaves as 3D clusters can be seen to be slimy in appearance. The relative strength of attachment of cells and debris was investigated by comparing hand washed, Figure 23 (C), with un-washed, Figure 23 (D). It is apparent that no significant difference can be seen between both.

In Figure 23 (E), high numbers of single bacterial cells (appropriate size and shape of bacterial bacilli) and clusters can be seen to be present close to stomata, and many are present on the guard cells. Figure (F) shows a large mass of slimy debris and bacterial cells, which are approximately 40 µm wide (black arrow) in the region of a stomata (red arrow).

3.3.3 EDIC microscopy to assess the structure and microenvironment of the watercress leaf matrix, before and after washing.

In this Section, the structure and microenvironment of the watercress phylloplane matrix was investigated using EDIC microscopy. This was then compared to the lettuce and spinach phylloplanes examined previously.

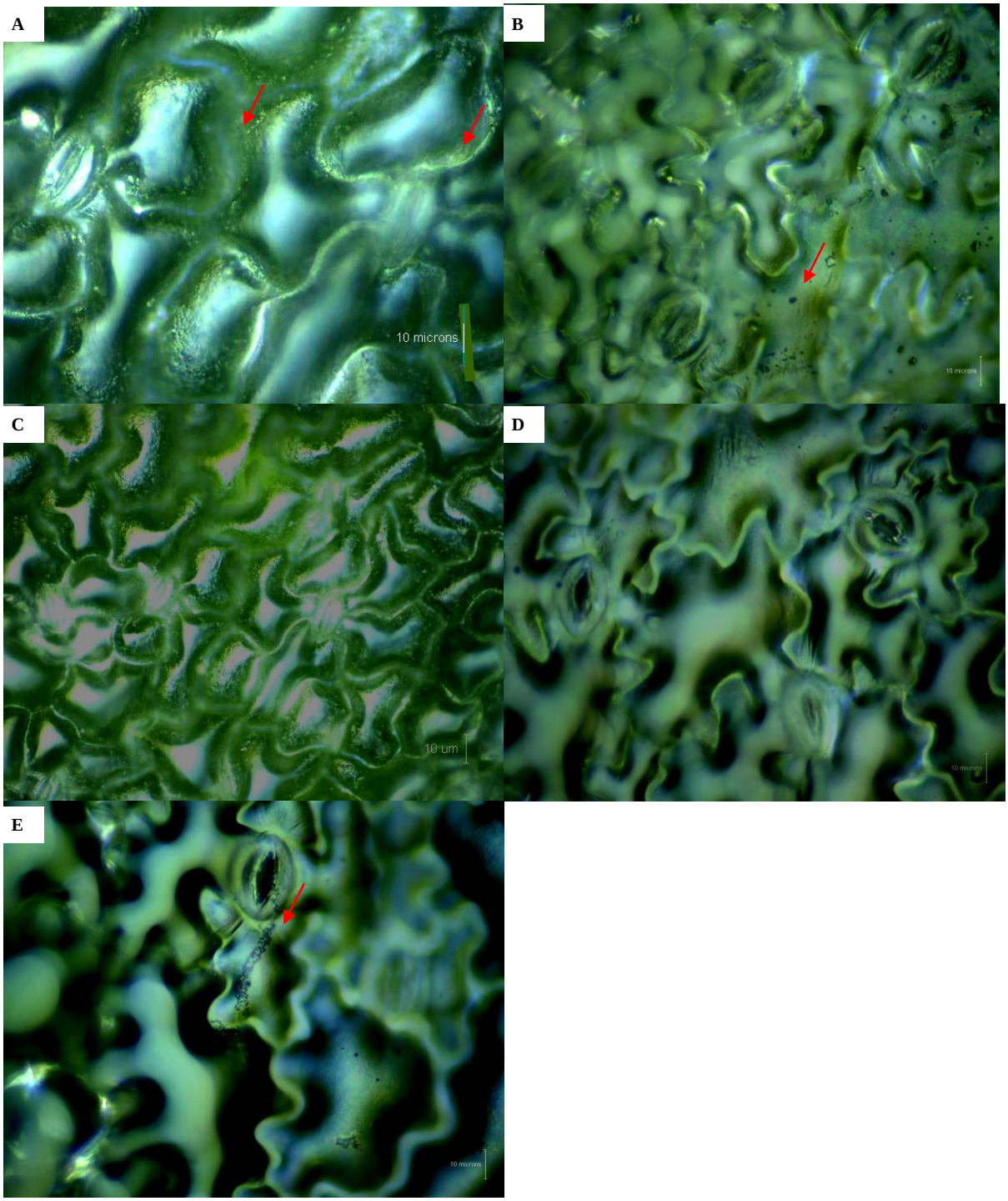


Figure 24. EDIC micrographs of watercress leaves: (A) Epidermal cells and stomata, some contamination and debris that is mainly located in the cell margins is apparent (red arrows); (B) Epidermal cells, some contamination can be seen to be present in the centre of the epidermal cells (red arrow); (C) Epidermal cells, different samples are contaminated to different degrees (D) Un-washed leaf sample; (E) The same leaf sample as Figure D, that has now been hand-washed, strongly attached contamination leading into the stomata is visible (red arrow) and on average the same degree of contamination is apparent across both unwashed and washed leaf samples. Bars depict 10 µm.

As for lettuce and spinach leaves, the EDIC microscope was able to produce high quality images of the watercress phylloplane. In Figure 24, the epidermal cells can be seen to be more irregular in shape to those of spinach and lettuce leaves and the area of each cell is smaller. This would allow for greater crevices and grooves for attachment of bacteria in a set sample; and compared to spinach leaves, most bacterial contamination (red arrows) appears to be in these cell margins rather than across the whole cell, as is apparent in Figure 24 (A). Although some contamination and debris could be found in the centre of the epidermal cells, as in Figure 24 (B, red arrow), this was much less apparent and was close to stomata. The degree of debris and contamination is less than for spinach but greater than that of lettuce leaves, this may be due to the environmental conditions in which it is grown.

From Figure 24 it is apparent that the degree of contamination by debris and/or bacteria is quite variable across different fields of view, on same leaves and different leaves. This variability was also apparent on spinach leaves; however, the variability appears to be greater on watercress leaves. Figures 24 (A), (B) and (C) are contaminated to different degrees although all three are control leaf samples and are from the same batch of leaves.

Figure 24 (E) shows a typical field of view when watercress was washed by hand; Figure 24 (D) is the same leaf sample before washing. The degree of contamination on average can be seen to be the same, no significant difference was apparent as for spinach leaves. The debris also had slime-like appearance and was strongly attached to the phylloplane. In Figure 24 (E) a stomata with debris (red arrow) that is strongly attached is apparent, since this sample has undergone a hand wash and the debris has still not been removed. Several individual cells that appear to be the size and shape of bacteria can be visualized within and around this slimy debris, it is highly likely that this is a mass of bacteria that have formed biofilm and are entering the stomata.

3.3.4 Determination of live and dead endogenous microflora on the lettuce phylloplane using BacLight™ viability.

The aim of this section was to examine the location of live and dead bacteria on the surface of the lettuce leaf matrix, using the BacLight™ viability kit and the EDIC/EF microscope. The stains used in this kit were evaluated for use in staining and viewing cells on complex surfaces such as that of the phylloplane.

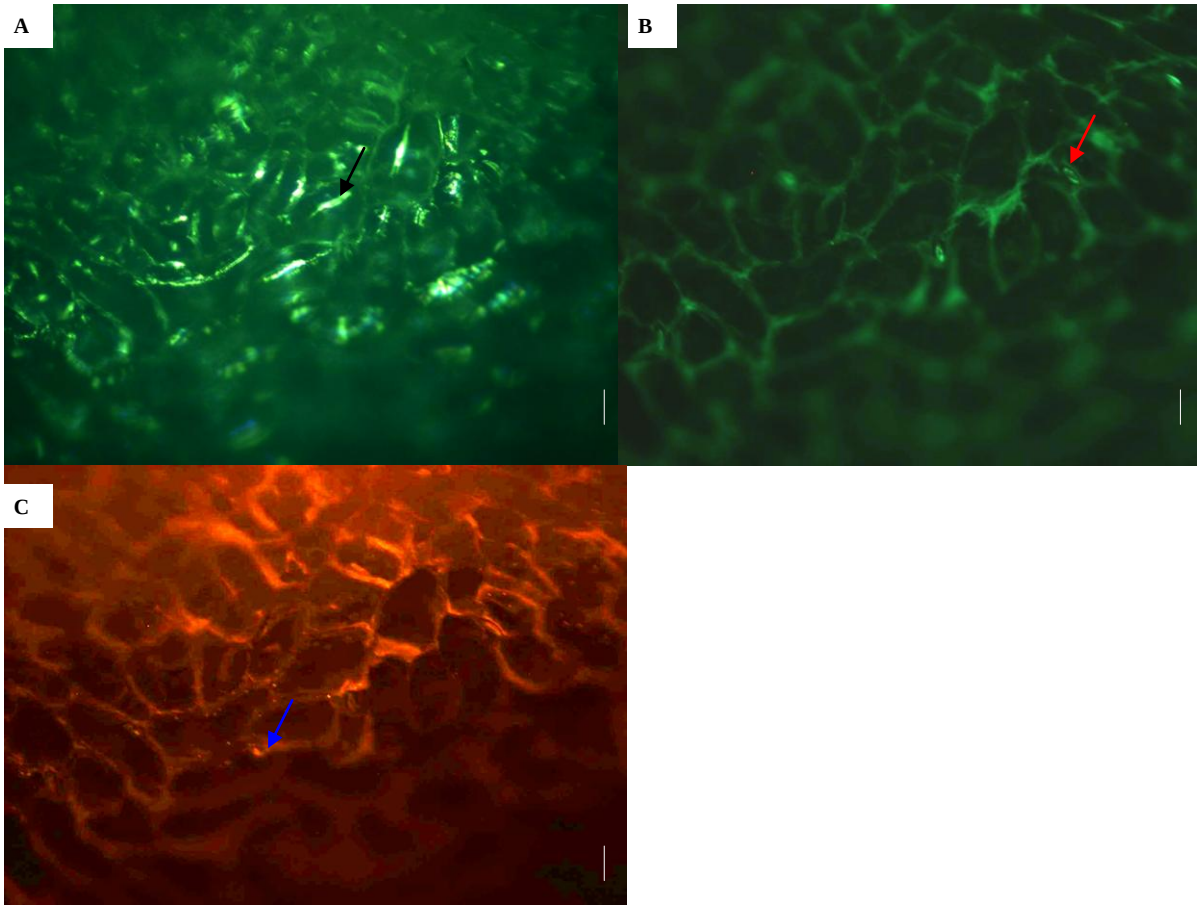


FIGURE 25: EDIC/EF micrograph of lettuce leaf, (A) Control - unstained leaf. Chlorophyll can be seen in the centre of most cells (black arrow), EDIC; (B) BacLight™ stained leaf viewed using FITC filter. Green, live natural microflora are visible around the edges of the lettuce cells and the stomata (red arrow). FITC filter. (480/500 nm). (C) BacLight™ stained leaf viewed using TRITC filter. Red, dead natural microflora are visible around the edges of the cells and the stomata (blue arrow). TRITC filter. (490/635 nm). Bars depict 10 µm.

The BacLight™ assay describes bacterial viability. The LIVE/DEAD BacLight™ bacterial viability kits use mixtures of SYTO 9 green-fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. These stains differ in their spectral characteristics and in their ability to penetrate healthy bacterial cells. The SYTO 9 stain labels all bacteria in a population, including those with intact and those with damaged cell membranes. Propidium iodide penetrates only those bacteria that have damaged membranes. Therefore, in the Figures above, bacteria with intact cell membranes have stained fluorescent green (live bacteria, red arrow) whereas those with damaged cell membranes have stained fluorescent red (dead bacteria, blue arrow). The TRITC filter set was used for viewing the dead bacteria stained by propidium iodide and the FITC set for viewing bacteria that stained green; bacteria can be deemed live if they stained green but not red.

In Figure 25 (B) and (C), it can be seen that the natural microflora on lettuce can mainly be found in the epidermal cell margins (in the grooves); this appears to be the case for both live and dead cells. In Figure 25 (C) the TRITC filter set was used and although individual and groups of cells that are the shape and size of bacterial cells can be seen, background fluorescence is greatly apparent. The red fluorescence of propidium iodide is close to that of chlorophyll (black arrow) and therefore, it is likely that when the TRITC filter set was used the high degree of background fluorescence was due to this. A similar result is evident in Figure 25 (B), where although individual cells are apparent, there is still a high degree of green background fluorescence; this may be due to the penetration of the SYTO-9 stain into the leaf interior, particularly the cell wall areas.

3.3.5 Comparison of the surface of watercress and spinach using EDIC/EF microscopy and BacLight™.

The aim of this experiment was to visualize the surface of watercress and spinach and the location of bacteria that are live or dead and to evaluate the use of the BacLight™ kit for the study of the phylloplane in combination with the EDIC/EF microscope.

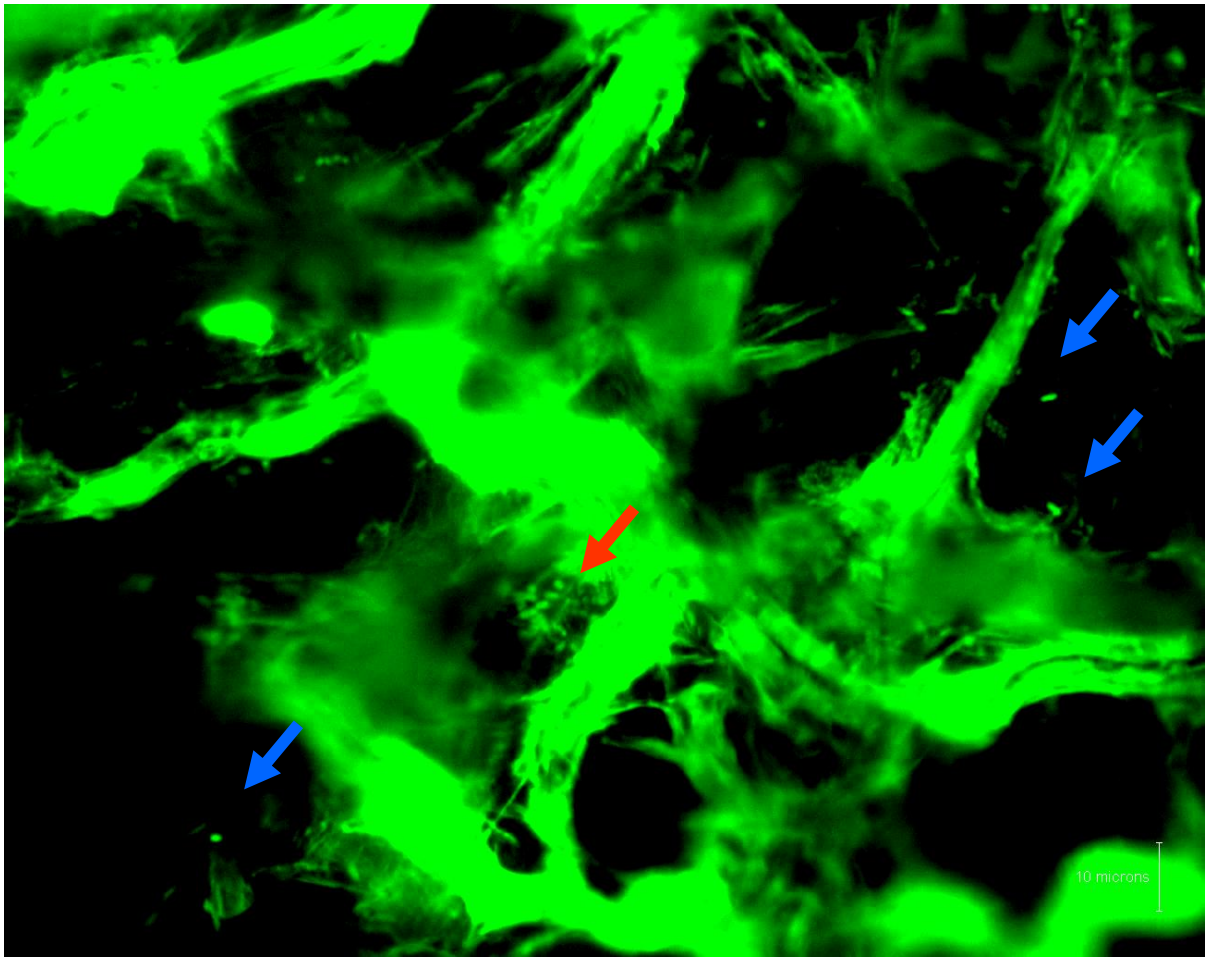


Figure 26: Photomicrograph of the watercress phylloplane; live microflora visible using the FITC filter set and the BacLight™ assay. Single bacteria are apparent in the centre of leaf cells, these may be more easily removed by washing/disinfection (blue arrows). The clusters of bacteria around the edges of leaf cells (forming biofilms) may be difficult to remove by washing/disinfection (red arrow). Bar depicts 10 µm.

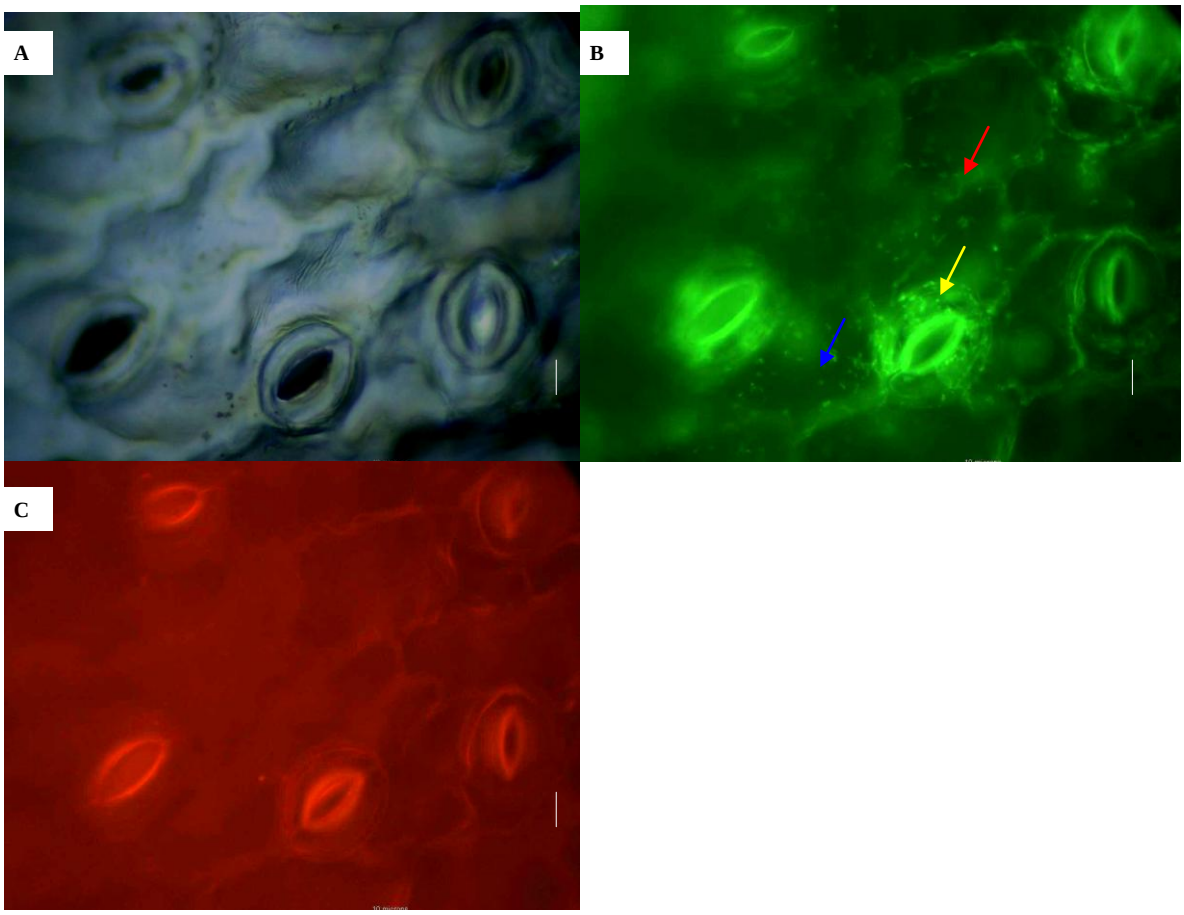


Figure 27: Live/dead microflora on the spinach phylloplane visible using BacLight™. (A) EDIC micrograph of the spinach phylloplane. (B) SYTO-9 stained leaf visualised using the FITC filter set. Single bacteria in centre of leaf cells may be more easily removed by disinfection (blue arrow). The aggregates of bacteria around the edges of leaf cells may be difficult to remove by disinfection (red arrow). There tends to be greater contamination around stomatal regions (yellow arrow). C: Propidium iodide stained spinach phylloplane visualised using the TRITC filter set; a high degree of chlorophyll autofluorescence is apparent. Bars depict 10 µm.

(a) Watercress with no treatment, stained with BacLight™.

Live microflora are visible in Figure 26, using BacLight™ staining and the EDIC/EF microscope with the FITC filter set. In each field of view through the microscope, it was apparent that the degree of contaminations varied greatly; some micrographs showed a high degree of bacterial contamination, others much less. In Figure 26, single bacteria that are shown with the blue arrows are visible more towards the centre of the leaf cells; these should be more easily removed by disinfection treatments. Clusters of bacteria can also be seen as the red arrows, these groups of bacteria are more frequent around the edges of the plant cells. It may be that these clusters of bacteria have formed biofilms and will be more difficult to remove by disinfection treatment. The clusters of bacteria could be shaken off

by vibrational means using the Pulsifier, and then they will be 'free' to be attacked by the disinfectants, as investigated in the next Chapters.

For watercress, the degree of auto fluorescence from the cytoplasm was very high; therefore, micrographs of the leaves before and after wash treatment using the TRITC filter for visualising dead bacteria had to be disregarded. A wash treatment was also incorporated into the protocol and the leaves stained, followed by viewing under the FITC filter set for live bacterial comparisons to Figure 26 above. No clear difference could be seen between the pre-hand wash images and the post-hand wash images (results not shown). This may be because it was difficult to view such differences when the microbial load on the leaf is already quite high or it may simply be the case that hand-washing does not remove large quantities of the microflora, which are therefore attached strongly.

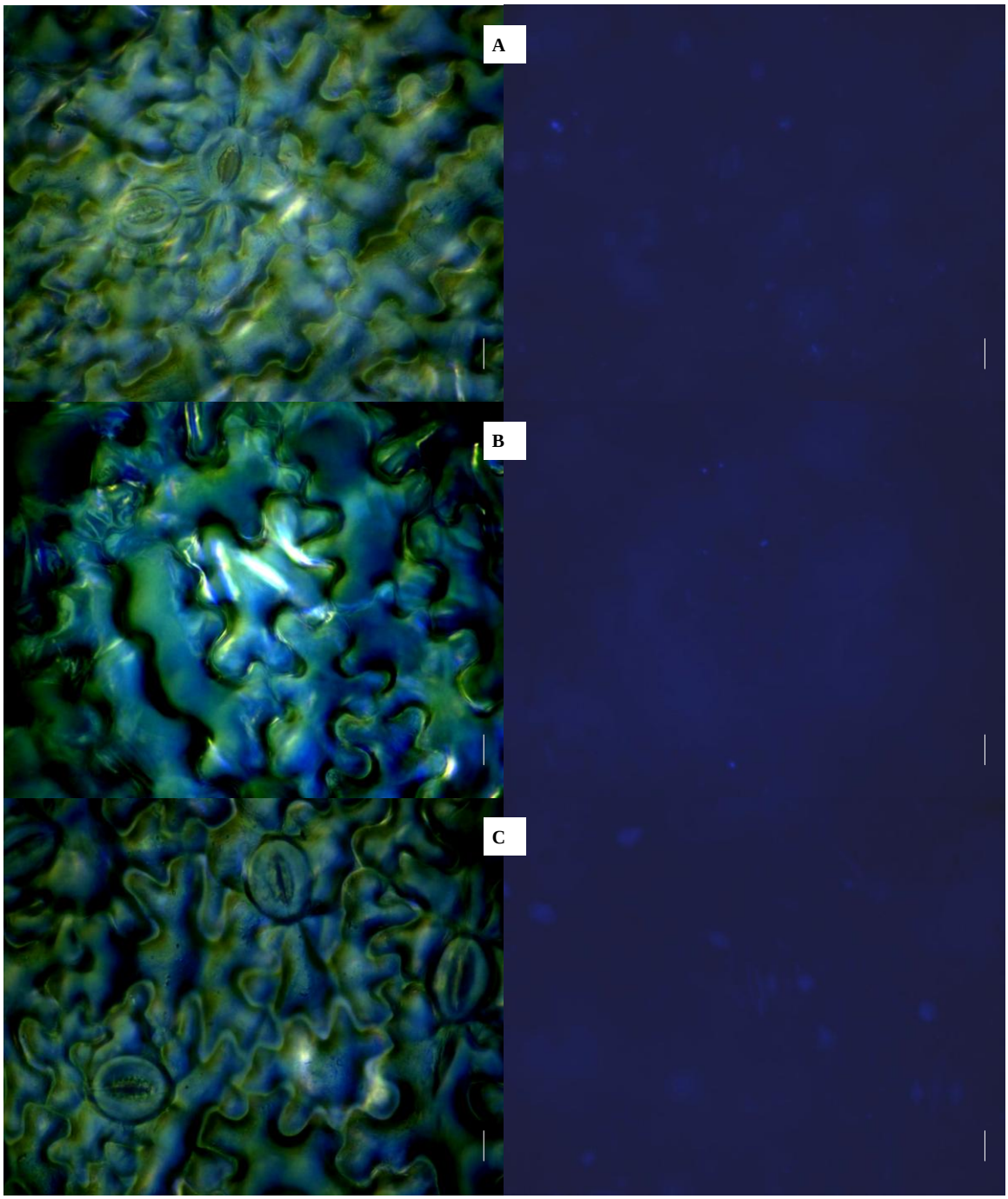
(b) Spinach with no treatment, stained with BacLight™. Magnification x100.

Figure 27 (A) to (C), shows a typical image obtained with the use of the BacLight™ kit on spinach leaves. In each field of view through the microscope it was apparent that the degree of contaminations varied greatly, some micrographs showed a higher degree of bacterial contamination than others. In Figure 27 (C), it is apparent that as for watercress and lettuce leaves propidium iodide staining and the use of the TRITC filter set results in a high degree of background fluorescence. However, some individual bacterial cells can be seen that are therefore dead. SYTO-9 staining and the use of the FITC filters set results in a much lower degree of background fluorescence and more individual bacterial cells are apparent. Clusters or groups of these live bacterial cells can be seen to be located close to stomata (red arrow), and individual cells (blue arrow) can be seen across most epidermal cells and are not confined to the cell margin or stomatal areas (yellow arrow).

The leaves were hand washed and viewed under the microscope to observe differences in localization of live or dead cells, as for the previous sets of results, the images viewed using the TRITC filter set showed a high degree of autofluorescence and minimal bacterial cells could be seen. Using the FITC filter set, live bacterial cells could be seen, and on average there was no clear difference in numbers between washed and unwashed samples. This suggests that live bacterial cells are attached strongly to this surface, and agree with the results produced above where this was apparent when viewing the phylloplane directly using EDIC microscopy.

3.3.6 Evaluation of the use of DAPI staining on the watercress and spinach leaf surface and its potential to quantify the number of cells naturally on the phylloplane.

The cyan fluorescent, nucleic acid stain DAPI, was evaluated for use as a quantitative tool for the detection of bacteria on the watercress or spinach phylloplane.



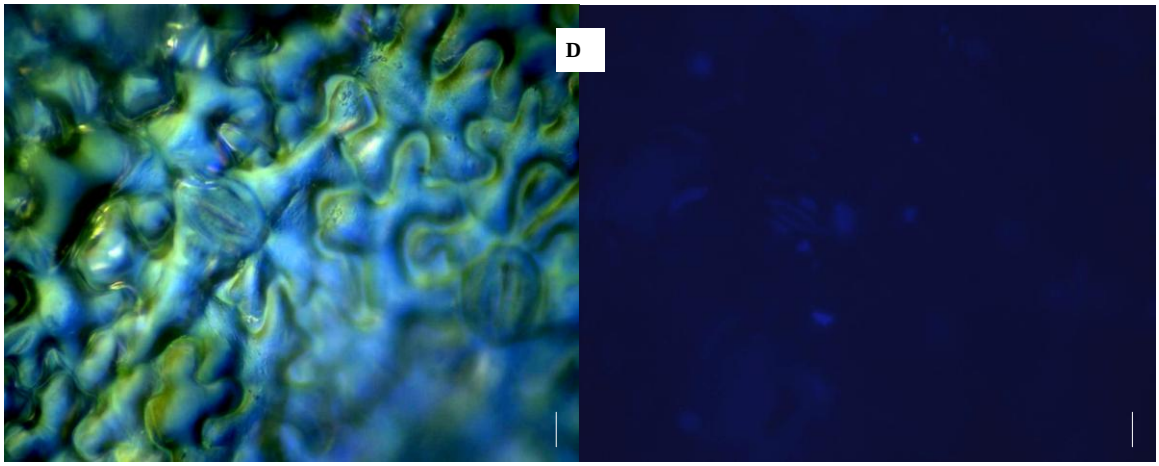


Figure 28: Use of DAPI staining to visualize the indigenous bacteria on the watercress phylloplane. EDIC/EF micrographs showing: (A) Watercress leaf stained with DAPI for 15 minutes, some bacteria are visible, however, these are not clear and all the bacteria on the surface do not appear to be being stained. (B) Watercress leaf hand-washed for 30 seconds and then DAPI stained for 15 minutes, on average no clear difference is seen between washed and unwashed samples, the level of bacteria being seen is still very low. (C) Watercress leaf after DAPI staining for 18 minutes, dull areas of staining of the nuclei of the epidermal cells are visible. (D) Watercress leaf hand-washed for 30 seconds and subsequently stained with DAPI for 18 minutes, again no or few bacterial cells are visible, there being high levels of background staining of the epidermal cell nuclei (fluorescent areas). Bars depict 10µm.

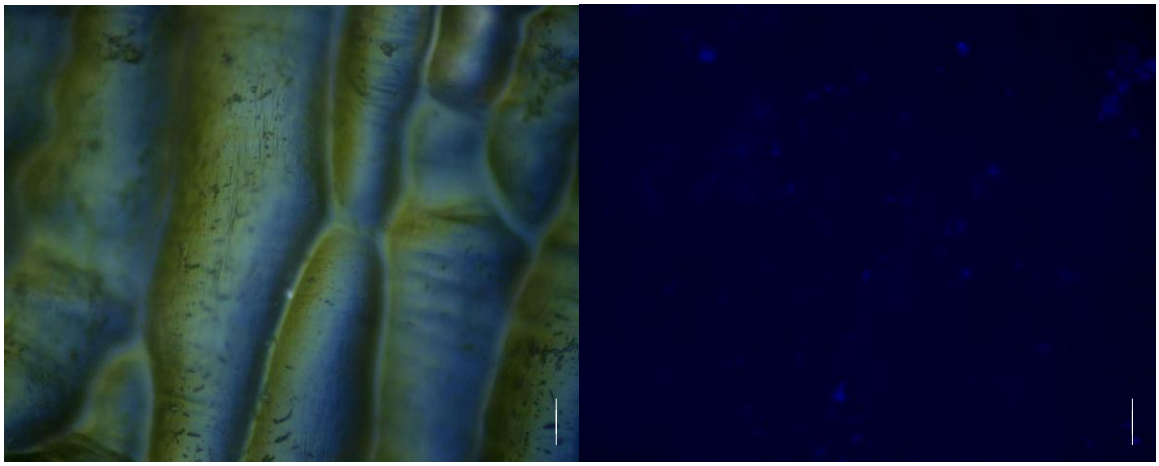


Figure 29: Use of DAPI staining to visualize the indigenous bacteria on the thicker spinach phylloplane. Spinach leaf surface stained with DAPI, EDIC (left) and EF (right) images. Spinach leaf stained with DAPI for 15 minutes, the bacteria have been stained as single cells and aggregates. Regions that are visible as contamination under bright light can be seen to be stained partially by DAPI as bacteria, the remaining regions are strongly implied to be slimy exopolymer material. Minimal background fluorescence from the epidermal nuclei is visible. No clear difference was apparent after a 30 second hand-wash. Bars depict 10µm.

Initially, the watercress samples were stained for 15 minutes in order to stain the bacterial cells; however, as is visible in Figure 28A, few cells were stained in this incubation period

and those that were stained fluoresced faintly, making it impossible to count the cells. A subsequent 30 second hand-wash step (Figure 28B), produced very little difference in the images obtained, again the level of staining of the bacteria was very low. The staining incubation period was then increased to 18 minutes to hopefully allow for more bacterial staining. As shown in the images obtained in Figure C and D, 18 minute incubation resulted in the nuclei of the epidermal cells being stained, making it hard to distinguish these from any bacterial cells and the same being the case in the samples stained after a 30 second hand-wash.

The watercress leaf is thin and this may be the reason why it was difficult to use the DAPI staining protocol; to confirm if this may be the case, the thicker spinach leaf was stained with DAPI to determine if it worked on this phylloplane. As can be seen in Figure 29, the thicker spinach leaf can be DAPI stained and bacteria can be more clearly seen in the phyllosphere. When comparing the fluorescence image back to the bright field image, it is apparent that the bacterial cells naturally occur in the grooves and crevices as well as some being centralised. In the bright field image, areas that can be seen as contamination can now be confirmed to be bacterial in nature due to their staining by DAPI. The bacterial cells are visible single and as aggregates, the single cells may be quantified on this salad leaf type. It is also evident that the larger aggregates of contamination that appear to be 3D in nature (can be seen due to the use of the EDIC microscope which uses the intact sample without the use of cover slips or fixing) are not entirely bacterial, these areas are more likely to be areas of exopolymer, suggesting the formation of biofilm in the phyllosphere. When the spinach leaf matrix was hand-washed for 30 seconds, on average no clear difference could be seen in the colonization of bacteria, suggesting that the natural microflora is strongly attached to this surface. These results confirm the suggestion that DAPI staining problems with the watercress phylloplane are due to the fact that the watercress leaf surface is thin. The thicker spinach phylloplane is more suitable for study using DAPI and quantitative comparisons may be possible.

3.3.7 Use of the EDIC/EF microscope to view the locations of inoculated GFP *Salmonella* Thompson on the watercress phylloplane and the potential to quantify levels.

The EDIC/EF microscope was evaluated for use with GFP labelled *Salmonella* Thompson, to observe patterns in colonization on watercress leaves and to quantify levels of

contamination. Samples sizes of 25 g were inoculated with pathogen for 12 hours followed by direct observations using the EDIC/EF microscope.

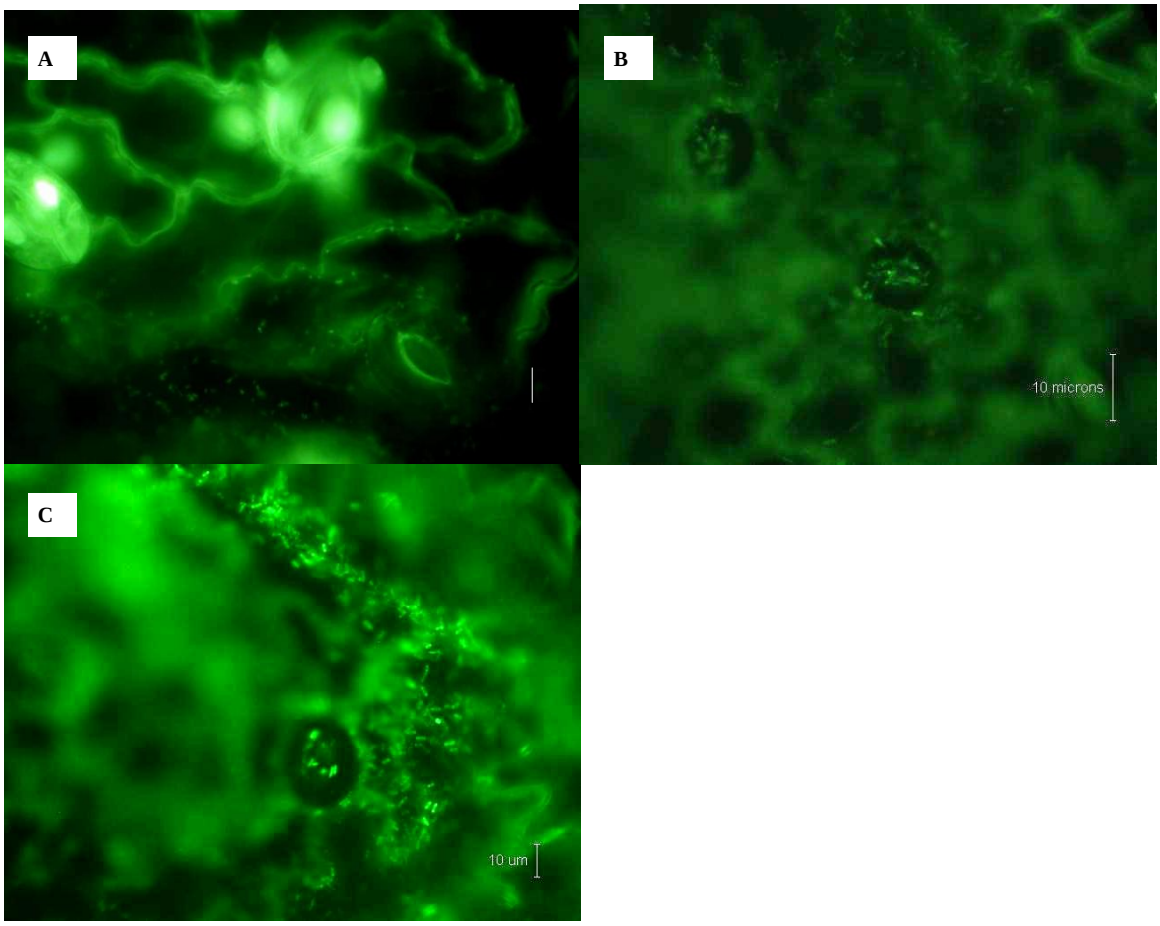


Figure 30 EDIC/EF micrographs of GFP *Salmonella* Thompson on the surface of watercress leaves. Image A and C show that in some regions of the leaf the *Salmonella* locate specifically in the cell margins. Image B shows 7 *Salmonella* cells in the vicinity of a stoma. Bars depict 10µm.

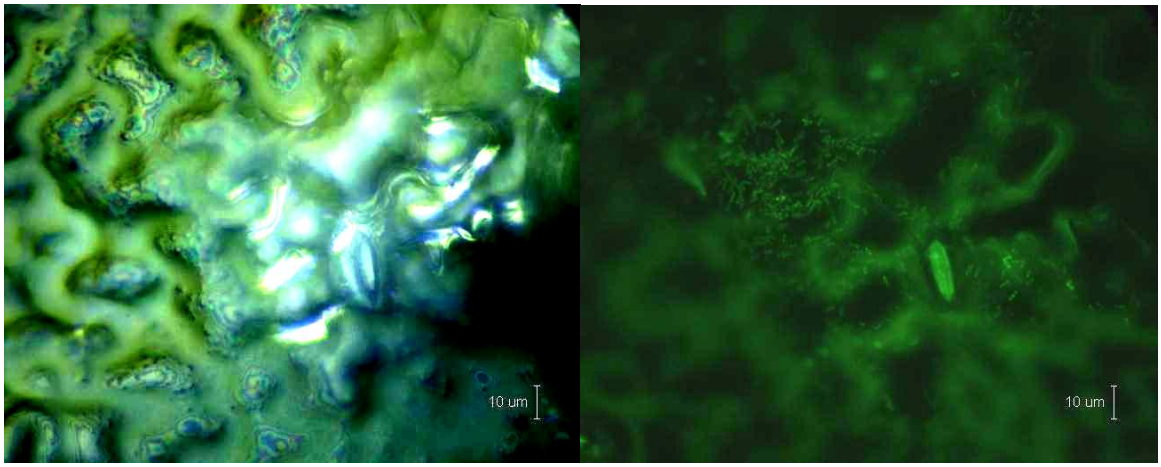


Figure 31: EDIC and EF micrographs of the watercress leaf surface inoculated with GFP *Salmonella* Thompson. The two images can be easily compared to see that the *Salmonella* have located preferentially in the cell margins. Bars depict 10µm.

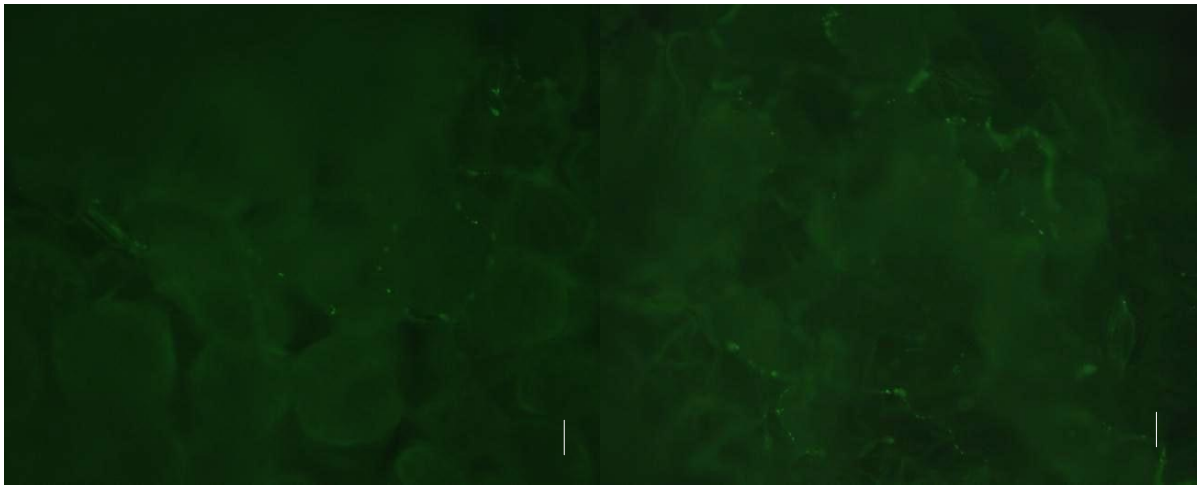


Figure 32: EF images of the watercress leaf surface inoculated with GFP *Salmonella* Thompson, the same sample as in Figures 30 and 31. The *Salmonella* cannot be visualized clearly and it is apparent that these A and B are greatly different from each other and from Figures 30 and 31. Due to the great differences in one sample on the levels of *Salmonella* visible, it is not possible to use this methodology for the quantification of *Salmonella* in the watercress phyllosphere. Bars depict 10µm.

Figure 30 shows that as for the endogenous microflora, the majority of inoculated GFP *Salmonella* was found in the margins and the stomatal regions of the leaf. In Figure 30 (B), approximately seven *Salmonella* Thompson can be seen directly on top of a stoma. In Figure 31, the EDIC and EF images clearly show the preference of the pathogen to travel to preferred niches on the phylloplane.

It can be seen from the large differences averaged over a series of sample areas, described in Figures 30, 31 and 32, that quantitative enumeration of inoculated GFP *Salmonella* Thompson is difficult using the 25g samples with 225 ml inoculum solution; this is due to the large variations in the numbers seen in each field of view on different leaves in the 25 g sample.

After a 30 second hand-wash, on average no difference could be seen (results not shown) compared to the un-washed leaves; it was apparent that some fields of view had considerable microbial contamination, others very little, and some in between, and therefore, evaluation of these results pre- and post-treatment using this protocol was difficult.

3.3.8 Comparison of the Confocal Microscope with the EDIC/EF microscope and its use to establish if GFP labelled *Salmonella* Thompson is internalized into watercress leaf surfaces.

The confocal microscope was compared to the EDIC/EF microscope to establish if one was more efficient than the other. GFP *Salmonella* Thompson was inoculated onto a single watercress leaf surface (due to the difficulties observed previously in the inoculation of larger samples) and allowed to incubate for 30 minutes. The leaf was then covered with a coverslip and viewed using the confocal microscope. Possible internalization after a minimal incubation period was investigated.

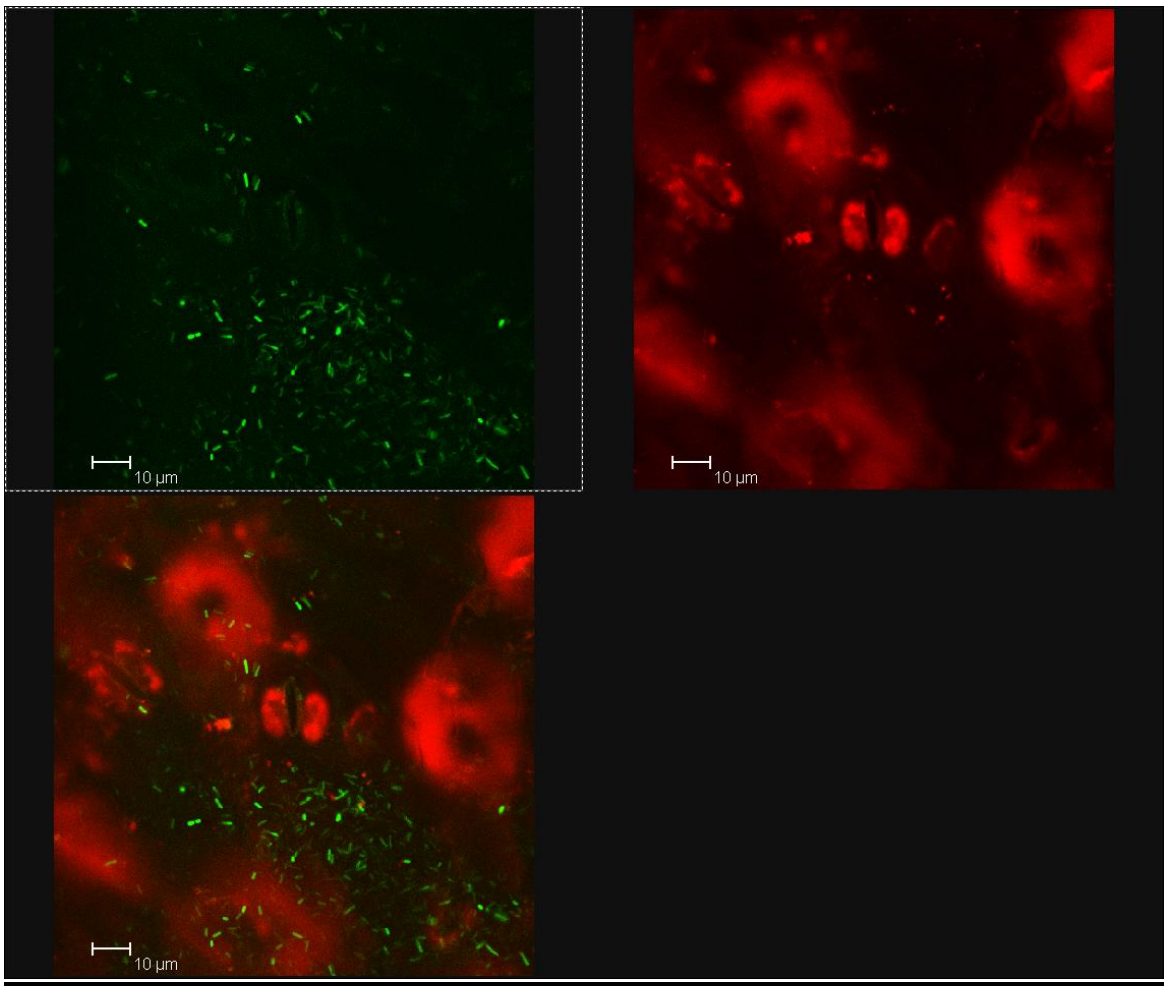


Figure 33: Visualization by LSCM projected z series to view GFP labelled *Salmonella* Thompson on the watercress leaf surface. Sample inoculated 30 minutes prior to imaging. It is evident that the pathogen has not had time to all locate fully to the stomatal regions. Surface colonization patterns can not be fully made compared to EDIC/EF microscopy. Bars 10µm.

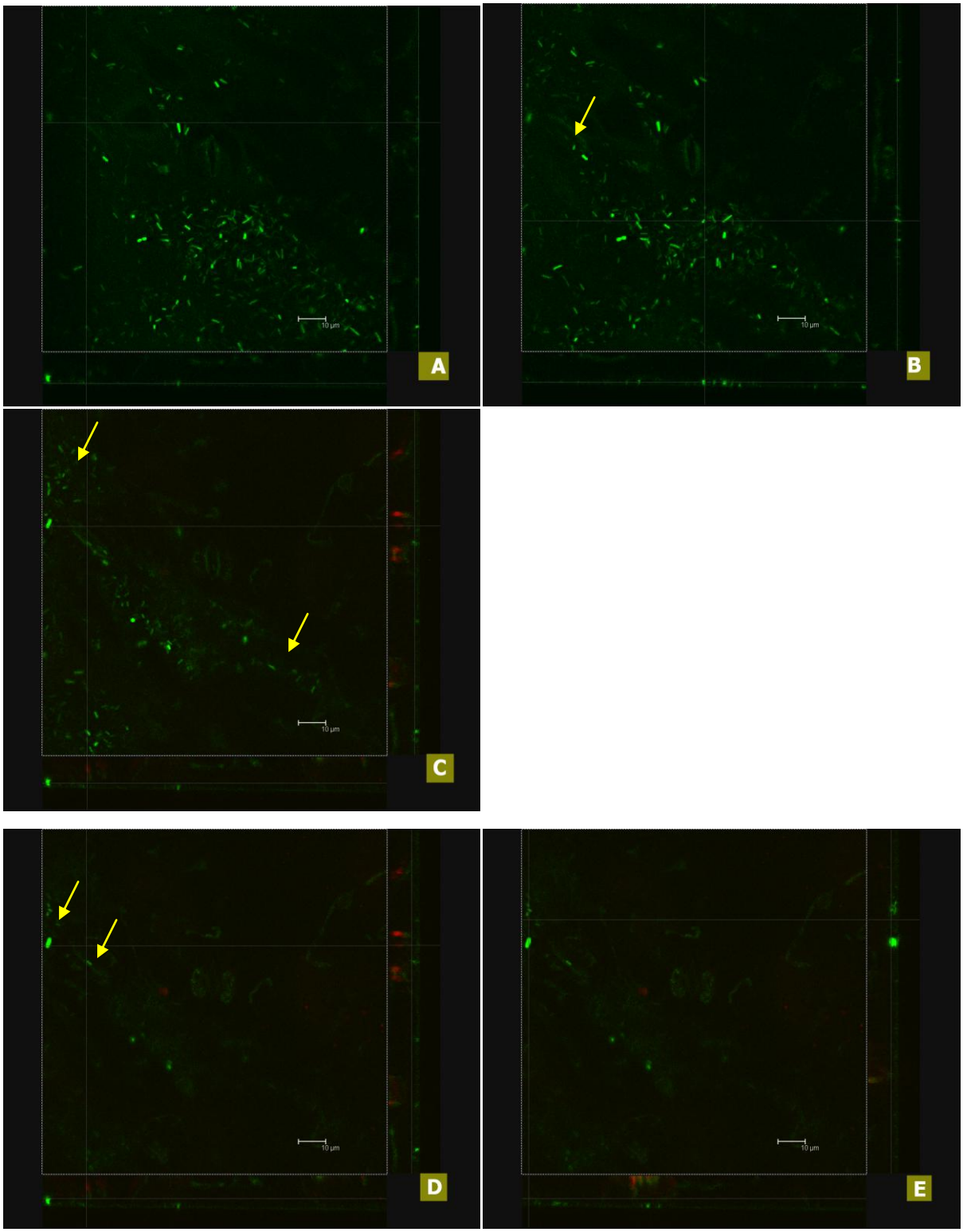


Figure 34: Use of the confocal microscope to show that GFP *Salmonella* Thompson is internalized into the surface of the watercress leaf. Optical sections through the leaf of the watercress in orthogonal view. Each image shows one optical section taken parallel to the leaf surface (large frame) and two images perpendicular to it (small frames in the lower part and on the right of each image). The reconstruction is based on the same data set (z-stack) as in Figure 33. The parallel optical section in Image 34 A was taken on average approximately 0.7 µm below the surface of the leaf. The corresponding section in Figure 34 B was taken at a

level of ca. 1.1 µm, in Figure 34 C of ca. 1.6 µm, in Figure 34 D of ca. 2.0 µm, and in Figure 34 E of ca. 2.5 µm below the leaf surface. Bars 10µm. The images suggest that *Salmonella* internalization is approximately 2 µm after an incubation period of 30 minutes. Yellow arrows depict bacteria that have been internalised, and the bars denote 10 µm.

Figure 33 shows that compared to the EDIC/EF microscope, the images produced by use of the confocal microscope are not as informative. Regions of grooves or cell margins are not visible and some stomatal regions are obscured by background fluorescence of the leaf. In general, sample preparation takes longer when using the confocal microscope and samples need to be fixed, thereby losing their original integrity. The whole process involved to produce an image is much more time consuming and required greater expertise and training. From the images obtained, however, it is still evident that in general after a 30 minute incubation period, *Salmonella* Thompson has not had sufficient time to move to the stomatal regions; compared to results produced previously after a 12 hour incubation of a 25 g sample.

The results show that those few pathogens that had sufficient time to migrate to the stomata began to internalise by 30 minute incubation (approximately 1% of the population). The images in Figure 34 show optical sections parallel to the leaf surface in different focal planes, as well as transverse sections in orthogonal view. From the images obtained it is apparent that *Salmonella* Thompson is found in higher concentrations on the surface of the leaf as would be expected (Figure 33); the stomata being taken as a reference feature to detect the surface. From optical sections that lie below the leaf surface (Figures 34 A, B, C, D and E) it is apparent that fewer bacteria are present. An overlap can be seen in the image sections, however, due to variability's in the strength of the fluorescent signal the section that the bacteria are actually present in can be distinguished. Figures A and B are images that were taken approximately 0.7 µm or ca. 1.1 µm below the surface of the leaf and it is apparent that high numbers are present here, the bacteria appear to be in aggregates and some are very close to a stomata. When comparing Figures 34 A and B with Figure 33, it is apparent that the high level of bacteria visible in the sectioned images is in fact due to an overlap of the sections. Fewer bacterial cells are apparent compared to the top surface (Figure 33) and those visible have lower fluorescence. In Figure 34 B, some bacteria can be seen to be internalised to this level, since they are fluorescing more strongly compared to the higher sections (yellow arrow). Figure 34 C was taken at ca. 1.6 µm and

shows fewer bacteria, however, green-fluorescence and rod shapes which are indicative of bacteria are visible, which suggests that these are the cells that have been internalized, particularly those that are different to Figure 33 and 34 A, B, C, D and E or the degree of fluorescence is greater (yellow arrows). Figure 34 D and E show optical sections deeper within the leaf (ca. 2.0 µm and of ca. 2.5 µm below the leaf surface respectively) and considerably less fluorescence is apparent; however, to the left of these images, obvious rod shapes (yellow arrows) can be seen, which are in the region of a stomata. Therefore, the images suggest that *Salmonella* internalization is approximately 2 µm after an incubation period of 30 minutes.

3.4 Chapter Three Discussion

The aim of this section of work was to characterize the phylloplane of each leaf matrix and evaluate the use of the EDIC/EF microscope with BacLight™, DAPI staining and GFP-labelled *Salmonella* Thompson, to visualize the phylloplane in a manner not done previously. The use of this microscope to view the phylloplane of three different salad leaves can be concluded to be highly efficient, the procedure is rapid, non-destructive and requires minimal or no sample preparation. The images produced are of high quality and bacterial contamination, as well as debris that are likely to be exopolysaccharide biofilm material, is visible.

The EDIC images showed clearly the xylem and phloem of lettuce leaves. Bacteria gain entry into the leaf through wounds in the surface of the leaf or stomata; they then enter the vascular system of the leaf from the mesophyll through the parenchyma of the bundle sheath and spread throughout the leaf. This leads to greater nutrient availability (rather than being confined to one area) and less competition amongst the bacteria. The bacteria multiply and cannot be efficiently washed by food industry cleaning procedures; they succeed in their mission for survival by subsequently entering the intestines of an individual who has eaten the contaminated salad leaves. The intestines then contain optimal conditions for growth of these bacteria.

The images show that there is little intercellular space on the phylloplane and the epidermal cells are packed very closely, therefore, preventing water loss and acting as a barrier to fungi and pathogenic bacteria. Stomata can also be seen; these allow gas exchange (but at a cost of water loss) and are regulated by the guard cells that surround the openings. All of

these grooves can serve as niches for bacterial residence. From examining lettuce leaves under magnification, it was found that stomata appear to be present on both the inside and the outer side of the leaves. Due to this, pathogens can enter the leaf through both the upper and lower surfaces - due to splashes from the soil (lower surface) or direct faecal contamination, such as from birds (upper surface).

Stomata could not be seen to be present on the inside or the outside of the white stalk side of lettuce. Only epidermal cells could be seen at x100 and x400 magnification. Bacteria would therefore be expected to be found around the edges of the epidermal cells. Entry into the leaf would be expected to only occur in these areas if the leaf was damaged. This area of the leaf could be used as a control when investigating the entry of bacteria into leaves via stomata.

From these microscopy pictures the structural environment that pathogens may encounter if they were to be on the leaf surface is evident. The edges of the epidermal cells would allow some protection from environmental stresses, explaining the greater occurrence of bacteria in these regions. However, it appears that the optimal place for the pathogens to travel to would be the stomata, since it is in this area that nutrients may be available, also, it is in this area that pathogens may be able to gain access to the inside of the leaf surface. High numbers of single and clusters of bacterial cells could be seen to be present close to stomata and many were present on the guard cells. Cuts or abrasions in the surface of the leaf may also attract pathogens in the same way and for the same reasons, as had been previously reported in the literature (Seo and Frank, 1999; Solomon *et al.,* 2002).

In general, different leaf surfaces were found to be contaminated to different degrees, spinach samples containing the greatest amount of debris and bacteria and/or biofilms; followed by watercress and then lettuce. It was apparent that lettuce leaves contain minimal debris and/or minimal areas of contamination; it is likely that smaller levels of contamination are confined to the edges of the epidermal cells, rendering them more difficult to observe clearly. As suggested previously, this may be due to the different ways in which each of these leaves are grown, and also may be due to the differences in the size and shape of the epidermal cells of each type of salad leaf. Although spinach and lettuce leaves are larger than watercress leaves, the watercress epidermal cells are smaller in size, and therefore, in a set area contain a greater proportion of grooves and crevices. Lettuce

leaves, particularly those from the centre of the lettuce, are more protected from the environment, and therefore, have less surface area available for immediate contamination from the soil. Lettuce generally have their outer layers shed off, and therefore, result in 'cleaner' produce, whereas spinach is grown in soil with minimal leaf shedding and maximal contact with the environment. Watercress is grown in a water environment so it may be that the general currents and water movements do not allow for large debris or bacterial aggregates to remain on the leaf surface, those that become lodged into cell margins being the ones that go on to form larger aggregates. The degree of contamination by debris and/or bacteria was quite variable across different fields of view, on same leaves and different leaves. This variability was greatest on watercress leaves. This may be due to the way in which individual leaves have been exposed; some may be folded onto other leaves or they may be overlapping, leading to less exposure to contamination from the environment. This highlights the importance of viewing at least ten fields of view, each on six different leaves, to ensure this variance is accounted for.

EDIC images were taken before and after hand-washing for 30 seconds, no differences were apparent between each, therefore suggesting that the debris and bacterial cells present on the phylloplane are strongly attached. The debris that is present on the leaves can be seen to be slimy in appearance, which may suggest the presence of biofilm exopolysaccharide. This would be important with the consumer in mind who would only wash by hand. Single and clusters of bacteria were not removed by the wash and the suggestion of the presence of biofilms could render the cells more resistant to further industrial detachment and decontamination procedures.

In conclusion, the results presented show that the phylloplane can be viewed directly to produce high quality images, without any sample staining, or preparation such as fixing or dehydrating, as has been shown previously for other matrices and pathogens, using the EDIC microscope (Keevil, 2003). Although phylloplane contamination studies have been previously published with similar observations to those presented in this Chapter, the studies have not previously used the EDIC microscope. Published work has shown the natural colonising patterns of the microflora on the phylloplane using LSCM and SEM (Carmichael *et al.*, 1999; Monier and Lindow, 2004 Yadav *et al.*, 2005). However, using EDIC/EF microscopy has been shown to be more advantageous since it is rapid and images can be taken and viewed almost instantaneously, therefore, the errors involved in leaf

sample's changing, particularly due to the increased light or heat exposure, are minimized. The other problem faced when viewing the phylloplane by use of currently worldwide used SEM or LSCM for leaves, is that the fixing and dehydrating of the leaf samples beforehand may alter the microenvironment and/or the debris locations and sizes that are present, so the images produced may not be accurate; also, these are very costly procedures and motile bacteria cannot be observed.

From the microscopy images obtained after BacLight^{TM} staining and use of the EDIC/EF microscope it can be established that live bacteria tend to concentrate around the edges of the epidermal leaf cells or in the stomata. The degree of auto fluorescence from the cytoplasm was very high, when using the propidium iodide stain and the TRITC filter for dead bacteria, particularly for the thinner watercress leaf, so the images were not clear. SYTO-9 staining and the use of the FITC filters set resulted in a much lower degree of background fluorescence and more individual bacterial cells were apparent. From SYTO-9 stained leaf micrographs it was apparent that the natural microflora on the phylloplane can mainly be found in the epidermal cells margins (in the grooves). This may be because live bacteria are motile, and therefore, are able to migrate to preferred niches to improve their chances of survival during stress; stomata would allow nutrient availability and also by internalization could protect the microorganisms from disinfectants or environmental stresses. It is possible that bacteria are located in these regions because the stomata and other niches have irregular contours resulting in the passive collection of the cells randomly. However, this study is the first to show that dead bacteria were mainly found in the centre of the surface of leaf epidermal cells, singularly, whereas, live bacteria tended to be around the edges of these cells or near stomata, in groups or clusters. This suggests that the localisation of bacteria in certain niches may be an active process since it is the live bacteria that tend to be found in the optimal niches in greater quantities.

Pulsification is most likely required to remove clusters of cells that may also be forming biofilms (as investigated in the next Chapter). Biofilm formation would protect and aid in the survival of bacteria in the fluctuating environment and would protect cells against disinfection treatment. The EDIC images presented in this Chapter suggest the formation of biofilm on the phylloplane; slimy exopolymer-like material is apparent in association with bacterial cells. Although the presence of biofilm on the phylloplane has been documented with the use of LSCM, SEM and epifluorescence (Gras *et al*, 1994; Morris *et*

al., 1997; Carmichael *et al.*, 1999), there are numerous draw backs of using these techniques, such that, that they do not allow for the detection of the phylloplane directly, the procedures could alter the consistency of the phylloplane and the images would be more difficult and time consuming to obtain. EDIC or EDIC/EF microscopy allows for the production of pseudo-3D images, this is due to the fact that the leaves are not dehydrated, fixed or crushed with a cover slip. This results in the more accurate detection of areas on the phylloplane that may be biofilm, due to the direct imaging of the morphology and depth of any contamination compared to SEM or LSCM.

From the results it could be established that using BacLight™ staining for comparative studies of pre- and post- wash treatment of leaves was not useful, particularly when investigating dead bacteria on the phylloplane. In general, the images show that qualitative comparisons are more applicable than quantitative results for live bacteria; this is due to the high population of live bacteria found residing on the phylloplane – particularly in some areas of the leaf. Therefore, direct quantitative comparisons either may not be possible or would not be accurate. It was difficult to observe clear differences between treatments, the next set of work (Chapter 5) involved the use of cell culture methods to establish whether a difference is actually evident when leaves are treated differentially. It may be that the high overall numbers of naturally residing cells on leaves camouflage the reduction in cells after treatment, since the bacteria are removed from across the whole sample.

Limitations of the BacLight™ staining method are that under certain conditions bacteria that have compromised cell membranes may be able to recover and reproduce; these may be scored as dead in this assay. Some bacteria with intact cell membranes may be unable to reproduce in nutrient medium, and these would be scored as live. Although the typical images obtained allow for the comparison of the live microflora on the phylloplane to be made qualitatively; this staining procedure would be difficult to use for quantitative imaging due to the high degree of background fluorescence, particularly when using propidium iodide to view dead bacteria. Previous studies have attempted to use the BacLight™ kit, however, only with the use of LSCM microscopy, which is both destructive and time-consuming; these published studies showed the kit to be advantageous for qualitative examination of the phyllosphere rather than quantitative assessment (Fett and Cooke, 2005). Key topographical characteristics of the phylloplane are not visible

using LSCM and so the EDIC/EF microscopy technique shown here is more advantageous in this analysis.

DAPI staining leads to bacteria fluorescing in the cyan region of the spectrum and therefore, background fluorescence is minimised. However, DAPI staining was shown to be difficult or impossible on the thin watercress leaf, since the nuclei in the epidermal cells were readily stained, thereby making it difficult to distinguish the bacteria on this phylloplane. DAPI staining of the thicker spinach phylloplane was shown to be more advantageous, such that individual bacterial cells could clearly be seen; refinements in the protocol (such as the reduction is sample size from 25 g phylloplane to 1 leaf) could potentially also allow for quantitative enumerations. When comparing EDIC images to EF images of the spinach phylloplane stained with DAPI, it was apparent that the aggregates of bacteria that appeared to be slimy in nature and indicative of biofilm areas, were not entirely bacterial in nature, and therefore, may be the exopolymer matrix - this further suggests that these areas are actual biofilm. As for direct EDIC imaging alone and EDIC/EF combined with BacLight^{TM} staining, hand-washing did not appear to affect the level of bacterial contamination on the spinach phylloplane; this suggests that the bacteria are strongly attached to the salad matrix. Although DAPI staining has been previously reported in the literature (Schreiber *et al.*, 2005), images of stained bacteria as revealed in this Chapter, have never before been shown.

The images obtained after inoculating the watercress phylloplane with GFP-labelled *Salmonella* Thompson, show that as for the endogenous microflora, the majority of inoculated GFP *Salmonella* was found in the margins and the stomatal regions of the leaf. As for the endogenous microflora, this would allow for the entrance of this pathogen into the leaf, allowing for protection against environmental stresses and in terms of food safety it may be protected from disinfection processes. This pattern in leaf surface coverage is likely to be an active process rather than the passive physical entrapment of cells in niches since the inoculation was done in suspension, therefore, it would have been likely for the bacterial cells to have equal access to all areas of the leaf surface. The surface area of the main region of epidermal cells is greater than that of the margins so in fact a greater deposition may have been likely in these areas if it was a passive process; however, this has been shown not to be the case. *Salmonella* flagella mutants could be used to verify if this is the case in future work.

The advantage of using GFP-labelled bacterial strains in combination with EDIC/EF microscopy is that the way in which pathogens specifically colonize the phylloplane can be seen, and there is no loss of information due to poor recovery, sample damage or time delay. However, from the micrographs obtained, it can be seen that quantitative enumeration of inoculated GFP *Salmonella* Thompson is difficult using the 25g samples with 225 ml inoculum solution; this is due to the large variations in the numbers seen in each field of view on different leaves in the 25 g sample.

After a 30 second hand-wash, on average, no difference could be seen in *Salmonella* contamination compared to the un-washed leaves; some fields of view had considerable microbial contamination, others very little, and some in between. It would be expected that after 12 hour incubation time the *Salmonella* had become attached to the watercress leaf surface very tightly and so would be difficult to be removed. Barak *et al* (2002) have previously suggested that *Salmonella* attach to the phylloplane very tightly; this has been reported to be approximately 1000 times more tightly than *E. coli*. The results indicate that the protocol used in this study (in terms of weight of phylloplane) is advantageous since it mimics the sample sizes used in food industries and in sample preparation procedures, such as stomaching or pulsification; however, when inoculating samples and viewing the leaves directly *in situ*, this protocol is not applicable due to great variations in the availability of leaves to the inoculum. A further study is now underway, on the colonization of *Salmonella* Thompson *in situ*, using EDIC/EF microscopy, however, the sample sizes have now been reduced to single leaves.

The results allowed for the comparison of the use of LSCM and epifluorescence microscopy. Epifluorescence was rapid and produced high resolution images of the phylloplane containing the inoculated *Salmonella* Thompson. Although LSCM images were also of high quality, the process was very time-consuming taking up to four times the time required for that of epifluorescence images, this would be a problem since it could result in loss of information if fluorescent stains are used, due to fading of the fluorophores. In general, LSCM requires fixation of samples, however, in the protocols used in this study this stage was by-passed and despite this the images obtained were of high quality. The greatest problem with LSCM is that it is an expensive procedure and it requires time to scan the z-plane and produce images, furthermore, it is more difficult to use and greater

training is required beforehand (Keevil, 2003). Unlike EDIC/EF microscopy, LSCM is unable to produce high quality bright field images of the leaf surface. As for the protocol used by Brandl and Mandrell in 2002, to view cilantro leaves, chlorophyll autofluorescence was used in the present study in order for leaf cells to be visible. This is not as beneficial as EDIC/EF micrographs, since the images are not clear, although stomatal areas can be seen, other surface characteristics, such as that of debris, contamination, leaf damage, and regions of possible biofilm cannot be visualized and temporal comparisons cannot be made.

The main disadvantage of the EDIC/EF microscope is that leaves cannot be visualized subsurface, unlike for LSCM. Section 3.3.8 allowed for the detection of *Salmonella* Thompson that had gone subsurface close to stomatal regions by use of LSCM. In the literature, except for the study of *Salmonella* Thompson on cilantro leaves (Brandl and Mandrell, 2002), the visualization of internalization of pathogens into the leaf matrix has not been cited, and never before for the watercress phylloplane. The results presented by Lapidot A. *et al.* (2006), also suggested that the ability of *Salmonella* to penetrate plant tissue was a more important factor in the protection of *Salmonella (*in cilantro leaves). The results presented in this Chapter show that *Salmonella* internalization was apparent after only a 30 minute incubation time, and the degree of internalization was approximately 2 µm in the region of stomata. This internalization would offer protection of *Salmonella* from the environment and also from disinfection procedures. Approximately 1% of inoculated *Salmonella* cells were shown to be internalized after only a 30 minute incubation time; therefore, if internalization rates are the same over time, a 12 hour incubation period might result in approximately 27% of cells internalizing; 12 hours is modest when considering that the salads may be growing in the field for many weeks. This suggests that *Salmonella* internalization must be investigated further if improvements are to be made in food safety. The results here suggest that *Salmonella* internalization is a contributing factor to the spread of foodborne disease.

Further investigations are currently being carried out in a PhD project specifically investigating the *in situ* quantification of microorganisms, possibly using smaller sample sizes and a variety of different stains, as an extension to the work presented in this study. This will be useful in that cell culture techniques may not allow for the detection of viable but non-culturable species, although the extent to which this is the case remains to be investigated. The disadvantage of *in situ* quantification with stains such as DAPI is that

the viable number is not counted for; only the total numbers are recordable, so it is not possible to determine the proportion of live cells which is vital in food science. However, as seen in this Chapter the great advantage in the use of EDIC/EF microscopy is that the colonization patterns of naturally occurring microflora and contaminating pathogens can be rapidly observed. In this project advances in microbial cell culture techniques that are currently used by industries worldwide were used alongside EDIC/EF microscopy for visualising spatial patterns of colonization across the phyllosphere. In the next Chapter, improved recovery techniques from the phyllosphere were studied in order to minimize losses at the recovery stage by industries and research groups worldwide. Watercress leaves were used as the model system throughout the rest of this project as requested by industrial sponsors and as supplied by Vitacress Salads.

CHAPTER FOUR

COMPARISON OF THE PULSIFIER AND STOMACHER TO FACILITATE CHARACTERIZATION OF THE NATURALLY OCCURRING MICROFLORA AND PATHOGENS ON DIFFICULT PHYLLOPLANE MATRICES

4.1 Introduction

The next objective of this project was to compare the efficiency of the Pulsifier with the food industry standard Stomacher to detach and characterize natural microflora and foodborne pathogens from difficult matrices such as the watercress phylloplane. This would enable optimization of the operating conditions since, to date, these have not been investigated for any food matrix. At present, the current industry adopted method is to use the 'paddle-type' processor, the Stomacher, which uses mechanical forces to homogenize or semi-homogenize food matrices, with the release of bacteria; this inevitably causes severe damage to the sample. The Stomacher was introduced in 1972 (Sharpe and Jackson, 1972), to supersede the then used Homogenizer which completely blended samples; subsequently, the Pulsifier was evaluated for use in 1998 (Fung *et al.*, 1998). The Pulsifier combines shock waves and intense stirring to drive microbes into suspension, but leaving the sample intact. Fung *et al.* investigated the efficiency of the Pulsifier compared to the Stomacher; all samples were naturally contaminated, i.e. were not spiked with pathogens; and a variety of matrices, except for the watercress phylloplane, were studied for total aerobic counts on tryptic soya agar (Fung *et al.*, 1998).

The aim of this Chapter was to extend the work done by Fung *et al.* in 1998 and Wu *et al.* in 2003, to determine if the Pulsifier is more efficient than the Stomacher in the recovery of heterotrophic microflora (using R2A as the agar recovery medium, which has a low nutrient concentration and may thereby resuscitate possible stressed, sublethally damaged cells) and coliforms (using VRBA, the current industry accepted agar recovery medium) from the watercress phylloplane. The optimal working conditions of the Pulsifier compared to the Stomacher were also investigated in terms of length of treatment time. If the efficiency of detachment varies with length of treatment, then the comparisons made in previously published studies may not be entirely accurate if this parameter was not considered; as such it should be important to investigate the optimal operating conditions for cell release using pulsification (or stomaching) for each sample matrix type before use. Previously, Sharpe *et al.* in 1972, stated that a 15 second stomaching was adequate for most foods, in data that was not shown, therefore, suitable working conditions of the Stomacher were also investigated (Sharpe and Jackson, 1972). Subsequently, the pulsification or stomaching conditions that appeared to be optimal for the watercress phylloplane as discovered in this Chapter, could be used throughout the rest of this project. Preliminary studies performed in this project showed that when pulsifying samples the approximate

quantity of air contained in the bag could vary quite significantly, resulting in distinct differences in the noise level. The noise level was lower if there was less air in the bag and considerably higher if there was more air in the bag. For this reason, the effect of the quantity of air in the sample bag prior to use was also investigated in terms of recovery of coliforms and the physical quality of the phylloplane. The current published work on the use of the Pulsifier does not state if air was incorporated into the sample bags prior to use, or if air was manually removed, so it may be assumed that this factor was not considered throughout those studies. The EDIC/EF microscope combined with the BacLight™ kit (to distinguish live/dead cells) and DAPI staining (to identify the total microflora) on the phylloplane post-pulsification were used in this Section to observe any changes in colonization and release patterns *in situ* after treatment. In the published comparative studies performed previously, viable pathogen levels were not studied (Fung *et al.*, 1998; Wu *et al.*, 2003) and neither was the watercress phylloplane, which is the matrix of study in this project. Consequently, *Salmonella* Thompson, which is an important pathogen in causing gastrointestinal illness worldwide, was inoculated onto the watercress phylloplane and the efficiency of detachment post-pulsification investigated using Rambach agar. Comparisons were also made of phylloplane damage pre- and post-stomaching or pulsifying using optical density of the aqueous phase and visual examination of the phylloplane matrix. An optimized procedure for sample preparation and pathogen release would have direct consequences to ensure microbiological quality of products in the food industry and also for protocols that are utilized after recovery, such as filter methods and microscopy.

4.2 Methodology

Please refer to Chapter Two.

4.3 Results

4.3.1 Comparison of the Pulsifier to the Stomacher for recovery efficiency of coliforms from the watercress phylloplane.

The aim was to make comparisons between the efficiencies of the Stomacher and Pulsifier on the release of naturally occurring coliforms from the watercress phylloplane, using VRBA. The effect of length of treatment time when using the Stomacher or the Pulsifier on recovery of coliforms from the watercress phylloplane was also investigated.

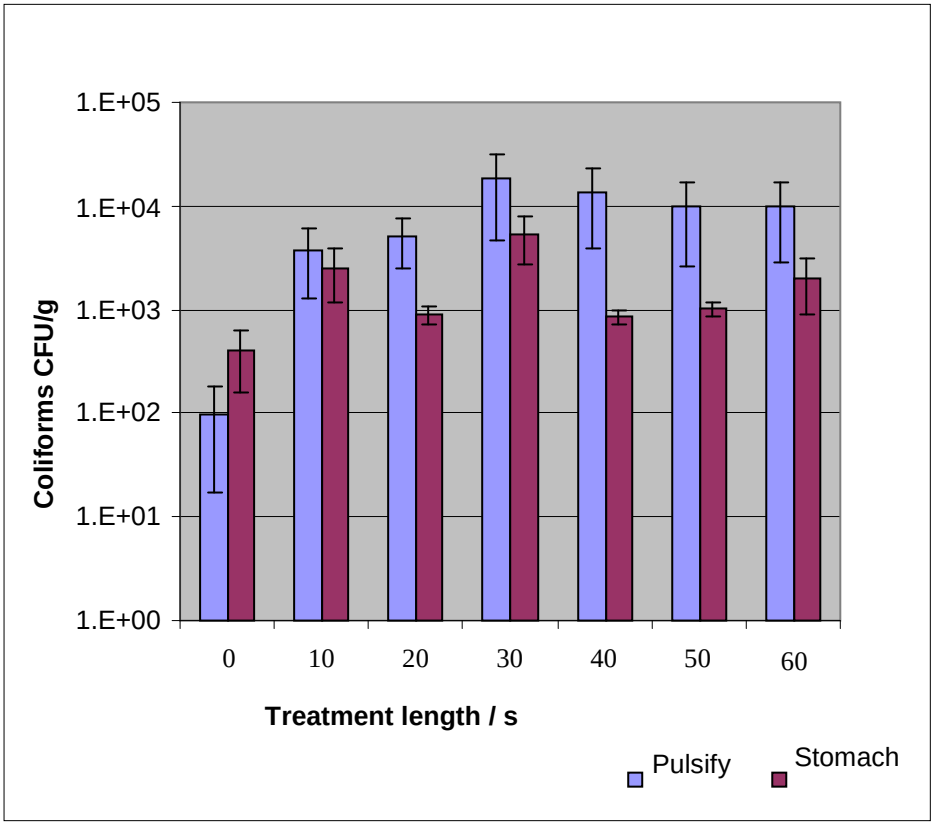


Figure 35: Log numbers of coliforms recovered from the watercress phylloplane (in suspension), after either pulsification or stomaching treatments for time lengths between 0 and 60 seconds. A 30 second treatment length appears to be optimal for pulsification and stomaching. Error bars are representative of the ± standard error of the mean of three experiments for each treatment

Statistical analysis showed that there was a significant difference between the number of coliforms recovered after pulsification across the seven lengths of treatment, shown by a one-way ANOVA ($p=0.0018$). This indicates the importance of testing the optimal treatment time for a sample prior to pulsifying. A one way ANOVA showed the numbers of coliforms recovered from the phylloplane with the differing stomaching treatment lengths not to be significant ($p=0.2208$). Therefore, lower treatment lengths could be used, however, as showed by the data in Figure 35, there is still an increase in the numbers of coliforms recovered, and the highest recovered is after 30 seconds of stomaching, therefore, this was used throughout the remaining studies. There was also a significant difference between pulsification and stomaching, tested using a two way ANOVA ($p=0.001$). P values less than 0.05 were regarded as significant.

From the results described in Figure 35 it is clear that variations in lengths of treatment time effects the numbers of cells recovered after both pulsification and stomaching; a 30

second length being optimal for the 25 g / 225 ml watercress sample to PBS buffer ratio used. A clear difference between coliforms recovered from the watercress phylloplane (in suspension) after stomaching or pulsifying is evident. The Pulsifier is more efficient, both in terms of recovery (Figure 35) and also sample quality, with much less debris produced as shown by visual analysis (Section 4.3.3).

In the results presented in Figure 35 it is evident that after a 30 second pulsification, 4-log coliforms were released from the watercress, into suspension; this being approximately 0.7-log greater than a 30 second stomaching treatment, and on average 2.2-log greater than with no treatment. A 30 second stomaching step could be seen to increase coliform recovery by on average 1-log compared to no stomaching treatment. This suggests that stomaching is still a useful procedure for sample preparation and cell recovery, however, for the watercress phylloplane it is not as efficient in recovery of coliforms as the Pulsifier; and the post-stomached sample is also considerably more damaged.

From the Stomacher data obtained (Figure 35), it is evident that the numbers of coliforms recovered after 10 seconds was relatively high, although not as high as after a 30 second treatment. By 20 seconds pulsification length, there was a 0.5-log lower plate count of the coliforms compared to 30 seconds of treatment. After 40 seconds of treatment, a 0.7-log drop in recovery of coliforms is evident, compared to 30 seconds of stomaching. Although there is a slight increase in coliform recovery after 60 seconds of stomaching, the level does not reach that which is obtainable after 30 seconds.

From the data presented in Figure 35, it is apparent that 30 seconds is the most efficient treatment length for recovery of coliforms from 25 g samples of watercress with 225 ml PBS buffer solution, using the Pulsifier. Between 10 and 30 seconds there appears to be a continuous rise in the numbers of coliforms extracted, then after a peak at 30 seconds the numbers recovered steadily declines. The amount of chlorophyll in the bag had also increased during this time. After 60 seconds the rate of reduction in recovery can be seen to be lower.

4.3.2 Coliform recovery from the watercress phylloplane after pulsification when comparing the effect of air or no air in a sample bag.

The aim of this Section was to investigate the effect of the level of air in the sample bag, on the recovery of coliforms from the watercress phylloplane and the quality of the phylloplane post-treatment. The suspension post-pulsification was plated into VRBA and the coliforms counted after incubation.

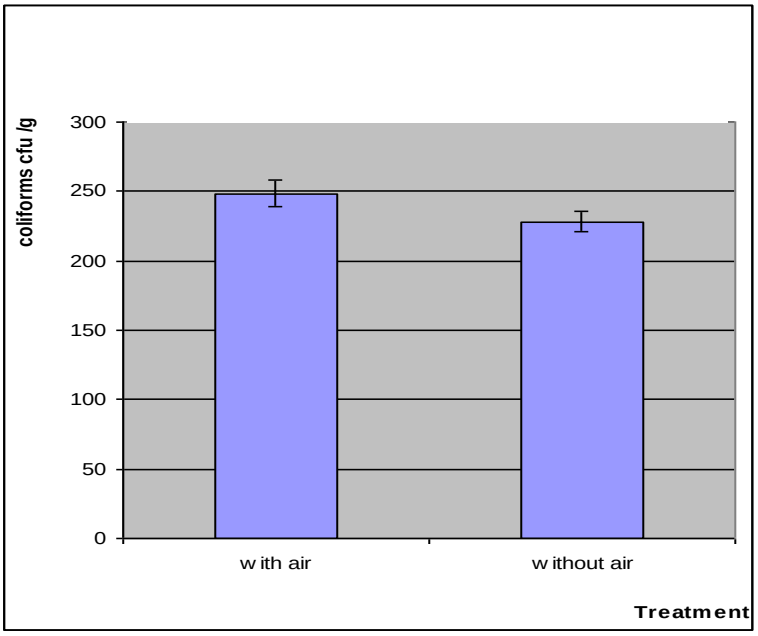


Figure 36: Numbers of coliforms recovered from the watercress phylloplane (in suspension) after a 30 second pulsification treatment with or without air in the sample bag. Pulsifying with air can be seen to result in a higher recovery of coliforms. Error bars are representative of the ± standard error of the mean of six experiments for each treatment.

In terms of microbial counts only a slight difference between the samples with or without air can be seen (Figure 36); on average an 8 % increase in numbers of coliforms recovered is evident when the sample bag contained air. Statistical analysis showed that there was no significant difference between the number of coliforms recovered after pulsification, in the presence or absence of air, using a paired t-test ($p<0.05$).

4.3.3 Investigation of the watercress phylloplane integrity after a variety of treatments. Changes in OD of the supernatant recovered after specific leaf treatments.

The aim of this section was to use optical density to make comparisons of the suspension produced after different treatments of the watercress phylloplane, thus indicating the level

of phylloplane integrity and damage. Published work has already compared the level of damage using membrane filtration rates. These studies concluded that food suspensions prepared by the Pulsifier filtered between 1.3 times to 12 times more rapidly than those by the Stomacher (Sharpe *et al.*, 2000). In the present study, optical density was used to compare the clarity of the suspension after different treatments, extending the work done by Wu *et al.* (Wu *et al.,* 2003). This would give a clear indication of the level of leaf damage, which is of particular interest to the food industry if the Pulsifier mode of action is to be potentially (after further investigations, as detailed in Chapter 5), used as chemical-free means of sanitizing leaf samples. The watercress leaf quality post-treatment was also visualized and described, to evaluate leaf conditions pre- and post- treatment. Treatments analyzed were a 30 second PBS treatment, a 30 second hand wash, a 30 second pulsification with air, a 30 second pulsification without air and stomaching for 30 seconds.

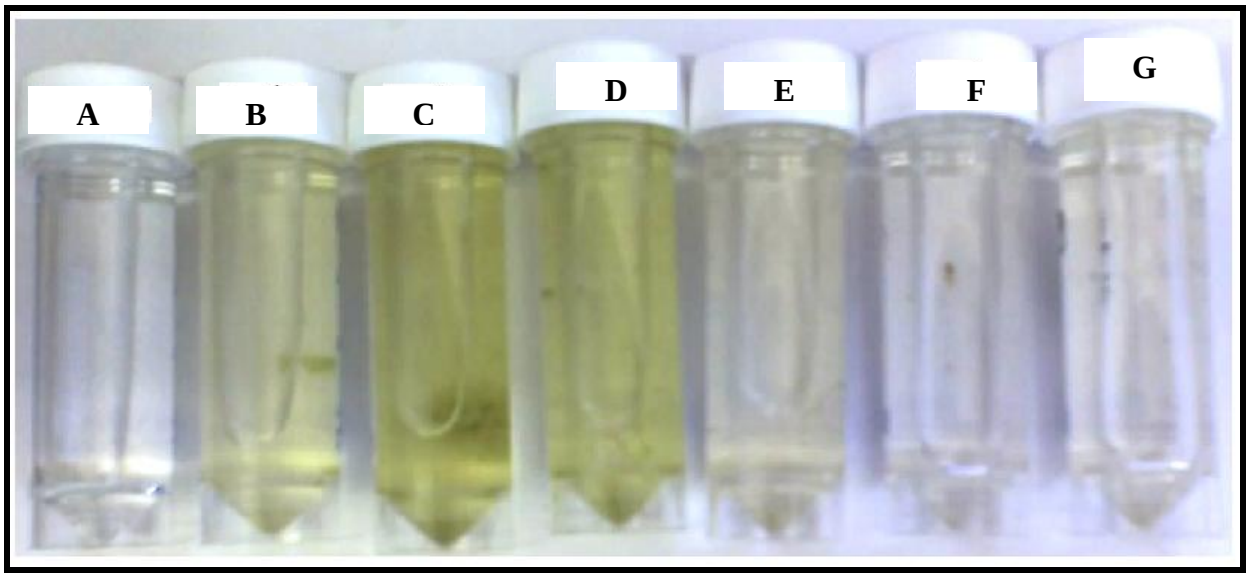


Figure 37: Effect of air with pulsification, no air with pulsification, stomaching, handwash or PBS wash - on the colour of suspension obtained. A = 30 seconds with PBS; B = 30 second pulsification with air example 1; C = 30 second stomaching; D = 30 second pulsification with air example 2; E = 30 second pulsification without air; F = 30 second hand-wash; G = 30 second pulsification without air. The hand-wash and pulsification without air result in a similar suspension colour.

Table 4: Effect of 30 seconds with PBS, 30 second hand-wash, pulsifying with air, pulsifying without air, and a 30 second stomaching step, on the optical density of the suspension post-treatment. The optimal treatment for recovery appears to be a 30 second pulsification without air.

Phylloplane Treatment	Average Optical Density (OD) 620 nm	Leaf Condition
30 second PBS	**0**	**Intact leaf**
30 second hand-wash	**0.097**	**Intact leaf, some debris removal**
Pulsify 30 seconds without air	**0.114**	**Mainly intact leaf, some debris removal, some leaves slightly scarred**
Pulsify 30 seconds with air	**0.301**	**Not as much damage as pulsification, although individual leaves are scarred and bruised**
Stomach 30 seconds	**1.027**	**Very thin leaves, break easily into smaller pieces, almost homogenised.**

A: **B:** **C:**

Figure 38: Photographs showing the effect of (A) 30 seconds with PBS; (B) 30 second pulsification without air and (C) 30 second stomaching step, on watercress leaf quality. The above leaves were of the same batch, and were in diluent, 25 g sample to 225 ml PBS. The leaf can be seen to be drastically damaged post-stomaching. The quality of the leaf post-pulsification has slightly deteriorated compared to the sample with only a 30 second wash in PBS (A), however, only to a level that produces minimal debris and/or chlorophyll loss.

From the post-treatment suspension images shown in Figure 37 it is apparent that each of the treatments results in a different effect on the leaf. Levels of chlorophyll release are visually distinctive and the OD values in Table 4 quantify this damage. If the phylloplane is to remain as intact as possible then it is advantageous if there is minimal loss of chlorophyll. The greatest difference in OD is apparent between stomached phylloplanes and those phylloplanes that were hand-washed (a difference of 0.930 OD), followed by

stomached phylloplanes and those that were pulsified without air (a difference of 0.912 OD). The next greatest difference is evident between hand-washed phylloplanes and those phylloplanes that were pulsified with air (a difference of 0.1863 OD). The watercress samples that had the most similar OD were those that were hand-washed and pulsified without air, with a difference of 0.017. No significant differences in OD's were apparent between hand-washed or pulsified samples without air ($p>0.05$); a significant difference was apparent between all other treatment conditions ($P<0.05$). Paired t-tests were performed and p values less than 0.05 were regarded as significant.

It is evident that a 30 second hand wash, a 30 second pulsification and a 30 second pulsification without air (Figure 38 E, F and G respectively) result in similar levels of leaf damage. Due to the results presented in the previous sections it is, therefore, advantageous to pulsify a sample for 30 seconds, since this leads to the greatest coliform recovery and as seen here, minimal leaf damage.

From the results it was also evident, that extra air in a sample bag did have an affect on pulsification. The first notable difference was that the noise level of the Pulsifier was greatly louder compared to when there was no air in the sample. When viewed through the transparent door on the Pulsifier, the phylloplane also appeared to be being vigorously shaken compared to without air, and more bubbles and froth appeared to be formed. Although, upon examination of the post-pulsified samples with air, the leaves were still in better condition compared to when the Stomacher was used, and this is apparent in the suspension images in Figure 37 and the corresponding OD results. From the colours of the suspensions obtained it was clear that when air was incorporated during pulsification, more chlorophyll was removed from the watercress phylloplane; it was evident that the level of leaf damage and chlorophyll release was more variable for each sample (Figure 37 B and D). The watercress sample also appeared to be more damaged, with considerable more debris release. In contrast, when air was manually removed from the sample bag and the sample subsequently pulsified, on average the colour of the suspension could be seen to be almost the same as that of the watercress sample with no pulsification or stomaching and just a 30 second wash in PBS (Figure 37 E and A, respectively), indicating minimal loss of chlorophyll; the phylloplane also remained intact.

Upon comparing the suspensions produced after a 30 second stomaching treatment and a 30 second pulsification treatment (Figure 37 C and F respectively), it is clear that, even if only examining leaf damage and chlorophyll release alone, a pulsification step is more advantageous. The results in this section can be seen to show that in terms of minimal chlorophyll release, low optical density of suspension, and minimal watercress leaf damage, if a release mechanism was required then with a 1 in 10 ratio of sample to buffer, a 30 second pulsification without air was the optimal procedure. These visual differences in the leaf quality, for watercress washed in PBS for 30 seconds, watercress post-pulsification (without air) and watercress post-stomaching can be seen in Figure 38.

4.3.4 Comparison of the Stomacher and Pulsifier for recovery of total naturally occurring heterotrophs from the watercress phylloplane.

The aim was to compare the efficiencies of the Stomacher and the Pulsifier in release of the total naturally occurring heterotrophic bacteria from the watercress phylloplane; counts were compared to a 30 second wash in PBS and a 30 second hand wash. Low nutrient R2A medium was utilized to possibly resuscitate any stressed, sublethally damaged cells.

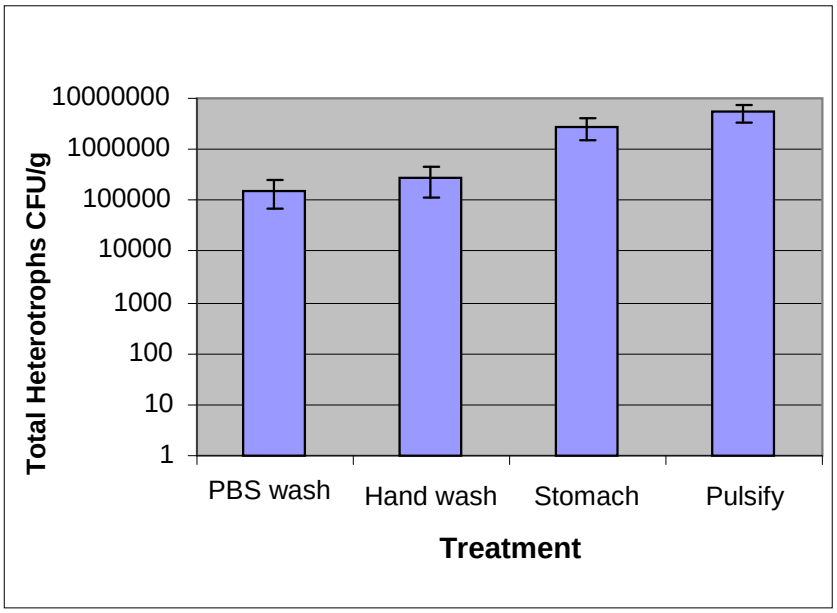


Figure 39: Log numbers of total heterotrophs recovered from the watercress phylloplane (in suspension) after either a 30 second wash in PBS, 30 second hand wash, a 30 second pulsification or a 30 second stomaching treatment. Pulsification treatment can be seen to recover the greatest number of heterotrophs. Error bars are representative of the ± standard error of the mean of six experiments for each treatment.

Statistical analysis showed that there was a significant difference between the number of total heterotrophs recovered between a 30 second wash in PBS and pulsification ($p=0.0081$), a 30 second wash in PBS and stomaching ($p=0.0182$), hand wash and pulsification ($p=0.0096$), hand wash and stomaching ($p=0.0257$). No significant difference could be seen between the 30 second wash in PBS and hand wash ($p=0.5135$) and between stomaching and pulsifying ($p=0.3674$). These were shown by an unpaired t-test. P values less than 0.05 were regarded as significant. The results suggest that either a stomaching or a pulsifying step significantly increases the recovery of heterotrophs from the watercress phylloplane. The fact that pulsification results in a cleaner suspension post-treatment and the phylloplane remains intact still strongly suggests this is the best method to use for sample preparation.

The results presented in Figure 39 show that an increase in approximately 1.5-log recovery of total heterotrophs is apparent after a 30 second pulsification treatment compared to the 30 second wash in PBS. The difference in the increase in recovery of heterotrophs after pulsification treatment compared to after stomaching treatment appears to be less compared to the previous studies investigating coliform recovery. The maximal level of viable (including any stressed, sublethally damaged) cells recovered is close to approximately 7-log and this is after a 30 second pulsification treatment. From the results it is evident that a simple hand wash is not an effective means for detaching microorganisms from the watercress phylloplane; this is important to consumers that rely on this procedure for sanitizing their salads. The fact that approximately 5-log heterotrophs were recovered from the phylloplane (in suspension) after no treatment suggests that these were the cells that were loosely attached.

4.3.5 Evaluation of the use of the EDIC/EF microscope to view the natural microflora on the phylloplane, after no treatment, pulsification or stomaching treatments.

The aim of this Section was to view the *in situ* effects of no treatment, pulsification or stomaching treatments, on the colonization patterns of the naturally occurring microflora on the watercress phylloplane. The EDIC microscope was used to view the *in situ* effects using bright field illumination; the BacLight^{TM} kit was used to view the colonization pattern of live or dead bacteria and DAPI staining was used to view the colonization pattern of the total microflora on the phylloplane. In this Section, the spinach (instead of the watercress)

phylloplane was investigated due to the conclusions established in Chapter Three. In particular, the spinach phylloplane is more contaminated initially, compared to the watercress phylloplane, and therefore, results in clearer EDIC images. Due to the spinach leaf being thicker (see Chapter 3) it can also be stained with DAPI to view the total microflora.

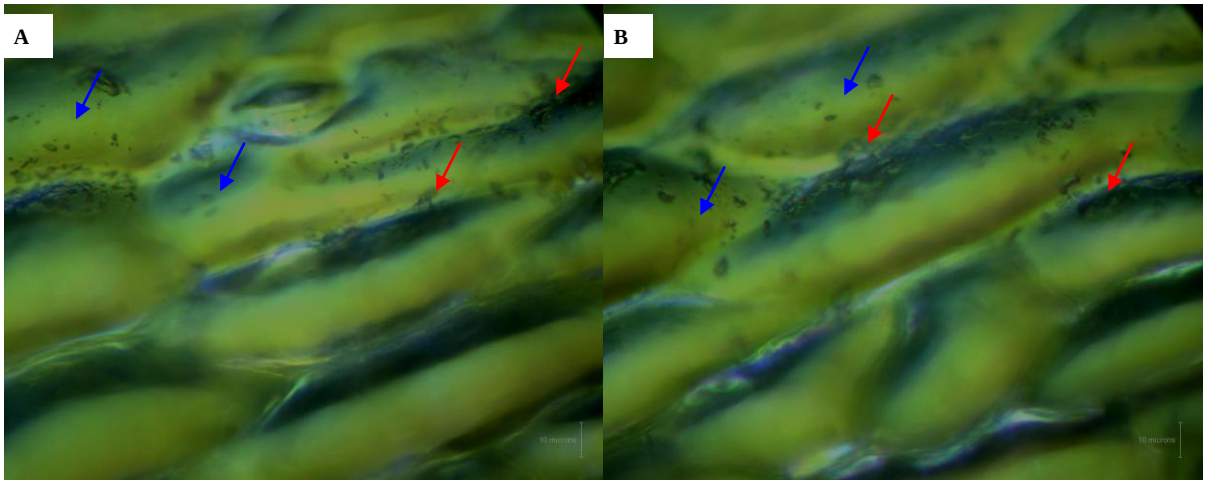


Figure 40: The *in situ* visualization of the spinach phylloplane using the EDIC microscope. (A) pre-pulsification; (B) post-pulsification. Blue arrows show singular rod-shaped bacteria, that can be seen to be located more centrally on the epidermal cells; and red arrows show 3D aggregates of bacteria (or biofilm), these being located more towards the edges of the cells. No significant difference can be seen when viewing the two different samples *in situ* using EDIC microscopy. Bars 10 µm.

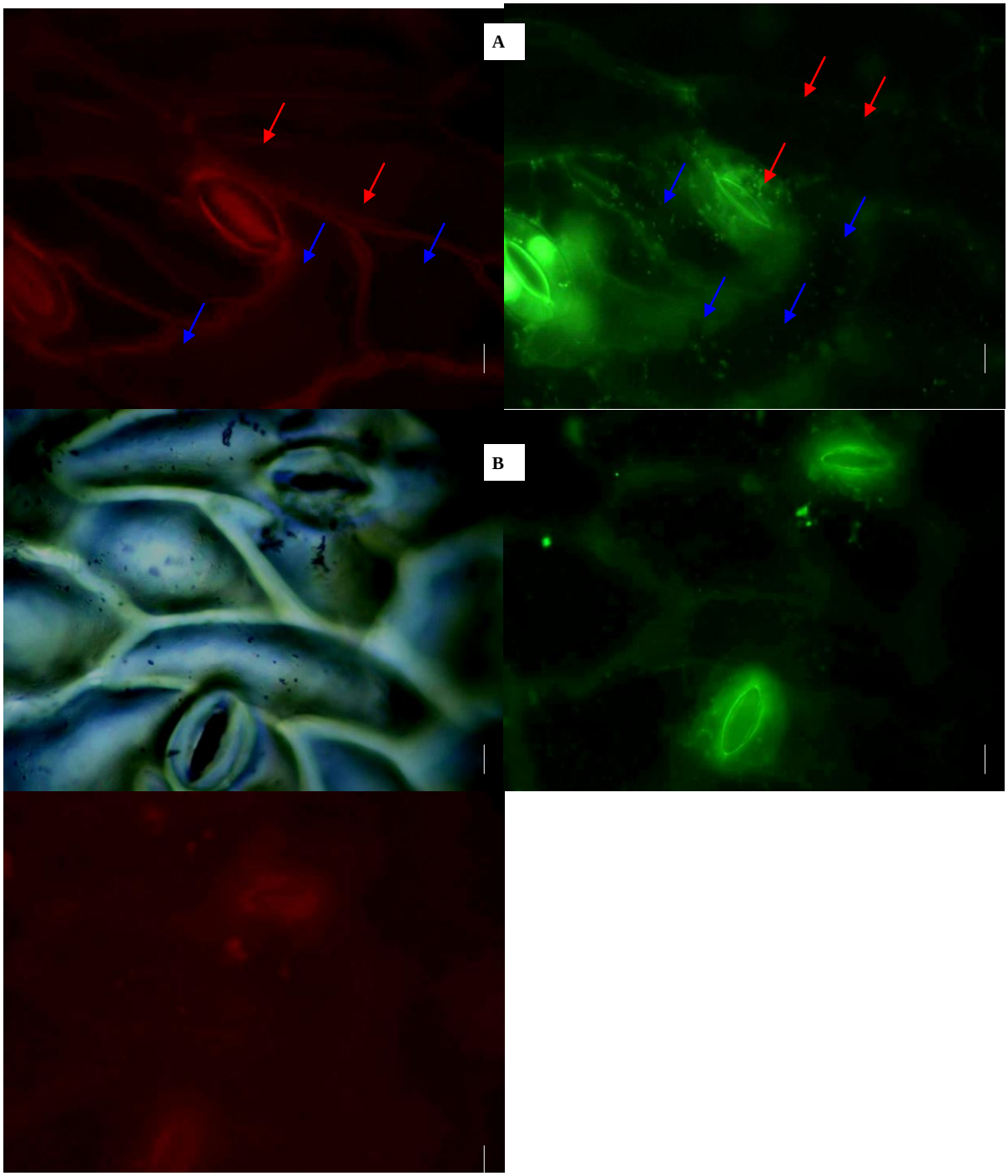


Figure 41: The *in situ* visualization of the spinach phylloplane using the EDIC/EF microscope. (A) Pre-pulsification; dead cells stained red using propidium iodide and live cells green (left to right respectively); (B) post-pulsification; left to right: EDIC image, SYTO 9 staining live cells green, propidium iodide staining dead cells red. Blue arrows show singular bacteria, that can be seen to be located more centrally on the epidermal cells; and red arrows show 3D aggregates of bacteria (or biofilms), these being located more towards the edges of the cells. A drop in single live cells is apparent after pulsification. However, overall in 10 fields of view, no significant difference can be seen when viewing the two different samples *in situ* using EDIC/EF microscopy. Bars 10 µm.

A:

B:

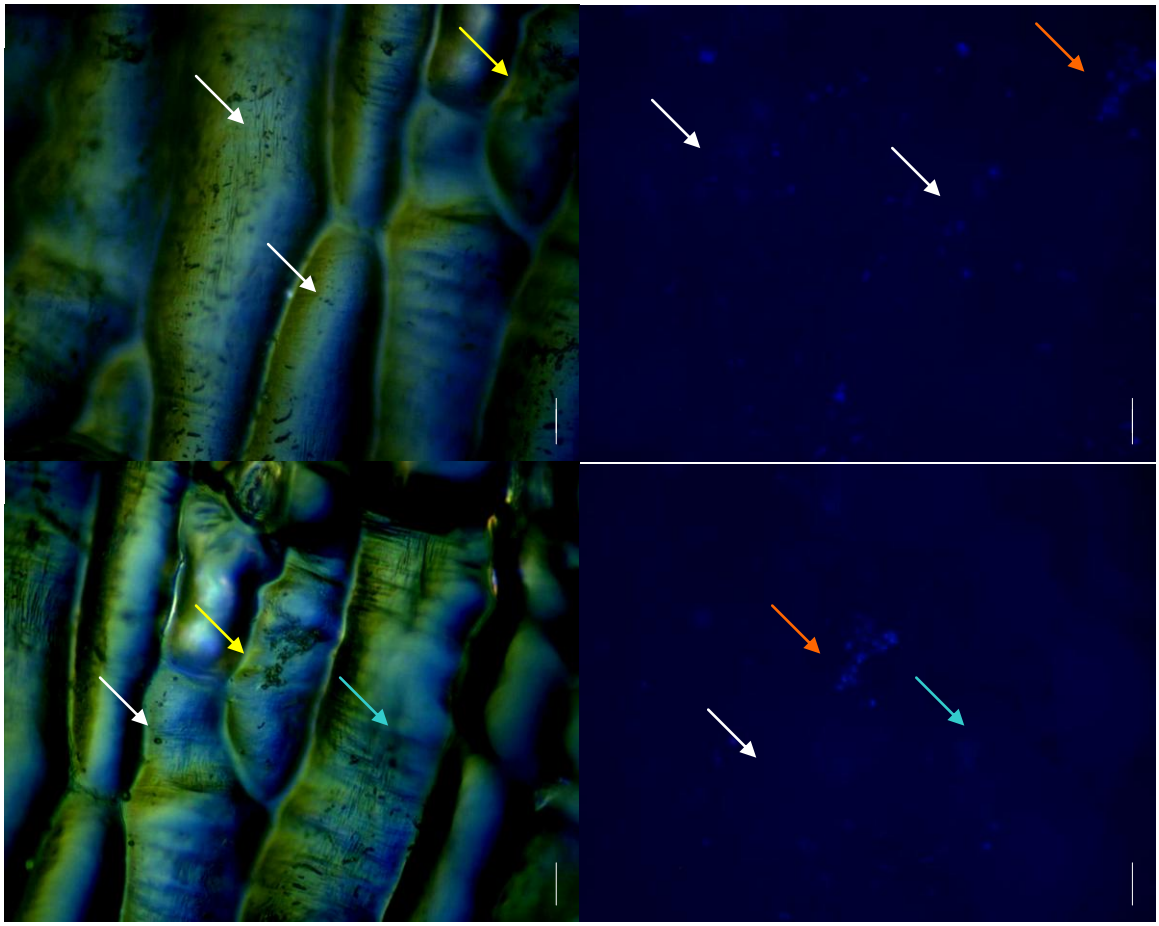


Figure 42. Use of DAPI staining to visualize the indigenous bacteria on the spinach phylloplane. Spinach leaf surface stained with DAPI, EDIC and EF images. Figure A: Spinach leaf stained with DAPI for 15 minutes, stained as single cells and aggregates. Figure B: Spinach leaf pulsified for 30 seconds and then DAPI stained, for 15 minutes. The yellow arrow shows an area on the EDIC image of contamination; the orange arrow shows the corresponding bacterial content; it can be seen that the level of bacteria is lower suggesting the presence of e.g. the exopolysaccharide structure of a biofilm. The white arrows show single bacterial cells, the quantity being higher on pre-pulsified samples compared to post-pulsified. The blue arrows show additional aggregated areas of bacteria that remained on the phylloplane post-pulsification. Bars are 10µm. A drop in single bacteria is apparent after pulsification, however, overall in 10 fields of view, no significant difference can be seen when viewing the two different samples *in situ* using EDIC/EF microscopy

The EDIC images in Figure 40 show the spinach phylloplane pre- (A) and post- (B) pulsification. The *in situ* visualization of the spinach (and the watercress) phylloplane proved to be impossible post-stomaching due to the damaged nature of the leaf post-treatment, therefore, possible *in situ* differences between stomaching and pulsification could not be visualized. Figure 40 A and B show that no significant difference can be seen in the phylloplane colonization patterns before and after pulsification. Figure 40 shows that single bacteria (blue arrows) and aggregates of bacteria (or biofilms; red arrows) are

present on the phylloplane. The single bacterial cells tend to be more located towards the centre of the epidermal cells and the aggregates in the grooves between epidermal cells.

Figure 41 shows the spinach leaf phylloplane stained with BacLight™ to view the live/dead ratio of cells using EDIC/EF microscopy. Figure 41 A shows the spinach phylloplane pre-pulsification; portraying the red, dead microflora and the green, total/live microflora (from left to right, respectively). Figure 41 B depicts the phylloplane post-pulsification; showing the leaf surface, live cells and dead cells (from left to right, respectively). It is apparent that a greater number of live bacteria are present on the phylloplane compared to dead microorganisms, although the level of background fluorescence by the cytoplasm is high (as explained in Chapter Three) which may interfere with results.

The EF images in Figure 41, confirm that single bacterial cells (blue arrows) tend to be more centralized on the phylloplane compared to the aggregates of bacteria (red cells), confirming what was portrayed by the EDIC images (Figure 40). This shows that cells preferentially migrate towards the grooves between the epidermal cells and once there, they are more likely to form complex 3D structural aggregates. An aggregate of green, live cells can be seen in Figure 41 A. This group of cells are situated directly on top of a stoma (see red arrow).

When observing the red arrows on the EDIC image compared to on the green live bacteria image in Figure 41 B; it can be seen that larger 3D areas indicative of bacteria are visible on the EDIC image compared to the EF green live bacteria image. This suggests that the 3D aggregate visible on the EDIC image is composed of material other than bacteria, its slimy appearance indicating it to be the slimy exopolysaccharide material characteristic of a biofilm. Pulsification has not removed this aggregate.

In the sample that has not been pulsified, the viable bacteria appear to be forming clusters or groups and also appear to be locating towards the cell margins, although there are many cells that are still in the centre of the epidermal cells and many that are still single. In the sample that has been pulsified it is evident that a large drop in the numbers of viable bacteria is seen and those that remain are located in the epidermal cell margins or as small clusters - these are the naturally occurring microbes that were not removed after the 30

second treatment and therefore, must have been more resistant to the pulsification detachment forces. The bacteria that were located in the centre of the epidermal cells and those that were single have been removed by the process. Many of the bacteria that were in niches such as around the stomata and grooves in the leaf surface have also been removed.

Figure 42 shows EDIC/EF microscopy images of the spinach phylloplane stained with DAPI to show total numbers of bacterial cells; Figure A showing the *in situ* colonization patterns pre-pulsification and Figure B showing those post-pulsification. The post-pulsified phylloplane (Figure B) can be seen to have lower levels of bacteria compared to the pre-pulsified sample. Most of the single cells (white arrows) appear to have been removed, therefore, suggesting their weaker attachment strength. The aggregates (blue arrows) appear to still remain on the sample after pulsification, thereby indicating the stronger attachment of the bacteria within these. As shown in the BacLight^{TM} images, the areas of aggregates or contamination that can be seen on the EDIC images (i.e. see Figure 42 A, yellow arrow) are not entirely composed of bacteria, since in the corresponding EF images, the entire 3D structure is not fully stained with the DAPI (see Figure 42 A, orange arrow). The fact that these areas are significantly strongly attached compared to other regions of bacteria, suggest the formation of a biofilm on the phylloplane.

4.3.6 Efficiency of *Salmonella* Thompson recovery from the watercress phylloplane using pulsification and stomaching treatments.

In this Section, comparisons were made between the efficiencies of the Stomacher and the Pulsifier for the release of inoculated *Salmonella* on the watercress phylloplane. *In situ* quantification of the watercress phylloplane, for *Salmonella* levels, after each of the treatments was attempted again. However, as in Chapter Three, this proved to be very inaccurate, due to the treatment sample sizes used in this study, therefore, viable counts using Rambach agar were exploited instead.

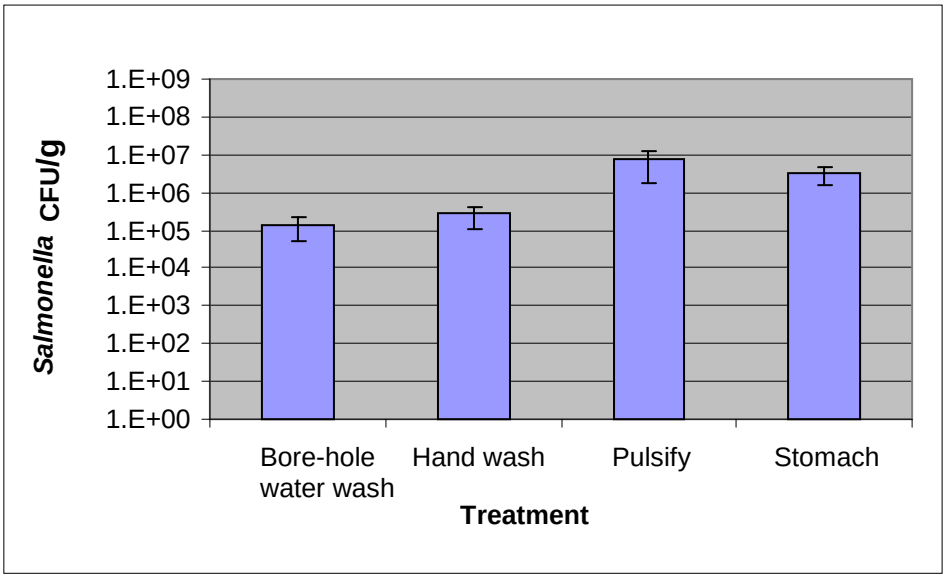


Figure 43: Log numbers of *Salmonella* Thompson recovered from the watercress phylloplane (in suspension) after either a 30 second wash in bore-hole water), a 30 second hand wash, a 30 second pulsification or a 30 second stomaching treatment. Approximately 8-log *Salmonella* was initially inoculated onto the watercress phylloplane. Pulsification has recovered the greatest number of *Salmonella.* Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

A statistical analysis showed that there was a significant difference between the number of *Salmonella* recovered between the inoculum level and the 30 second bore-hole water wash ($p<0.001$), thus suggesting that a significant number of *Salmonella* attached to the watercress phylloplane. No significance was found between the 30 second hand wash and the 30 second bore-hole water wash ($p=0.999$), 30 second hand wash and 30 second stomaching ($p=0.9489$), 30 second pulsification and 30 second bore-hole water wash ($p=0.2546$), 30 second hand wash and 30 second pulsification ($p=0.2714$), 30 second stomaching and 30 second bore-hole water wash ($p=0.9378$), and 30 no significant difference between 30 second pulsification and 30 second stomaching ($p=0.7768$). Although no significant difference is apparent between many of these treatments, as Figure 43 shows, *Salmonella* levels were shown to vary with each of the treatments. These were shown by an unpaired t-test. P values less than 0.05 were regarded as significant. Since the initial level of *Salmonella* is known and the level of cells attached is known, the efficiency of the Pulsifier to recover *Salmonella* could be calculated. On average, the efficiency of the Pulsifier to remove *Salmonella* Thompson from the watercress phylloplane can be calculated to be approximately 68 %, therefore, 32 % *Salmonella* surviving pulsification.

Approximately 8-log *Salmonella* was inoculated onto the watercress phylloplane. Figure 43 shows that approximately 5-log levels of *Salmonella* remained in suspension after a 30 second bore-hole water wash, suggesting that 7.5-log *Salmonella* had attached to the phylloplane after 12 hour incubation. The hand wash treatment also resulted in similar levels of *Salmonella* in suspension, thereby suggesting that hand washing does not necessarily detach a significant number of pathogen from the matrix (although a small increase in suspension is evident). An approximate 2-log increase in recovery post-pulsification is evident compared to the un-treated sample (30 second bore-hole water wash), resulting in approximately 7-log *Salmonella* recovered in total. Since the level of *Salmonella* inoculated initially into the sample was 8-log, this suggests that 7-log *Salmonella* remained attached to the phylloplane even after pulsification. Although a higher recovery of *Salmonella* was apparent post-pulsification compared to post-stomaching, this level was shown not to be significantly different.

4.3.7 Investigation of the differences in coliforms recovered from watercress imported from either Portugal or the USA.

The aim of this Section was to investigate any differences in the natural levels of coliforms on the watercress phylloplane, when imported from either Portugal or the USA. VRBA was used to enumerate the coliforms that were recovered from the watercress, and in suspension.

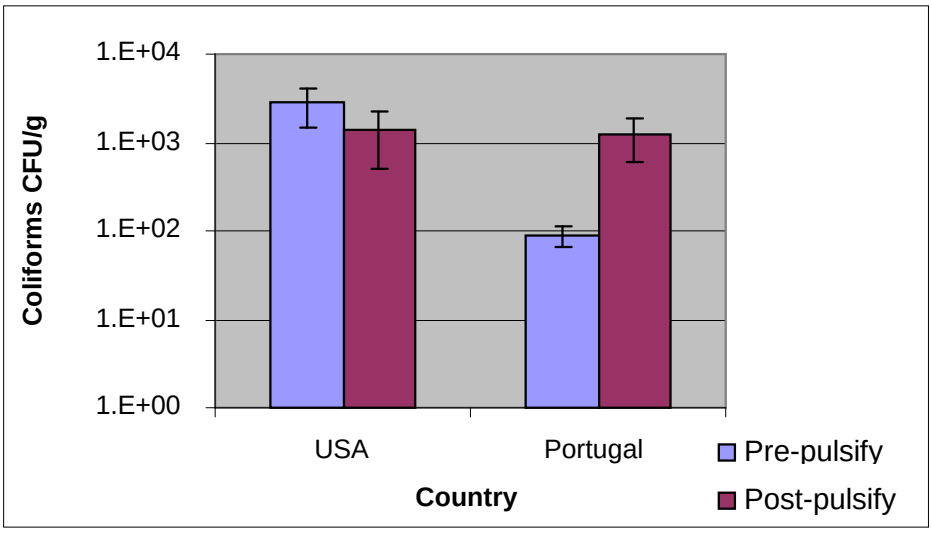


Figure 44: Coliforms recovered from watercress leaves (in suspension) that were imported from either the USA or Portugal. Pre- and post- pulsification coliforms numbers are evident; these show the loosely and strongly attached coliforms respectively. Error bars are representative of the ± standard error of the mean of six experiments for each country.

Statistical analysis showed that there was a significant difference between the pre-pulsification numbers of coliforms recovered between the two countries, shown by ANOVA. This indicates the potential problem of imported produce and the variations in levels of the natural microflora on salad leaves. No significant differences were apparent in the numbers of coliforms recovered post-pulsification between the two countries, and in the total numbers of coliforms recovered. P values less than 0.05 were regarded as significant.

The results presented in Figure 44 show that watercress from different farms across the world has varying numbers of coliforms present in the control samples. It is evident that watercress from the USA has higher background numbers of coliforms than those from Portugal. On average approximately 1.5-log more coliforms were recovered pre-pulsification from the watercress samples that were imported from the USA compared to those from Portugal. Although the post-pulsification levels of coliforms recovered is slightly higher from the USA phylloplanes, no significant difference was apparent.

4.4 Chapter Four Discussion

The aim of this Chapter was to compare the Pulsifier to the current industry standard Stomacher, in order to facilitate the characterization of the naturally occurring microflora and inoculated *Salmonella* Thompson on the watercress phylloplane. The aim was also to investigate varying operating conditions, i.e. that of treatment length, and presence or absence of air on the numbers of microorganisms recovered. Investigations were also made to determine if any differences in attachment strengths of coliforms exist, between the country that a phylloplane is imported into the UK from, i.e. between the USA and Portugal.

From the results in this Chapter it can be concluded that the Pulsifier is more efficient than the Stomacher in terms of less damage to the watercress phylloplane, the matrix remaining intact after 30 second treatment. This was shown by the optical density results and the photographed images of the leaf matrices pre- and post-treatment. This is important for industries when the sample is required to be left intact, and also is important for filtration or microscopy studies, since samples having undergone between a 10 and 30 second stomaching treatment were impossible to view under the microscope. Minimal damage is important, since cuts and abrasions to the leaf surface result in an increased likelihood of internalization of potential pathogens (Garg *et al.*, 1990; Seo *et al.*, 1999; Brandl *et al.*,

2002). The Pulsifier was also shown to be significantly more efficient than the Stomacher in terms of coliform recovery, possibly due to the fact that less debris is produced after pulsifying and therefore, less of the bacteria may remain trapped on this debris - more being free in suspension to be accounted for using the analytical procedure. It may also be that the intense shock waves produced by the Pulsifier are the correct frequency for optimal recovery of this particular organism from the watercress phylloplane, compared to the mechanical semi-homogenization using the Stomacher. In terms of total heterotrophs, although an increase in recovery was seen post-pulsification compared to post-stomaching, this was shown not to be significant. This could be because some microorganisms (such as coliforms, as shown by the data) may be more easily removed from the watercress phylloplane compared to other microorganisms, resulting in differences in the total heterotrophs being recovered between the two treatments not being significant.

The mechanisms by which the Pulsifier may be eliciting its effect on microorganisms have not been established to date. However, it may be that the shearing forces that are applied to the matrix surface result in forces of attraction between bacteria, and between bacteria and the phylloplane being weakened. As shown by the EDIC/EF images, although single cells are removed more easily, some areas of biofilm are still shown to be removed by the shearing activity of the Pulsifier. This attribute characterizes the phylloplane matrix, since it shows that these aggregates of bacteria are more firmly attached to the phylloplane compared to the singular cells. Since biofilm bacteria are known to be more strongly attached and resistant to removal than single bacteria, this provides further evidence for the existence of biofilms on the phyllosphere (Gormon *et al.*, 2001). Biofilm bacteria are thought to resist removal due to the bacteria exhibiting a different genotypic response to adherence (Gormon *et al.*, 2001) compared to their planktonic counterparts. Cells that are incorporated into a biofilm on the watercress phylloplane would also be surrounded by an exopolymer matrix, which would buffer any environmental changes and also any possible treatments used by industries for salad leaf sanitization (Monier and Lindow, 2005). Published studies have investigated factors that increase biofilm detachment and have reported that fluid shear stress does play a role in this (Picioreanu *et al.*, 2001). If even some areas of biofilm are being removed (not all as shown by the EDIC/EF images), then this is of great advantage since biofilms are a major problem in industries and as suggested previously, may be a major contributing factor to continued foodborne illness outbreaks despite industrial sanitation processes.

To date, the literature contains no information on the recovery of pathogens, such as *Salmonella*, from any matrix using the Pulsifier. The results presented in this Section show that pulsification is as efficient as stomaching in removal of pathogens from the phylloplane. The fact that a clearer diluent remains post-treatment and the sample remains intact post-pulsification suggests that the Pulsifier is overall more efficient. On average, the efficiency of the Pulsifier to remove *Salmonella* Thompson from the watercress phylloplane can be calculated to be approximately 68 %, 32 % *Salmonella* surviving pulsification. The 32 % that survived pulsification may have integrated into the naturally occurring biofilm structures that can be visualized by EDIC/EF post-pulsification. It is these protected cells that go on to cause gastrointestinal illness. Some of this 32 % of *Salmonella* that has not been recovered from the phylloplane may have been internalized, as shown to occur within 30 minutes of inoculation in Chapter Three. Chapter Three also deduced that inoculated *Salmonella* Thompson is also found on the regions of the phylloplane that already contains the indigenous bacteria, this having also been noted in the literature (Carmichael *et al*, 1999). Therefore, since the Pulsifier was shown to result in detachment of these biofilm cells, then any *Salmonella* cells that became attached would be expected to be removed at a similar time too. A distinct advantage that *Salmonella* cells have over *E. coli* cells is the ability to produce aggregative fimbriae (curli). Although *E. coli* does have curli genes, it has been reported that over 95 % have a single mutation resulting in absence of curli (Uhlich *et al*, 2001). Curli has been shown to be induced at low osmolarity and low temperature conditions, these are conditions that are found in the phyllosphere, therefore, it is likely that *Salmonella* is attaching to the watercress phylloplane by use of curli (Romling *et al*, 1998). The Pulsifier may be hypothesized to be eliciting its effect on the detachment of *Salmonella* from the phylloplane by acting on curli. If time were available, *Salmonella* curli mutants could be used to determine if this was the case. A study in 2006 investigating the attachment of *E. coli* to lettuce tissue suggested that bacterial processes (production of extracellular compounds, gene expression and motility) were not necessary for initial attachment, and that entrapment in niches was the most important factor initially; however, Solomon *et al* (2006) carried out this study specifically using *E. coli* and it may be that this species uses this described principle instead of curli.

The present study is the first to show the *in situ* colonization patterns of bacteria, using EDIC/EF microscopy combined with BacLight™ and DAPI. When viewing the leaf *in situ*

using the EDIC microscope alone, overall, no clear difference can be seen on the level of contamination pre- and post- pulsification. When investigating total heterotrophs recovered using R2A medium, it was apparent that almost 7-log recovery occurred post-pulsification, compared to a 30 second hand wash which recovered 5-log. The fact that this level of recovery does not result in differences in the EDIC images may suggest that the level of contamination, i.e. the quantity of indigenous bacteria, originally is very high, camouflaging the difference. It may also be due to the fact that 25 g watercress samples were compared in this study, smaller sample sizes of e.g. single leaves may result in fewer biological variations in the results. The fact that the Pulsifier is detaching up to 7-log of the total heterotrophs is important, since these, plus the coliforms, are naturally occurring in the phyllosphere and/or soil and therefore, are likely to have been on the phylloplane for long periods. The heterotrophs and coliforms may be likely to be more resistant and therefore, more strongly attached than pathogens such as *E. coli* or *Salmonella* which are newer residents on the phylloplane.

Staining with BacLight^{TM} to view the live/dead ratio of cells, and DAPI to view the total cells, combined with the use of EDIC/EF microscopy showed a reduction in contamination after pulsification, particularly in the single cells located centrally on the epidermal cells. Some, but not all, of the aggregates appeared to be removed post-pulsification. Overall, it was apparent that a larger number of live microflora was present on the phylloplane compared to dead microorganisms, although the level of background fluorescence by the cytoplasm was high (as explained in Chapter Three) which may have interfered with the results. However, it may be expected for levels of dead bacteria to be lower on the phylloplane due to diminished attachment and survival mechanisms compared to the live microflora which can attach, resist, and even locate to protected niches on the matrix. An aggregate of green, live cells can be seen in Figure 54 A. This group of cells was situated directly on top of a stoma. This is likely to be an active process by the individual cells, in order to move closer to the stomata, which can allow structural protection; this structure also exudes sugars and gases as potential nutrients; and also more importantly, serves as a possible entry point into the leaf interior. Internalization of any bacteria would result in protection from environmental fluctuations and also from any removal or disinfectant treatments employed by industries. When observing the aggregates on the EDIC images compared to the green fluorescent live bacteria images or the DAPI stained images, it could be seen that larger 3D areas indicative of bacteria were visible on the EDIC images

compared to the EF images. This suggests that the 3D aggregate visible on the EDIC micrographs is composed of material other than bacteria; its slimy appearance indicating it to be the slimy exopolysaccharide material characteristic of a biofilm. The fact that pulsification did not even remove the live bacteria in this aggregate, suggests that it is very strongly attached to the phylloplane, again indicating the presence of biofilm.

In the sample that had not been pulsified, the viable bacteria appeared to be forming clusters or groups and also appeared to be locating towards the cell margins, although there were many bacteria that were still in the centre of the epidermal cells and many that were still single. In the sample that had been pulsified, a large drop in the numbers of viable bacteria was evident and those that remained appeared to be located in the epidermal cell margins or as small clusters: these are the naturally occurring microbes that were not removed after the 30 second treatment and, therefore, must have been more resistant to the pulsification detachment forces. The bacteria that were located in the centre of the epidermal cells and those that were single appeared to be removed by the pulsification process. This is very likely due to the bacteria being more likely to be protected as part of an aggregate and these bacteria having increased protection mechanisms and attachment forces, compared to single cells. The fact that more bacteria are loosened from the matrix surface post-pulsification is advantageous because they are now in suspension and, therefore, no longer entrapped on the matrix. Many of the bacteria that were in niches such as around the stomata and grooves in the leaf surface were also removed. DAPI staining the spinach phylloplane showed the post-pulsified phylloplane to have lower levels of bacteria compared to the pre-pulsified sample, as for BacLight^{TM} staining above. Most of the single cells (white arrows) appeared to have been removed, therefore, suggesting their weaker attachment strength. The aggregates appear to remain on the sample after pulsification, thereby indicating the stronger attachment of the bacteria within these. These colonization patterns were apparent in some fields of view but not all; in leaves where this effect was not apparent, it may be that the leaves were overlapping each other or curled up, so they did not experience fully the effect of pulsification. For this reason, as an extension to the work presented here, it is important to develop a method by which pulsification could be performed on single leaves, to view the direct effect on the phylloplane, using EDIC/EF microscopy.

Pulsification of the phylloplane combined with EDIC/EF microscopy indicates the attachment strengths of bacteria in different locations of the leaf and the attachment strengths of bacteria that are either singular or aggregates, it also shows possible areas of biofilm formation on the phylloplane. Unfortunately, it was not possible to quantify the results due to biological variations, as discussed above, however, this may be possible in future work if the protocols used here were adapted to study single leaves.

For a sample size of 250 g the optimal treatment length for cell recovery is 30 seconds, this was for both the Stomacher and the Pulsifier. Although it is not entirely clear why a 30 second timing may be optimal, it is possibly due to a range of factors. Stomaching even at 30 seconds damages the sample, resulting in a semi-homogenized sample with high levels of debris; this is not ideal, however, stomaching for longer than 30 seconds produces even more debris. It is likely that the Pulsifier is more efficient in recovery of coliforms than the Stomacher because the increased debris that is produced by the Stomacher (due to its use of mechanical forces rather than vibrational forces) could be harbouring many of the cells. Stomacher treatment may be preferentially damaging the phylloplane rather than detaching cells from it, so when aliquots of the stomached sample are taken for plate counts it may be that many of the bacteria are on the large pieces of debris and have not been shaken into solution. The debris produced by the Stomacher make it difficult to pipette just the suspension of bacteria in buffer without pipetting pieces of leaf debris. The results suggest that bacterial cells are strongly attached to the surface of the leaf, even a 5 second pulsification treatment is not sufficient to detach large numbers of cells. However, if no pulsification treatment is included some cells are still detached, suggesting that all of the microbial population on the surface are not attached with the same strength, some being more loosely attached than others. If the cells do form biofilms on the surface of the leaf structure, then it may be that the top cells in the biofilm are detached more easily than the lower layers, as shown by some areas of biofilm being removed compared to others post-pulsification in the EDIC/EF images. Other bacterial cells that remain attached to the phylloplane post-pulsification are shown to be trapped in crevices or grooves or in the region of stomata, although levels of these single cells are considerably lower as stated above. The preferential attachment of microbes to grooves was shown in a study investigating wrinkled and smooth alfalfa seeds (Charkowski, 2001).

From the results obtained it can also be concluded that it is ideal if there is less air in the treatment bag when pulsifying or stomaching samples, since there is less noise, a lower likelihood of aerosol production and less damage to the matrix surface. Although this leads to a slightly lower bacterial recovery, the reduction has been shown not to be significant. The reason for this increase may be because in the samples that contain air there are more micro bubbles of air available to "hit" the phylloplane in the 30 second time period, so it would be likely for this to increase the level of bacteria dislodged from the phylloplane. There would also be a larger number of micro bubbles of air colliding with each other too, increasing the kinetic energy of all the molecules in general, which would therefore hit the phylloplane with greater force, this would destroy the sample more, but it would also allow for a greater number of potential pathogens from being removed from niches and crevices. This hypothesis would be particularly important if bacterial processes did not mediate survival in the phylloplane and if physical entrapment was the main cause for pathogen adherence as shown by Solomon and Matthews (Solomon and Matthews, 2006). On average approximately 8% more coliforms could be seen to be released from the phylloplane when air was incorporated into the sample bag. Although this may be important since in pathogenesis even a low inoculum level can lead to illness, in terms of the level of sample damage and quality of leaf it would be better not to incorporate air into the sample. For this reason, air was removed from samples prior to treatment in the remainder of this project.

From the results it can be seen that there are considerable differences with and without air in the sample bag; this factor would play a major role in comparative studies and it is therefore important for this to be standardised throughout a set of experiments. The design of the Pulsifier may need to be improved, i.e. to control the level of air incorporated into treatment bags. The results presented by Fung *et al.* in 1997, Sharpe *et al*. in 2000 and Kang *et al.* in 2001 did not account for the levels of air incorporated into the sample bag each time and neither did they investigate the optimal working time length of the Pulsifier or Stomacher for each matrix type, therefore, according to the results presented in this Chapter the conditions may not have been a fair test (Fung *et al.*, in 1997, Sharpe *et al.*, in 2000 and Kang *et al.*, 2001, Wu *et al.*, 2003). If time were permitted future work would involve collaborating with engineers to develop a Pulsifier that took into account these effects and increased its efficiency further. A project led by engineers at the University of Southampton is currently underway specifically to refine the mechanics of this instrument.

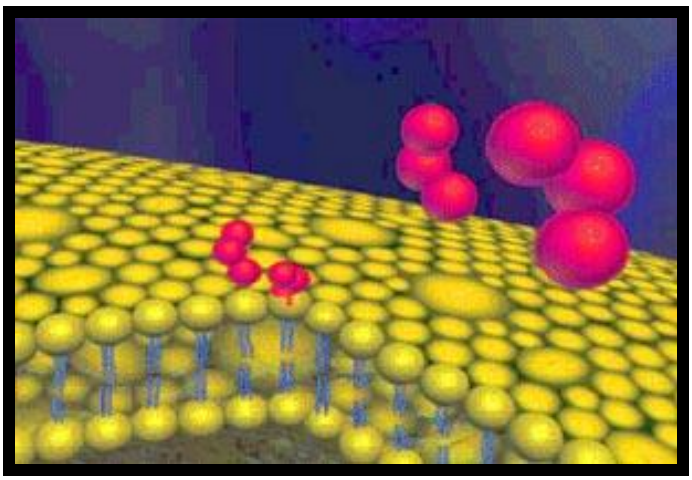
The results show that watercress from different farms across the world have different numbers of coliforms present in the control samples. It can be seen that watercress from the USA has higher background numbers of coliforms than those from Portugal. This may be due to a variety of factors, such as growth conditions, soil type, weather conditions, etc. This may also be affected by the location of the farms, how much irrigation water is used and where this water comes from. On average, approximately 1.5-log more coliforms were recovered pre-pulsification from the watercress samples that were imported from the USA compared to those from Portugal. This suggests that the numbers of coliforms on the USA phylloplanes that were more loosely attached were higher compared to the tightly attached numbers. Although the post-pulsification levels of coliforms recovered is slightly higher from the USA phylloplanes, no significant difference was apparent. This suggests that similar levels of the coliforms were more tightly attached to the watercress phylloplane between the two countries. These results may suggest a reason for the 2007 recall of watercress in Ireland due to imported watercress leaves from USA which had been processed in the UK i.e. that the *Salmonella* pathogens were easily detached and detected using conventional methods such that the hazard could be identified (FSAI, 2007).

Although investigating the numbers of coliforms present naturally on the leaf phylloplane is useful, since inoculating samples does not necessarily imitate environmental conditions, the limitation is that original numbers on the phylloplane are unknown, so the percentage coliform release efficiency by pulsification can not be calculated. If time was available, future work would involve investigating the potential use of FISH or PNA probes to view the *in situ* effects of naturally occurring microorganisms in the phyllosphere or the further development of the EDIC/EF microscopy methodologies described in Chapter 3. Despite the fact that this calculation cannot be performed and the actual level of efficiency of the Pulsifier in detaching the naturally occurring microflora is unclear, the results presented in this Section show that the Pulsifier is better than any other available microbial release agent to date. It was also shown that, on average, the efficiency of the Pulsifier to remove *Salmonella* Thompson from the watercress phylloplane was approximately 68 %, which is a significant amount. In further work it would be interesting to use mutants of *Salmonella* and thereby attempt to establish how the Pulsifier is eliciting its effect. *E. coli* could also be inoculated onto the leaf matrix to determine if the Pulsifier is effective on this microorganism, studies have reported that *Salmonella* attaches to alfalfa sprouts up to a

thousand times more that *E. coli*, so pulsification may remove *E. coli* from the phylloplane more easily (Barak *et al.*, 2002).

CHAPTER FIVE

DISINFECTION OF THE PHYLLOPLANE MATRIX. COMPARISONS OF CHLORINE, OZONE AND PULSIFICATION IN REDUCING LEVELS OF COLIFORMS, *E. COLI* AND *SALMONELLA*.



5.1 Introduction

The next objective of this study was to determine whether chlorine, ozone and pulsification treatments were suitable to disinfect the naturally occurring coliforms and *E. coli*, and inoculated *Salmonella*, from the watercress phylloplane. This was determined by using current industry approved plate count methods to enumerate initial levels of microorganisms, followed by the use of the Pulsifier to shear microorganisms from the watercress phylloplane to enumerate surface bound survivors. In addition, the Pulsifier and Stomacher were compared to elucidate their use in combination with filtration recovery techniques. The EDIC microscope was used to observe any *in situ* differences in colonization patterns of the microflora.

Experiments were conducted to establish whether the concentration of chlorine being used by food industries worldwide were the most efficient and if chlorine disinfection could be replaced entirely with other means of disinfection. Chlorination is the most commonly used fresh produce disinfectant; however, this processing may not be sufficient since previous studies have shown that significant numbers of pathogens survive on fresh produce treated with chlorinated water (Koivunen and Heinonen-Tanski *et al.*, 2005). Moreover, salad leaves cut during preparation release sap contents which in the presence of chlorine may form chlorinated compounds, such as chlorinated hydrocarbons and trihalomethanes (i.e. chloroform) that can produce eye and respiratory irritation and have been associated with cancer (Hery *et al.*, 1998; Simpson *et al.*, 2000; Komulainen, 2004). The microbial killing efficiency of the alternative disinfectant, ozone, on the watercress phylloplane was therefore, compared to chlorine, as was the practicality of incorporating an ozonator at the industrial scale. Ozone has already been established as an efficient means for antimicrobial water treatment installations worldwide without the production of hazardous residues (Rice *et al.*, 2000). In 2001, the U.S Food and Drug Administration (FDA) officially accepted ozone as 'Generally Recognised as Safe (GRAS)'; thereby enabling the legal use of the antimicrobial properties of ozone for the treatment, storage, and processing of foods, in both its gaseous and liquid phases. Ozone can therefore be potentially used in direct contact with minimally processed fruits and vegetables. Although ozone has been established as a powerful broad spectrum antimicrobial, in the literature there are wide variations in the reported sensitivities of even one organism (Broadwater *et al.*, 1973; Finch *et al.*, 1988; Kim and Yousef, 2000). This highlights the importance of food industries establishing ozone efficiency, prior to being incorporated

into a wash-line for fresh foods. Practical implications of ozone use on the industrial scale also needed to be considered, such as quality of produce post-treatment, maintaining ozone concentrations, and not exceeding the 0.1 ppm ozone concentrations at the factory level as this may irritate workers (Buckley *et al.*, 1975).

Finally, the use of the Pulsifier was investigated as a mechanical, rather than a chemical sanitizer for disinfecting the phylloplane matrix. The results presented in Chapter Four were expanded in this Section, in order to evaluate the use of the Pulsifier when incorporated into a wash process, rather than solely for the recovery of cells in sample preparation techniques. The phyllosphere was subsequently characterized further by investigating the effect of watercress age on numbers of coliforms recovered from the surface of watercress; this would also have implications on consumer storage of watercress.

5.2 Methodology

Please refer to Chapter Two.

5.3 Results

5.3.1 Determination of the effectiveness of hypochlorite treatment to reduce natural *E. coli* levels on the watercress phylloplane, using the Pulsifier for recovery.

The aim was to determine the *E. coli* count after either a wash in bore-hole water or 90 ppm hypochlorite, using the Pulsifier for recovery. Initially, a detection principle of direct plate spreading was utilised, however, this method was not sufficiently sensitive, and no *E. coli* were detected. Subsequently a filtration step was included, in order to concentrate any bacteria, and a positive result for *E. coli* was evident. The detection principle was based on membrane filtration concentration of recovered supernatant wash followed by overlay on TBX agar. This method was able to produce a detection limit of < 1 *E. coli* / 10 g. The concentration of the hypochlorite solution was measured using the DPD assay before and after treatment, in order to determine the drop in concentration after contact with the watercress leaves.

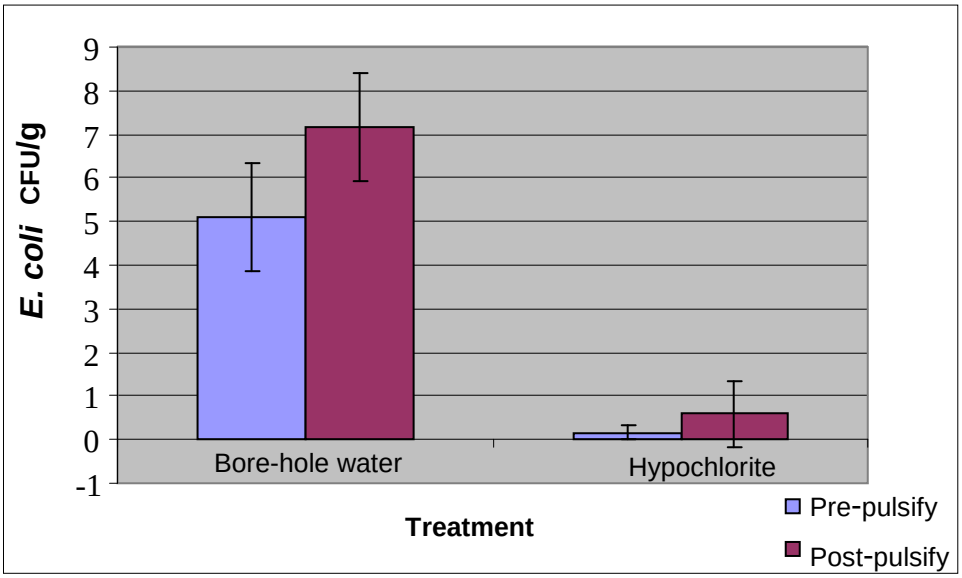


Figure 45: Numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension), after the phylloplane was treated with either bore-hole water or 90 ppm hypochlorite. Pre- and post-pulsification values show the bacteria that were loosely attached and strongly attached to the phylloplane, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

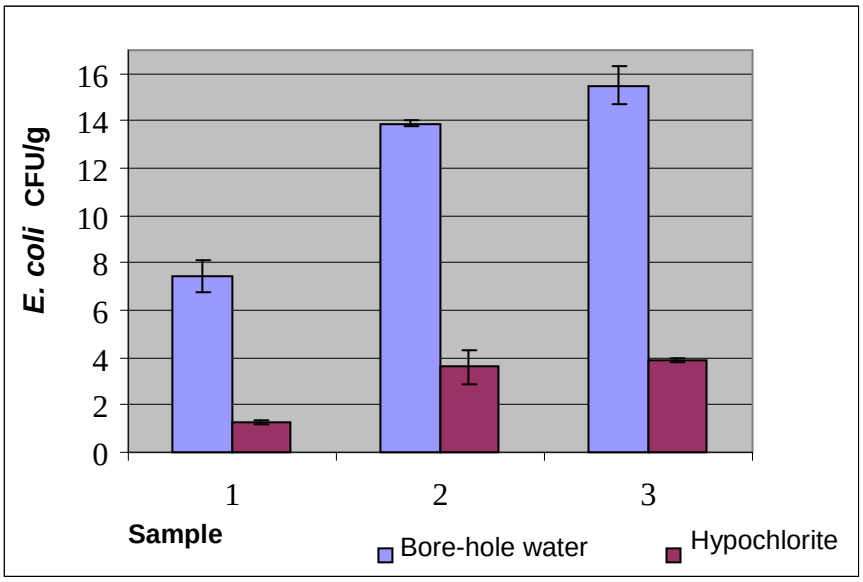


Figure 46: Total numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension), after the watercress phylloplane was either treated with bore-hole water or 90 ppm hypochlorite, using the Pulsifier for recovery. Biological variations can be seen between samples, as can variations in the efficiency of chlorine treatment. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference in the number of *E. coli* recovered between pre-pulsified and post-pulsified bore-hole water treatments ($p<0.001$).

A significant difference was also apparent between the pre-pulsified bore-hole water and hypochlorite treated samples ($p=0.0247$). No significant differences were apparent between the post-pulsified bore-hole water and hypochlorite treatments ($p=0.47$), or between the pre-pulsification and post-pulsification hypochlorite treatments ($p=0.0817$). These were shown by a paired t-test. P values less than 0.05 were regarded as significant. Significant differences were apparent between bore-hole water and hypochlorite treatments in all three individual samples ($p<0.05$).

From the results in Figure 45, it is evident that pulsification produces greater recovery of *E. coli* from the watercress phylloplane after a bore-hole water treatment and also after hypochlorite disinfection. It can be seen that when the watercress has been treated with 90 ppm hypochlorite none or minimal numbers of *E. coli* are detected on the agar; however, when the same sample of watercress is pulsified post-chlorination treatment, more *E. coli* are detected. Hypochlorite disinfection is shown to effectively kill *E. coli* that are 'loose' in the Pulsifier bag, i.e. those *E. coli* that have detached from the surface of the watercress and are in suspension, it is only less than 1 % of these *E. coli*, that are still surviving. From Figure 58 it is evident that approximately 82 % of the originally tightly attached *E. coli* were also detached and killed by the hypochlorite solution, but not by the bore-hole water solution. From the results it is evident that there is only a small decrease in the concentration of chlorine from 90 to 85 ppm after the 2 minute treatment time.

Biological variation can be seen in the results shown in Figure 46; in the three different samples, the same number of *E. coli* was not recovered each time. In the bore-hole water samples, in one sample approximately 16 cfu / g were recovered while in another 14, and in one sample only 8 cfu / g were recovered. In the sample where the *E. coli* recovered in total was approximately 8 cfu / g, 30 % of the cells that were detached and "loose" in the treatment bag still survived hypochlorite treatment; this may be because these cells were more resistant (Figure 46).

5.3.2 Determination of *E. coli* count from watercress phylloplane after bore-hole water and or hypochlorite treatments, using the Stomacher for recovery.

The aim was to determine the *E. coli* count from watercress, after a wash in bore-hole water or after a 2 minute hypochlorite treatment wash; using the Stomacher for analysis (the current food industry used methodology for cell detachment). As for the previous

Section, there were no background levels of *E. coli* in the bore-hole water, which was used as the diluent in the disinfection studies involving enumeration of *E. coli* numbers. As such, any *E. coli* found to be growing on the TBX plates could be assumed to be from the watercress samples supplied alone.

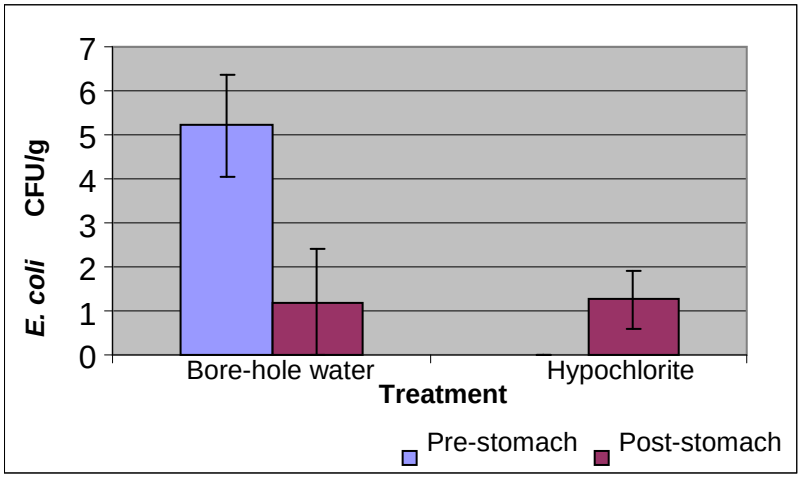


Figure 47: Numbers of viable *E. coli* in suspension, after watercress samples were treated with either a bore-hole water treatment or 90 ppm hypochlorite disinfection. Pre- and post-stomaching, values show the bacteria that were loosely attached and strongly attached on the watercress phylloplane, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

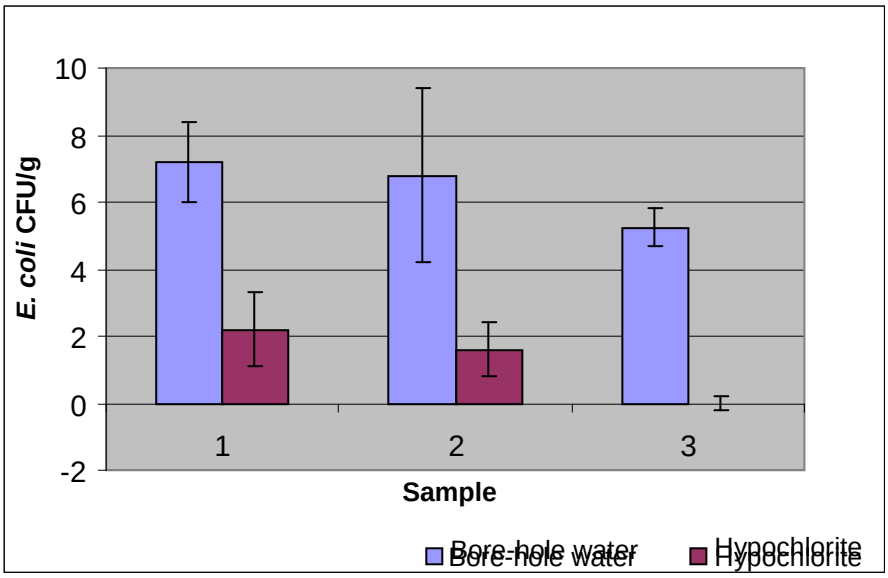


Figure 48: Numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension), after either a borehole water treatment or a 90 ppm hypochlorite disinfection, using the Stomacher for recovery. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference in the number of *E. coli* recovered between pre-pulsified bore-hole water and hypochlorite treatments (p=0.0460). No significant difference was apparent between the post-pulsified bore-hole water and hypochlorite treated samples (p=0.9612). No significant differences were apparent between the pre- and post-pulsified bore-hole water treatments (p=0.22) and between the pre-pulsification and post-pulsification hypochlorite treatments (p=0.1936). These were shown by a paired t-test. P values less than 0.05 were regarded as significant. No significant differences were apparent overall between bore-hole water and hypochlorite treatments in the individual samples (p=0.7127).

Stomaching is expected to produce greater recovery of microorganisms from watercress after bore-hole water (or PBS) treatment, as suggested by the results presented in Chapter Four and this may also be the case after hypochlorite disinfection. However, this trend was not seen in these results, since the analysis was very difficult. Due to the Stomacher damaging the watercress, the filters became clogged very easily, so the process was very slow. Inevitably small and large particles of the watercress were left on the cellulose nitrate membranes and after the 24 hour incubation time extensive fungus growth was apparent, making it extremely difficult to count the *E. coli* colonies. This shows that the Stomacher is not ideal for use when studying small numbers of bacteria and when filter equipment is required for bacterial concentration (Figure 47).

However, from the results it can be seen that when the watercress phylloplane had been treated with 90 ppm hypochlorite, none or minimal numbers of *E. coli* were detected on the agar (Figure 47 and 48). Hypochlorite disinfection appears to effectively kill *E. coli*. Subsequently, when the watercress phylloplane was stomached, any protected cells were released from the phylloplane. From the data shown in Figure 48 it is clear that biological variation among three different samples is apparent as for the previous experiment. The error bars are large due to difficulties in analysing results after using the Stomacher instead of the Pulsifier prior to filtration. Due to the difficulties in analysing the results it was not possible to make accurate conclusions from the data presented post-stomaching.

5.3.3 Enumeration of coliform count from the watercress phylloplane after bore-hole water or hypochlorite treatment using Pulsifier recovery.

The aim was to enumerate the coliform count from watercress, after a wash in bore-hole water or after a 2 minute hypochlorite treatment wash, using the Pulsifier for analysis. The detection principle used was direct inoculation (1.0 ml) of serially diluted wash supernatant into VRBA. The detection limit using this method was 10 coliforms / g. Initial investigations showed that there were no background levels of coliforms in the bore-hole water. The bore-hole water was the diluent in the disinfection experiments, so any coliforms found to be growing on the VRBA plates could safely be assumed to be from the watercress phylloplane.

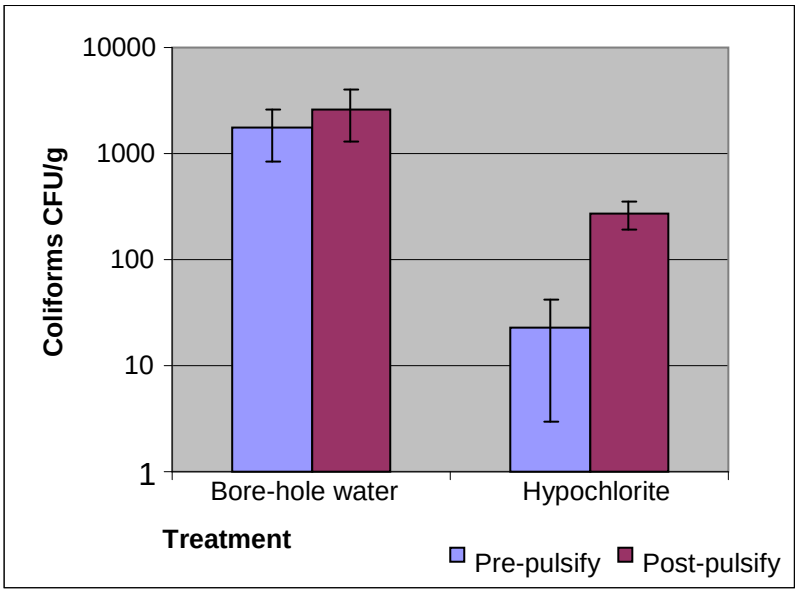


Figure 49: Numbers of viable coliforms recovered (in suspension) after bore-hole water or 90 ppm hypochlorite disinfection from the watercress phylloplane. Pre-pulsification and post-pulsification numbers show the coliforms 'loose' in suspension initially and therefore loosely attached, compared to those that were strongly attached to the phylloplane and needed shearing forces for detachment, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference in the number of coliforms recovered between pre-pulsified bore-hole water and hypochlorite treatments and post-pulsified bore-hole water and hypochlorite treatment ($p=0.038$ and $p=0.024$ respectively). Statistical analysis showed that there was no significant difference in the number of coliforms recovered between bore-hole water and hypochlorite treatments or

pre- and post pulsification, shown by a paired t-test (p=0.1124 and p=0.14 respectively). P values less than 0.05 were regarded as significant.

From Figure 49 it is apparent that pulsification produces greater recovery of coliforms from watercress after bore-hole water and also after hypochlorite disinfection. A 3.2-log increase in recovery is evident when the bore-hole water washed watercress leaves are pulsified. A 2.4-log increase in recovery is apparent when the hypochlorite treated leaves are subsequently pulsified. Approximately 1-log coliforms, that were initially shown to be strongly attached in the bore-hole water treated watercress samples (required pulsification for removal), can be seen to be released with hypochlorite disinfection and killed.

Figure 49 shows that hypochlorite disinfection results in an approximate 2-log kill of the coliforms that were already detached from the surface of the watercress and in suspension. However, approximately 1.4-log coliforms can be seen to survive hypochlorite disinfection prior to pulsifying.

5.3.4 Effectiveness of ozone treatment to remove and/or kill *E. coli* on the watercress phylloplane.

The aim was to determine the effectiveness of a 2 minute 0.2 ppm ozone treatment compared to a bore-hole water wash, in reducing numbers of *E. coli* on the watercress phylloplane. After treatment, the watercress samples were pulsified, in order to determine the number of *E. coli* that had remained strongly attached to the phylloplane post-disinfection.

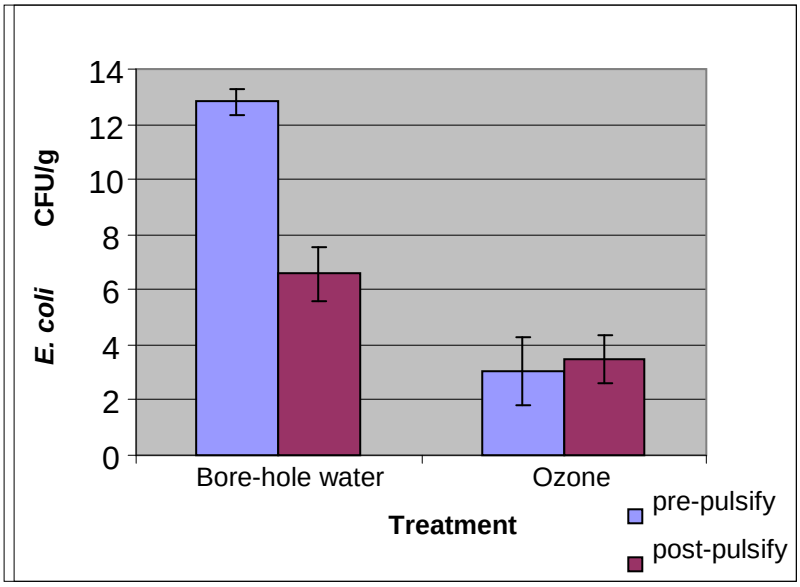


Figure 50: Numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension) after a bore-hole water treatment or 0.2 ppm ozone disinfection. Pre-pulsification and post-pulsification numbers show the coliforms 'loose' in suspension initially and therefore loosely attached, compared to those that were strongly attached to the phylloplane and needed shearing forces for detachment, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Table 5: Start and end concentration of ozone after a two minute treatment time of the watercress phylloplane. Minimal quenching effect is apparent in this batch of watercress.

Sample Number	Ozone concentration at START / ppm	Ozone concentration at END / ppm
1	**0.2**	**0.09**
2	**0.2**	**0.10**
3	**0.2**	**0.08**
4	**0.2**	**0.09**
5	**0.2**	**0.10**
6	**0.2**	**0.09**
Average	**0.2**	**0.09**

Statistical analysis showed that there was a significant difference between the total number of *E. coli* recovered after ozone treatment compared to the bore-hole water treatment ($p=0.0111$) and significant difference between bore-hole water pre- and post- pulsification

treatments (p=0.0107). There was no significant difference between ozone pre- and post-pulsification treatments (p=0.5379), shown by a paired t-test. P values less than 0.05 were regarded as significant.

Initially, the effect of the organic load on the concentration of ozone remaining in the treatment samples was investigated. The results in Table 5 show that after a treatment time of 2 minutes, there is still some ozone left in the treatment bag. Therefore, it is possible to treat a 25 g sample of watercress with 225 ml of 0.2 ppm ozonated bore-hole water since the quenching effect does not remove all of the ozone from the solution. On average 0.09 ppm of ozone remain after treatment.

As can be seen in Figure 50, pulsification has again been shown to produce greater recovery of *E. coli* from watercress after bore-hole water treatment (approximately 6 more *E. coli*) and also after ozone disinfection (approximately 4 more *E. coli*). Ozone disinfection appears to considerably reduce *E. coli* numbers compared to after bore-hole water treatment; approximately 80 % of the *E. coli* are killed with 0.2 ppm ozone treatment (Figure 50).

From Figure 50 it is apparent that approximately 18 % of loose *E. coli* (those *E. coli* that do not require pulsification for detachment) also survive ozone treatment and approximately 50 % of the *E. coli* that were initially shown to be strongly attached to the phylloplane (required pulsification for detachment) were removed by the ozone.

The watercress phylloplane was no different in appearance after the two minute ozone treatment; the leaves appeared as green and fresh as the bore-hole water treated samples.

5.3.5 The effect of watercress phylloplane age on the total numbers of *E. coli* detached after bore-hole water and ozone treatments.

The aim was to determine the effect of the age of the watercress phylloplane on the total numbers of *E. coli* detached into suspension, after either a 2 minute bore-hole water treatment or a 2 minute ozone treatment. Subsequently, the number of *E. coli* recovered were compared, after either a 24 hour or a 48 hour incubation, to determine if any stressed, sublethally damaged *E. coli* required extra time to grow and what effect the age of the watercress had on this.

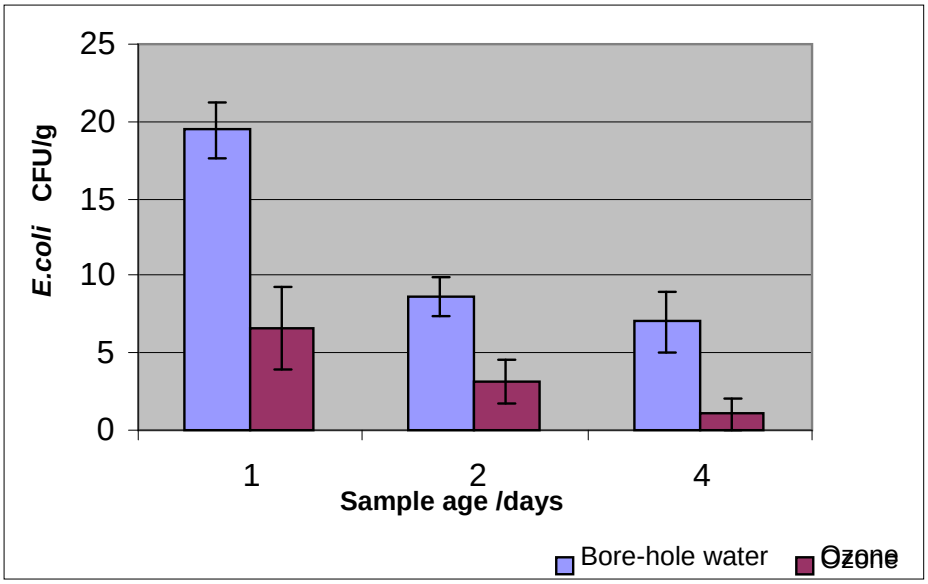


Figure 51: Numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension), after bore-hole water treatment or 0.2 ppm ozone disinfection, with varying age of the watercress samples. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

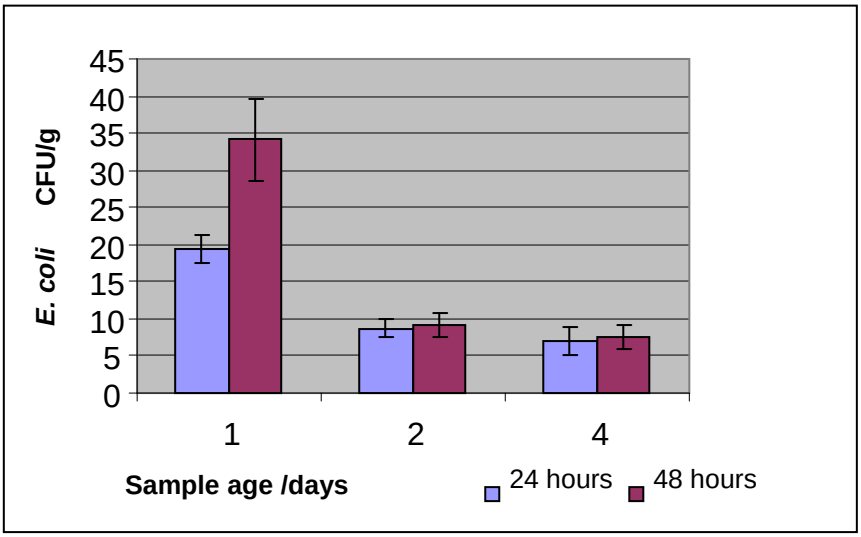


Figure 52: Numbers of viable *E. coli* recovered from the watercress phylloplane (in suspension), after a 24 hour incubation or a 48 hour incubation period of the TBX agar plates. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis of Figure 51 showed that there was a significant difference in the number of *E. coli* recovered after bore-hole water treatments on the three days (p=0.0001), and no significant difference after ozone treatments on the three different days (p=0.2721). A significant difference was apparent in the recovery of *E. coli* after bore-hole water or ozone treatments on day one (p=0.0042), but no significant differences between those on

day two (p=0.0987) and on day three (p=0.2351); as shown by a two way ANOVA. P values less than 0.05 were regarded as significant.

Statistical analysis of Figure 52 showed that in the 24 hour samples, there was a significant difference in the *E. coli* recovered (p=0.0001) across the three days; the same was evident in the 48 hour samples across the three days (p=0.0183). There was no significant difference in the number of *E. coli* recovered after 1 day between the 24 and 48 hour treatments (p=0.1837); no significant difference between these on day 2 (p=0.4226) and no significant difference on day four (p=0.1898). These were tested using a two-way ANOVA; P values less than 0.05 were regarded as significant.

The data shows that *E. coli* cells are killed with ozone, regardless of the age of the watercress; ozone treatment can be seen to result in lower numbers of *E. coli* in suspension. The results in Figure 51 show the effect of watercress storage age with total recovered numbers of *E. coli.* In the bore-hole water treated samples, it is evident that as the watercress phylloplane ages, the number of *E. coli* recovered from the phylloplane decreases. After bore-hole water treatment, compared to day 1 - approximately 60 % less *E. coli* were detected after day 2 and approximately 70 % less *E. coli* were detected after day 4. This effect was also similar for the ozone treated watercress, Figure 51 shows that after ozone treatment, approximately 25 % *E. coli* are killed in a 1 day old sample of watercress, compared to 29 % in a 2 day old sample and 22 % in a 4 day old sample.

As shown in Figure 52, after both treatment conditions (bore-hole water and ozone) it is evident that after a further 24 hour incubation on agar media (48 hours in total) at 37°C, there is an increase in the number of cells growing on the agar plates. This effect is most prominent in the samples of watercress that are one day old, in which up to 65 % of the total *E. coli* recovered were after the extra 24 hour incubation period. In the watercress samples that were 2 or 4 days old, up to 50 % of the total *E. coli* recovered were after the extra 24 hour incubation.

5.3.6 Effectiveness of ozone treatment in reducing coliform levels on the watercress phylloplane, using Pulsifier recovery.

The aim was to compare bore-hole water, ozone and hypochlorite treatments in the recovery of coliforms from the watercress phylloplane. The concentration of chlorine was

reduced to 50 ppm (previously 90 ppm); this was to determine if the concentration of chlorine could be reduced in food industries, whilst newer methods such as ozone treatment were being evaluated. The Pulsifier was used to release the cells for quantification of viable numbers remaining after treatment and VRBA was used to establish the coliform numbers recovered.

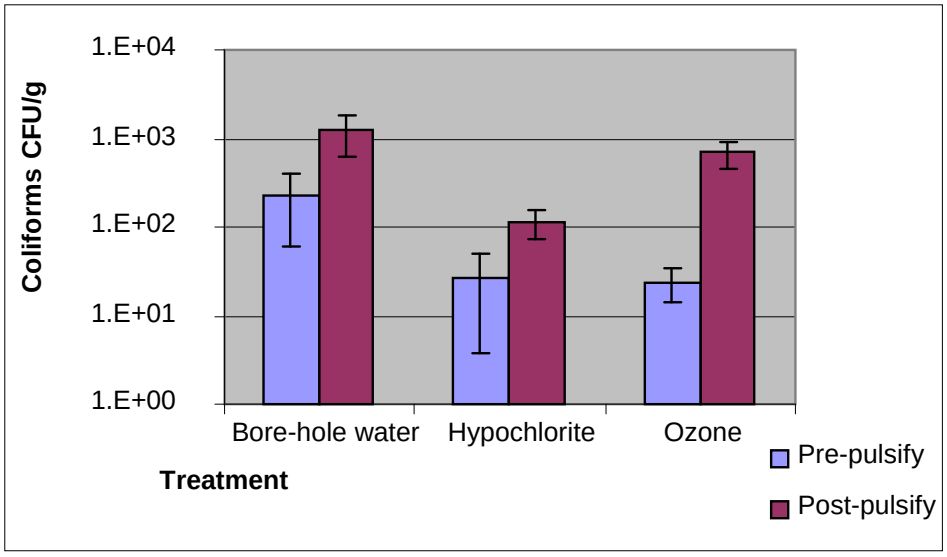


Figure 53: Numbers of coliforms recovered from the watercress phylloplane (in suspension), after either bore-hole water, 50 ppm hypochlorite or 0.2 ppm ozone disinfection. Pre-pulsification and post-pulsification numbers show the coliforms 'loose' in suspension initially and therefore initially loosely attached, compared to those that were strongly attached to the phylloplane and needed shearing forces for detachment, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

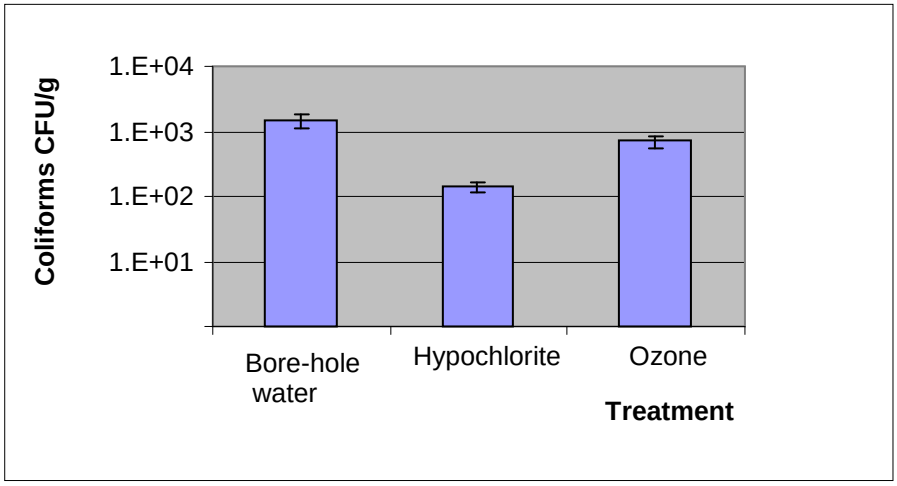


Figure 54: Numbers of total coliforms recovered from the watercress phylloplane (in suspension), after either bore-hole water, 50 ppm hypochlorite or 0.2 ppm ozone disinfection. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was no significant difference in the number of coliforms recovered in the pre-pulsification samples between the three treatments ($p=0.2988$) and in the post-pulsification samples between the three treatments ($p=0.0793$); as shown by two way ANOVA. P values less than 0.05 were regarded as significant. When analysing Figure 53, statistical analysis showed that there was a significant difference in the number of coliforms recovered comparing bore-hole water, hypochlorite and ozone treatments ($p=0.038$); as shown by two way ANOVA.

From the results in Figure 53, it is apparent that pulsification produces a greater recovery of coliforms from the watercress phylloplane in the bore-hole water treated samples and after hypochlorite or ozone disinfection, compared to pre-pulsification. In Figure 53 it can be seen that when the watercress had been treated with ozone or hypochlorite solution, less coliforms were detected on the agar, however, when the same sample of watercress was pulsified after treatment with the disinfectants, more coliforms were detected. In the bore-hole water treated watercress, 3-log more coliforms were recovered after pulsification, in the hypochlorite treated watercress 2-log more coliforms were recovered after pulsification, and in the ozone treated watercress 2.9-log more coliforms were recovered after pulsification.

From Figure 54 it is evident that hypochlorite and ozone disinfection seem to facilitate the detachment of some coliforms from the watercress phylloplane. In approximately 30 % of

individual samples analysed after ozone and hypochlorite treatment (results not shown), the numbers of cells recovered after pulsifying increased by between 1 and 2 logs, compared to pulsifying after bore-hole water treatment. When looking at the results more closely, it is evident that hypochlorite treatment facilitates the release of, and then kills, approximately 1-log coliforms compared to the bore-hole water samples. Although ozone treatment can also be seen to show this effect, the difference is a much lower 0.2-log. Figure 54 clearly shows that both disinfection types appear to considerably reduce coliform numbers compared to the bore-hole water treated samples. Ozone reduces the numbers of coliforms compared to the borehole water treatment; however, the difference is not as great as after hypochlorite treatment. Ozone disinfection does not appear to be as effective as hypochlorite disinfection, and the ozone treatment results were not very consistent with different watercress samples. Approximately 0.8-log more coliforms survived ozone treatment compared to hypochlorite treatment.

The watercress phylloplane was not altered in appearance or quality after either the two minute 50 ppm hypochlorite, or the 0.2 ppm ozone treatments; the leaves appeared as green and fresh as the bore-hole water treated phylloplanes.

5.3.7 Determination of background levels of *Salmonella* Thompson on the watercress phylloplane

In the next series of experiments the watercress phylloplane was spiked with *Salmonella* Thompson, since numerous outbreaks of foodborne illness have been caused by this microorganism worldwide. The watercress phylloplane was then subjected to different disinfection treatments. Therefore, it was necessary to determine the levels of *Salmonella* present naturally on the phylloplane before any spiking was performed, so that these numbers could be taken into account when analyzing the results.

Experiments using recovery on Rambach agar plates showed that there were no *Salmonella* species naturally present on the watercress phylloplane samples investigated. Therefore, when the leaves were inoculated with *Salmonella*, it could be assumed that there were no *Salmonella* background cells, and any counts obtained were those from the inoculations.

5.3.8 Effect of ozone or hypochlorite treatment on watercress inoculated with *Salmonella* Thompson.

The aim was to determine which treatment (ozone or hypochlorite) was optimal in reducing numbers of *Salmonella* Thompson that had been inoculated onto the surface of watercress. It was difficult to continuously check the concentration of ozone using the DPD assay; therefore, in order to counteract any quenching effect in the treatment bag, the treatment water was refreshed every 30 seconds. The high concentration of hypochlorite (90 ppm) used in the food industry was also reduced to a low level (20 ppm) to determine its effect on reducing the numbers of *Salmonella.* The second aim was to determine if the ozonator might be working correctly, since the manufacturer suggested that a strong bleaching of the DPD tablets was causing the level of ozone being recorded to be greatly lower than the actual level. If ozone was being incorporated into the solution as suggested, then numbers of *Salmonella* Thompson on the watercress phylloplane should in theory have been reduced.

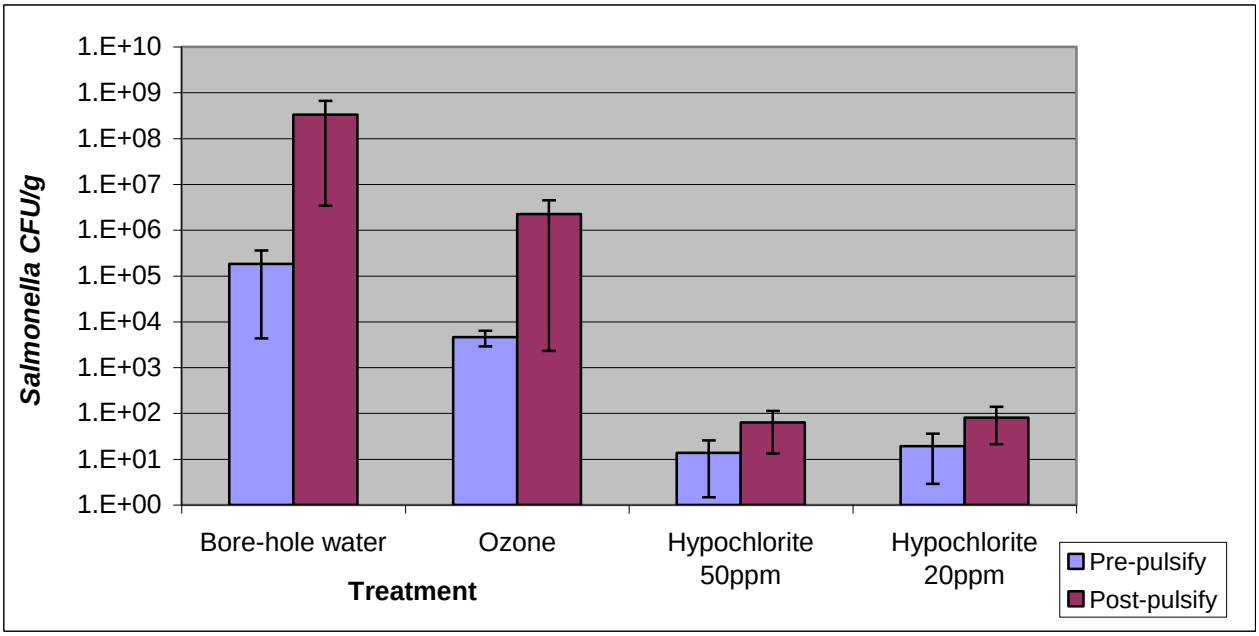


Figure 55: Numbers of viable *Salmonella* recovered from the watercress phylloplane (in suspension), after either bore-hole water, 50 ppm hypochlorite, 20 ppm hypochlorite or ozone disinfection. Pre-pulsification and post-pulsification numbers show the coliforms 'loose' in suspension initially and therefore loosely attached, compared to those that were strongly attached to the phylloplane and needed shearing forces for detachment, respectively. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Table 6: *Salmonella* recovered from the watercress phylloplane (in suspension) after ozone or bore-hole water treatment. No significant difference is evident between the results. The ozonator does not appear to be working.

Treatment	**Bore-hole water**		**Ozone**	
	Sample 1	**Sample 2**	**Sample 1**	**Sample 2**
No. of *Salmonella* CFU / g	2.37×10^2	2.79×10^2	2.50×10^2	2.85×10^2
No. of *Salmonella* CFU / g	2.23×10^2	2.15×10^2	2.37×10^2	2.93×10^2
Average no. of *Salmonella* CFU / g	$\mathbf{2.30 \times 10^2}$	$\mathbf{2.47 \times 10^2}$	$\mathbf{2.44 \times 10^2}$	$\mathbf{2.89 \times 10^2}$

Statistical analysis showed that there was no significant difference in the *Salmonella* recovered in the five different conditions pre-pulsification (p=0.4407) or post-pulsification (p=0.4469). There was significant difference when comparing the total numbers of *Salmonella* recovered after the five different treatments (p=0.0183). As shown using a two way ANOVA; p values less than 0.05 were regarded as significant.

A final inoculum concentration of approximately 10-log *Salmonella* was present in the treatment bags initially. The bore-hole water treatment results in Figure 55 show that approximately 5-log *Salmonella* were detectable in the suspension prior to any treatment; this suggests that 9.5-log *Salmonella* attached to the watercress phylloplane during the 12 hour incubation period. The results show that after only a contact time of 12 hours up to 8.65-log of the *Salmonella* were tightly attached. It is also apparent that without any chemical treatment, and after a pulsification treatment, approximately 1.5-log *Salmonella* remain attached to the phylloplane. A 0.2 ppm ozone treatment can be seen to result in an approximate 2-log kill in *Salmonella* levels. A 50 ppm or a 20 ppm hypochlorite treatment results in a 7-log kill in *Salmonella.*

The results in Figure 55 show that compared to bore-hole water treatment, hypochlorite treatment is effective in reducing the levels of inoculated *Salmonella* on watercress leaves. Reducing the concentration of the hypochlorite solution from 50 ppm to 20 ppm does not have a significant effect on the levels of *Salmonella* surviving treatment.

The quenching effect is not apparent for the hypochlorite solution (50 ppm or 20 ppm), since, even in the first treatment, no *Salmonella* were detectable. This also shows that within the first 30 seconds of treatment the hypochlorite solutions are capable of

inactivating all of the *Salmonella*. The DPD no. 1 tablets were also used to measure the concentration of the hypochlorite solution; the start and end concentrations of chlorine only dropped by 5 ppm over the 2 minute treatment period. As shown previously, a pulsifying step is able to recover extra *Salmonella,* such as those that are attached more strongly or possibly trapped in crevices or grooves and were therefore not attacked by the hypochlorite or ozone disinfection (Figure 55). An approximate 2-log lower recovery of *Salmonella* is apparent when pulsification is performed after ozone treatment compared to bore-hole water treatment. Hypochlorite disinfection appears to lead to an increase in recovery and subsequent kill of *Salmonella* too. Approximately 8.45-log *Salmonella* that were shown to be strongly attached in the borehole water treatments were shown to be removed and killed with hypochlorite treatment, prior to pulsification. This effect was observed in both the 50 ppm and 20 ppm treatments, to a similar extent.

The results in Table 6 show that ozone treatment did not appear to reduce the levels of *Salmonella* at all in these results; in fact levels were shown to increase (less than 5 %). Therefore, the ozonator is not functioning, not even at the level it was at the start of this phase of work. The DPD tablets that are showing that the concentration of ozone in the solution is very low to none are correct.

5.3.9 Effect of 0.1 ppm ozonated bore-hole water on numbers of coliforms naturally present on the watercress phylloplane, using an industrial scale model at Vitacress Salads.

Vitacress Salads obtained an industrial scale model of an ozonator and they cleared a production line, allowing for a 0.1 ppm ozone wash step to be incorporated into their wash system (instead of the conventional hypochlorite wash). The effect of the ozone step was determined by following the protocol prepared in Chapter Two; VRBA agar was used to monitor the effect of ozonation on coliform numbers.

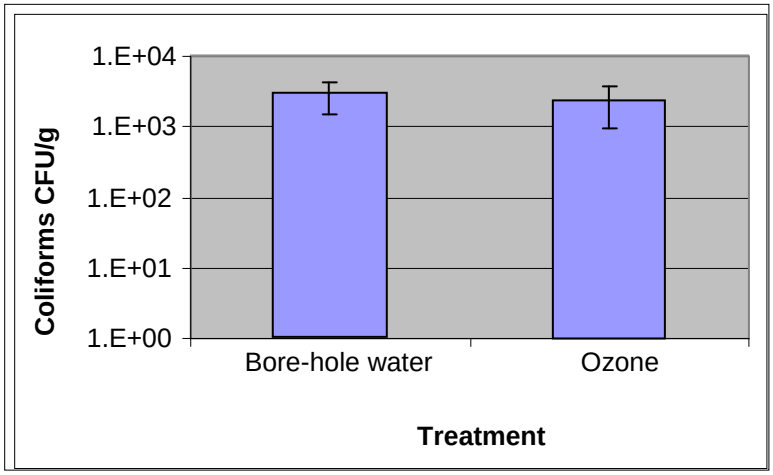


Figure 56: Numbers of coliforms recovered from the watercress phylloplane (in suspension), after either bore-hole water treatment or 0.2 ppm ozone disinfection. A corona discharge ozonator was used at the industrial scale. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

From the results shown in Figure 56, it is evident that the numbers of coliforms recovered after ozone treatment and after bore-hole water treatment is very similar and there is no significant difference between both treatments, as shown by a one way 2-sided paired T-test ($p=0.7974$). P values more than 0.05 were regarded as significant. This suggests that alternative methods of maintaining ozone concentrations at the large industrial scale needed to be looked into.

5.3.10 Determination of coliform subpopulation attachment strengths using the Pulsifier.

An investigation was carried out to determine coliform subpopulation attachment strengths on watercress, by using two pulsification steps. The protocol described in Chapter Two was followed. The first pulsification treatment shows the number of coliforms extracted from the watercress surface by a single 30 second pulsification. The second pulsification treatment allows for the determination of the number of coliforms that were "missed" by the first pulsification step, thereby indicating those coliforms that are very strongly attached to the matrix surface and require additional pulsification treatments.

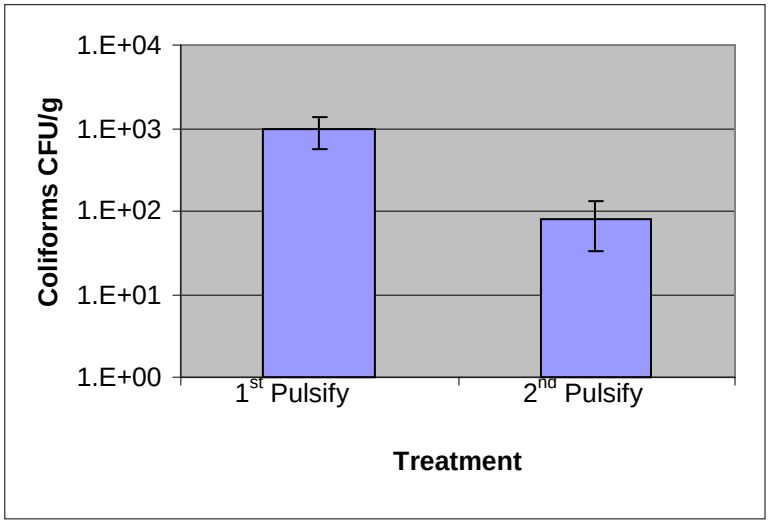


Figure 57: Numbers of coliforms recovered from the watercress phylloplane (in suspension), after either a single pulsification or after a second pulsification on the sample watercress sample. Error bars are representative of the ± standard error of the mean of six experiments for each treatment.

Statistical analysis showed that there was no significant difference in the coliforms recovered in the second pulsification compared to the first pulsification ($p=0.0863$), as shown using a paired t-test. This shows that although more coliforms are recovered after the second pulsification, this is insignificant compared to the fist pulsification. P values less than 0.05 were regarded as significant.

From Figure 57, although a large difference can be seen in terms of actual numbers (due to biological variation), a clear trend is apparent - that without the use of any chemical disinfectant, coliforms can be removed from the surface of the watercress samples. Also, after the second 30 second pulsification step the numbers of coliforms recovered have decreased by approximately 1-log. This suggests that most bacteria that are strongly attached are removed after the first pulsification step, however; approximately 2-log coliforms are very strongly attached and require a second treatment. The results show that when the bore-hole water is decanted off after a pulsification stage, most of the coliforms are removed from the phylloplane with the water.

5.3.11 Influence of a combined pulsification and hypochlorite treatment on coliform removal from watercress.

In the previous set of experiments it was apparent that a 30 second pulsification stage might be suitable in terms of decontamination of watercress of coliforms. The aim here was to investigate the numbers of coliforms recovered from watercress after a 30 second

pulsification treatment alone, compared to a 30 second pulsification followed by a 50 ppm hypochlorite treatment.

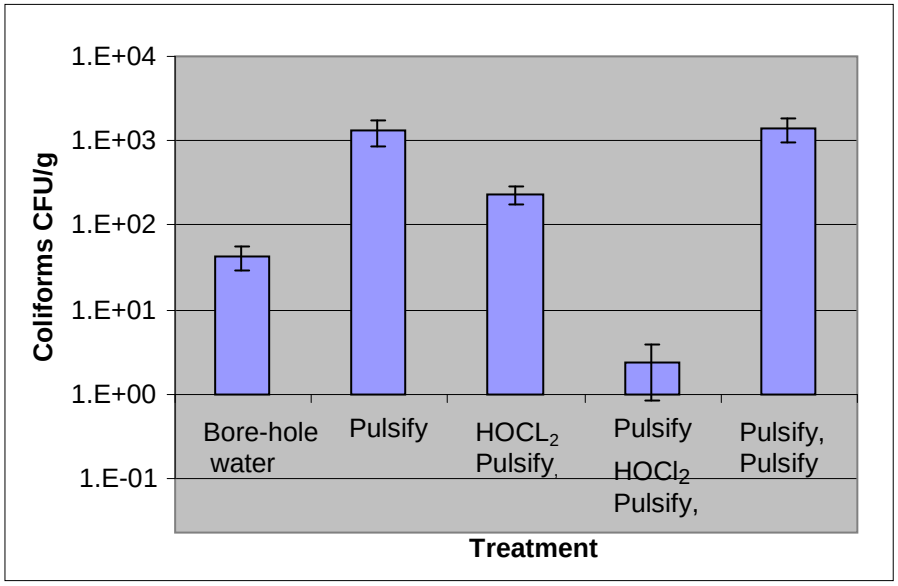


Figure 58: Numbers of coliforms recovered from the phylloplane (in suspension), after either: a 2 minute bore-hole water treatment (showing loosely attached coliforms), a 30 second pulsification (showing strongly attached cells), a 2 minute 50 ppm hypochlorite followed by a 30 second pulsification treatment (showing those coliforms that were not initially attacked by the hypochlorite), a 30 second pulsification followed by a 2 minute hypochlorite treatment and then a 30 second pulsification (showing the number of coliforms that were not attacked by an initial pulsification followed by hypochlorite treatment), and a 30 second pulsification followed by another 30 second pulsification (showing the number of coliforms recovered after two pulsification stages). Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference between each of the treatments performed, shown by a two way ANOVA ($p=0.0001$). There was no significant difference between a single pulsification treatment and two pulsification treatment shown by a two way ANOVA ($p=0.4469$). P values less than 0.05 were regarded as significant. From the results, as shown in Figure 58 it can be seen that, as expected from previous work, pulsification detaches bacteria from the surface of watercress. When the watercress is then subsequently treated with 50 ppm hypochlorite solution, the results show that all of the coliforms that were released into suspension by the Pulsifier are killed and therefore, no coliforms grow on the VRB agar (results not shown here).

When the hypochlorite treatment water was then decanted off, replaced with PBS and the watercress was then re-pulsified, on average less than 0.5-log coliforms can be seen to be

recovered. The results show that a 30 second pulsification stage prior to disinfection treatment results in less than 0.5-log coliforms (and even 0 in individual results) being recovered. From Figure 58, it is evident that approximately 2-log more coliforms are recovered from the watercress and killed when a pulsification step is performed prior to hypochlorite disinfection.

Figure 58 also shows that a slightly higher recovery from the phylloplane is evident after a second pulsification step; however, this difference is not statistically significant. The watercress phylloplane and leaf integrity was not altered to a great extent after pulsification.

5.4 Chapter Five Discussion.

The results suggest that microorganisms are protected on the surface of the leaf and that this may be the main reason why even after disinfection procedures, pathogens are surviving and proceeding to infect consumers. Pulsification produces greater recovery of *E. coli* and coliforms from watercress compared to stomaching in bore-hole water treated samples, as shown in Chapter Four; a similar pattern is evident when the phylloplane is treated with hypochlorite. Hypochlorite solution (and ozone) appears to preferentially attack those cells that are free in solution. Stomaching has been shown not to be ideal for use when using filter methods such as that used for concentrating *E. coli,* and in general, it has been shown to produce lower recovery of cells and greater sample damage.

Ozone was shown to be more effective than hypochlorite solution in initial experiments, 0.2 ppm ozone producing up to an 80 % reduction of *E. coli* in some samples and hypochlorite up to 77 %; however, some experiments did not show this due to difficulties in maintaining constant concentrations of ozone. Ozone treatment also appears to facilitate detachment and subsequent killing of cells that were originally tightly attached to the watercress phylloplane and could only be removed by pulsification.

The results suggest that ozone could have been ideal for use, since samples were also shown not to be damaged, however, it would be difficult maintaining ozone concentrations in an industrial environment. Ozone has been cited to be relatively unstable in aqueous solution, decomposing to oxygen in a first order reaction (Tomiyasu *et al.*, 1985). In the initial experiments, after a two minute treatment time, a starting solution of 0.2 ppm ozone

was shown to reduce to on average 0.09 ppm. This is ideal since as stated previously, within food industries, concentrations of ozone should not exceed 0.1 ppm. Therefore, the initial levels of 0.2 ppm would be quickly absorbed by the organic load and safe levels would be maintained, maximal levels recorded being 0.15 ppm. However, subsequent experiments showed that this effect differs with the performance of the ozonator and with the biological variations between each 25 g watercress sample tested. Sometimes initial recorded levels of 0.2 ppm ozone were down to less than 0.1 ppm or 0 ppm in a matter of seconds. The ozone was variably and sometimes rapidly quenched by the organic load once the leaves were immersed in the solution. Studies have shown that the quality of water also effects the stability of ozone in a solution; water that is used by food industries has generally been shown to contain high quantities of readily oxidizable organic and inorganic matter; for this reason, the same quality of water (from the same bore hole) was used throughout this study, including at the industrial scale (Kim *et al.*, 1998). Schulz and Bellamy in 2000 also showed that the design of the ozone-water contactors greatly affects the rate of solubilisation of ozone. Several studies have also shown that when ozone is bubbled through water, greater solubility occurs if bubbles are small in size since this results in a greater surface area of contact (Katzenelson *et al.*, 1974). When engineers incorporated this idea into their ozonator, the watercress matrix appeared to be damaged; the force of the bubbles colliding with the phylloplane resulted in holes in its surface. The solubility of ozone has also been shown to increase as the temperature of the water decreases; however, the temperature of the bore-hole water used in these studies was already at a low, approximately 4°C (Bablon *et al.*, 1991). The engineers were unable to produce an ozonator that could be reliably incorporated at the industrial scale. The prompt decomposition of ozone into harmless gases, which would be its main advantage, was shown to be a major drawback in this study, as well as difficulties of its dissolution in water. Due to the variations in the ozone concentration, the coliform killing efficiency was shown to only be on average 7 %, whereas 50 ppm hypochlorite was shown to reduce coliform levels by on average 33 %.

Ozone has been reported to attack numerous cellular constituents in the literature; including, proteins, lipids, cell membrane respiratory enzymes, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm. Likewise, numerous mechanisms of action have been reported for ozone, including the direct effects of molecular ozone, or those of the reactive oxygen species by-products, i.e. OH^- and O_2^-. Ozone has been shown

to elicit its effects on bacterial cell membranes by oxidation. Ozone oxidizes fatty acid chains, glycoproteins, glycolipids and membrane bound enzymes; this results in increased permeability and therefore leakage of the cell contents, followed by cell lysis (Murray *et al.*, 1965). *Salmonella Enteritidis* was shown to have disrupted cell membranes after treatment with ozone (Dave, 1999). Ozone leads to the oxidation of the sulfhydryl groups on enzymes and the double bonds of unsaturated lipids, resulting in the disruption of normal cellular activity (Chang, 1971). Chlorine has been reported in the literature, to selectively destroy certain enzymes, whereas, ozone acts generally, oxidising all enzymes (Sykes, 1965). *E. coli* has been reported to be killed by ozone due to its oxidising action on dehydrogenating enzymes in the respiratory system (Ingram and Haines, 1949). Studies have shown that ozone asserts effects on the nucleic material in bacterial cells too. Ozone had been shown to modify nucleic acids *in vitro*, with thymine being the main target (Ishizaki *et al.*, 1981).

Overall, it can be concluded from the results that coliforms are killed by hypochlorite disinfection by approximately 1-log; and sometimes by ozone disinfection (possibly not always, due to the quenching effect or the ozonator not producing solutions of constant ozone concentration). It can also be said that coliforms are protected on the leaf surface and are removed by pulsification after all treatments. Biological variations were apparent in all samples; therefore, it was not ideal to use different 25 g samples to compare the same factor. The same 25 g sample should be used with an acceptable number of replicate analyses, as was done in these experiments. This could possibly also explain why when GFP-*Salmonella* was inoculated onto 25 g samples of the watercress phylloplane, there were large variations in the distributions, when leaf surfaces were viewed *in situ*, and why values do not necessarily reflect what is happening, and will not reflect this unless the replicate is very high. The results showed that recovery was variable within the same treatments. Variations of the physical structure of individual leaves would affect this, as would original conditions that each leaf was exposed to in the field. When leaves are not artificially inoculated with a microorganism in the laboratory (i.e. the *E. coli* results in this study), natural variations can occur, for example, it is possible that some *E. coli* may have been attached for longer than others, and some *E. coli* may have physiologically adapted, or have been in a more suitable niche for protection.

Comparing the data from Section 5.3.3 (coliform counts) with that in Section 5.3.1 (*E. coli* counts) it is evident that coliforms tend to be recovered in higher numbers than *E. coli*. The coliforms also tend to be more tightly attached than the *E. coli*, a lower percentage being recovered initially than for *E. coli*, whereas a higher percentage of coliforms are extracted from the surface of the watercress after pulsification treatment. This may be because the *E. coli* is most likely from faecal contamination and therefore, these cells are more recent and therefore have not yet established fully their defence mechanisms to any stress, also they may not have yet reached any niches for protection. The coliforms appear to be more tightly attached, the coliforms as discussed previously can also be soil bacteria and are not entirely indicators of faecal contamination, and therefore, coliforms would be more environmentally adapted and robust; this would also explain why they may occur in higher numbers compared to *E. coli* since they have had more time to grow in numbers.

The results show that as watercress ages, the number of *E. coli* recovered decreases; this may be because the cells are dying, or they are attaching more tightly to the phylloplane (since even after pulsification the numbers recovered are very low). If the cells are attaching more tightly to the phylloplane this could be a problem in terms of disinfection, since as shown in this work, disinfection treatment would be unable to attack the cells whilst they were protected on the surface. When the *E. coli* were allowed an extra 24 hour incubation time, more cells were shown to grow; this indicates the presence of sublethally damaged cells. The results showed that more stressed, sublethally damaged *E. coli* were apparent in the one day old watercress compared to the two or four day old watercress. This may reinforce the suggestion from the results that in the older watercress samples, the reason why less *E. coli* were detected was because they had died, or had attached more strongly (not because they were stressed or sublethally damaged).

Further work in the Environmental Healthcare Unit is looking into enumerating stressed sublethally damaged cells that possibly may not be cultured. Preliminary results indicate that this may be a reason why foodborne pathogen outbreaks are still occurring, since some pathogens are undetected when they are sublethally damaged and are growing at a slower rate. Current adopted methods used for cell recovery are out-dated: improvements would involve allowing longer incubation times and also use of the Pulsifier in replace of the Stomacher.

Initial studies showed that unlike for *E. coli*, the watercress phylloplane is not contaminated with low levels of *Salmonella* naturally. There may be numerous reasons for this, the main one being that irrigation water and soil are less contaminated with this pathogen initially compared to with *E. coli*. It can also be that *Salmonella* is less able to adapt to survive in the phyllosphere than *E. coli*. Studies have shown that unlike most plant associated bacteria, including *E. coli*, *Salmonella enterica* is unable to assimilate sucrose, which is one of the main sugars present in leaf exudates (Lin, 1996). This may be a reason why there have been greater numbers of *Salmonella* outbreaks on fruit compared to salad leaves (Harris *et al*., 2003). Despite this, outbreaks of *Salmonella* on fresh produce still occur; for example, watercress that had been imported into the UK from Florida was recalled in January 2007 due to contamination with *Salmonella* (FSAI, 2007). Studies have shown that some *Salmonella* species attach to alfalfa sprouts ten times more than natural plant associated bacteria (Barak *et al.*, 2002). This current study showed that *Salmonella* was able to attach to the watercress phylloplane at levels of 8.5-log *Salmonella* per 25 g of watercress, when the inoculum level was 10-log; this level of attachment could be a problem to consumers if irrigation water or soil was greatly contaminated initially. The typical infective dose of most pathogens is low, for example it is 5000 cfu for *S. enterica*, this therefore suggests that outbreaks can occur when the soil in even minimally contaminated. The results showed that 8.65-log *Salmonella* Thompson were strongly attached after a 12 hour incubation, suggesting that within this time frame the *Salmonella* had moved or adapted in such a way that they were able to survive any subsequent disinfection treatment, or adverse conditions, and they were only removed from the surface of the watercress by pulsification. The results also showed that without any chemical treatment, but after pulsification, 1.5-log *Salmonella* remained tightly attached to the phylloplane. This 1.5-log may either be very strongly attached, or the *Salmonella* may have been internalized as shown in Chapter Three.

Inoculation studies performed using *Salmonella* Thompson show that the hypochlorite concentration used by the food industries could potentially be reduced from 90 ppm to 20 ppm, this still having similar 2-3 log reductions in pathogen loads. This would be very useful since it would reduce the excess production of trihalomethanes which have been shown to have long- and short-term health effects. Reducing the concentration would also be more cost effective, since less of the chemical would be used, and lower chlorine concentrations would reduce the corrosion of piping and containers used by food industries.

The limitation of the present study is that it is not possible to elucidate if the 20 ppm hypochlorite is sub-lethal to the *Salmonella.* If so, it may mean that some of the *Salmonella* may not have been detected on the Rambach agar since they were stressed and sublethally damaged, and not necessarily because they had been killed (Dodd *et al.,* 2007). This sublethal damage may also be the case for the 90 ppm hypochlorite that is used by food processing industries at present. Currently, Rambach agar is the ISO approved method for *Salmonella* detection, used by food industries worldwide. Therefore, if sublethal damage is a problem, then current adopted methods are out-dated and need to be improved. This was beyond the scope of this project due to time constraints; however, if time were available then the further development of the EDIC/EF microscopy techniques in Chapter 3 would result in direct *in situ* counts of all bacteria. Elongation of bacteria that are growing (and are thereby alive), combined with EDIC/EF microscopy, has recently been shown to be useful for detecting sublethally damaged bacteria (J. Warner, Personal Communication). If time were available then qualitative *in situ* comparisons of the phylloplane pre- and post-treatment would have been performed using EDIC/EF microscopy, as shown in Chapters Three and Four.

Further experiments, including the use of proteomics, must be performed in order to analyse the *Salmonella* after the 20 ppm hypochlorite treatment. Although 2-3 log kills were evident, it is possible that the *Salmonella* that were not killed may become more resistant to further hypochlorite disinfectant treatments due to any sub-lethal effects. Bacteria are capable of adapting to adverse conditions by inducing stress-proteins, these minimise the harmful effects of the environment. The risk of infection from bacteria is thought to depend both the number of potentially harmful bacteria on food and the on the ability of these microorganisms to recover from sub-lethal stresses encountered during food processing (Mangalappalli-Illathu *et al.,* 2008). *Salmonella* has been reported to develop chlorine resistance in numerous ways, including, catalase production, decreased activity of membrane bound dehydrogenases, and decreases DNA damage (Mokgatla *et al.,* 2002). These alterations would result in reduced hydroxyl radicals and singlet oxygen, both of which react with the active form of hypochlorite sanitizers, hydrochlorous acid, causing cellular inactivation and improve DNA repair (Mangalappalli-Illathu *et al,* 2008). Studies have however shown that adaptation of bacteria to sub-lethal concentrations of anti-microbials occurs gradually, i.e. depending on the duration of exposure (Langsrud *et al.*, 2004). Therefore, before lower concentrations of chlorine are used, such as 20 ppm, it is

essential to investigate any sub-lethal effects on any potential pathogens. Although, the sub-lethal concentration of chlorine would have to be investigated for each pathogen, since in theory the 90 ppm hypochlorite concentration used by food industries may be sub-lethal. A further problem with this phenomenon is that molecular mechanisms that are providing bacterial resistance to anti-microbials may further provide cross-protection against antibiotics (Braoudaki *et al.*, 2004; Sampathkumar *et al.*, 2004).

Due to the difficulties faced in maintaining ozone concentrations, comparisons between the efficacy of ozone and hypochlorite on the numbers of *Salmonella* recovered from the watercress phylloplane were not possible. The literature presents variable efficacies of ozone and chlorine on food matrices. A recent study by Chaidez *et al.* in 2007 has shown no significant difference in the reduction of *Salmonella* Thompson inoculated on tomato surfaces when comparing the efficacy of spraying ozone and hypochlorite solutions. In comparisons of the efficacies of these chemical disinfectants on immersion of tomatoes, hypochlorite was shown to be the most effective treatment (Chaidez *et al.*, 2007). The results in this current study show that hypochlorite and ozone solutions (but not bore-hole water) were able to facilitate the detachment of *E. coli* from the watercress phylloplane. This may have been due to an 'accumulative' release pattern, i.e. the less tightly attached cells are killed and thereby loosened from the phylloplane by the hypochlorite or ozone - due to the release of these cells, the dynamics of the phylloplane change, causing more cells to be detached off in a subsequent pulsification. If the biofilm or aggregates on the phylloplane were being attacked by the chlorine or ozone, then the cells killed would most likely have been the top layer or areas that are more available; the *E. coli* or *Salmonella* that were below or were surrounding the killed cells may not have had the opportunity to be attacked by the disinfectant initially, however, a subsequent pulsifying step would make them easier to be removed. This could explain why there was an increase in *E. coli* and *Salmonella* recovered after pulsifying, in the hypochlorite or ozone disinfected watercress samples, but not in the bore-hole water treated samples. The concentration of hypochlorite was shown to reduce during the 2 minute treatment duration, from 90 to 85 ppm. This was most likely due to the organic loading in the solution. The watercress leaves increase the organic load in the solution, and these quench the chlorine. This effect was also shown by Taormina and Beuchat in 1999; in their series of experiments, the free chlorine reduced by 90 % from 200 ppm to 20 ppm after treatment for 15 minutes of alfalfa seeds.

The results show that chemical disinfection could be completely replaced with pulsification treatment alone. Although a synergistic effect could be seen between chemical treatment and the detachment of cells from the watercress phylloplane, the disadvantages of chemical treatment outweigh the positive attributes. This study shows that the Pulsifier could be used effectively to remove potential pathogens from phylloplane food surfaces with 2-3 log reductions in pathogen load; this is without sample damage and also without the production of harmful by-products. This reinforces the fact that pathogens are protected on the leaf surface (possibly due to induction of their own stress-response factors); they can be loosened from the surface by use of the shearing forces provided by the Pulsifier. Pulsification may disrupt biofilms and also detach bacteria that are trapped or wedged in grooves and crevices, large volumes of water are then capable of washing the bacteria away to very low or zero concentrations.

When analysing the results produced after two pulsification steps to recover coliforms, it is evident that the first pulsification step is effective in recovery of the attached coliforms. Assuming that both pulsification steps removed the majority of coliforms from the phylloplane, the total number present was approximately 3.2-log. Therefore, the first pulsification detaches 3-log (or 94 %) and the second detaches 2-log (or 6 %). It remains to be seen whether the 6 % were strongly attached, had physiologically adapted on the leaf or whether they were different species of coliforms. Future experiments should speciate the coliforms recovered at each step to answer this question.

The literature shows that aqueous ozone is more effective on smooth samples, such as fruits than wrinkled samples with groves and crevices (Achen *et al.*, 2001). In general, ozone has been shown to reduce levels of *E. coli* or *Salmonella* Thompson by approximately 2-log amounts when free in suspension (Finch *et al.*, 1988; Farooq *et al.*, 1983). The results presented here show that the watercress phylloplane is a wrinkled surface and is a difficult matrix to decontaminate without the use of shearing forces to extract potential pathogens and epiphytic bacteria into suspension. An extension to this study would be to investigate the mechanisms by which the Pulsifier is eliciting its effect on the phylloplane. Bacteria have been shown to attach to biological surfaces in a variety of ways including the use of electrostatic and hydrophobic or hydrophilic interactions and also simply physical entrapment (Frank, 2001). Mutants in cell surface moieties involved in attachment would attempt to show the mechanism of action of the Pulsifier; or it may be that physical

removal of entrapped bacteria is the main mechanism of initial attachment to the phylloplane, as suggested by Solomon and Matthews (2006).

In conclusion, these data show that disinfection treatment of salads such as watercress is not always effective because potential bacteria are protected on the phylloplane. These bacteria are either trapped in crevices or grooves in the surface of the leaf, or they have formed biofilms. The bacteria or pathogens may have formed biofilms of their own, depending on the length of time they had been on the surface for; or they may have integrated into biofilms formed by naturally occurring bacteria (Lapidot *et al.*, 2006).

The novel use of the Pulsifier as part of a non-chemical means for sanitizing food surfaces appears promising. This is important in terms of consumer concern, and also for fresh produce that is to be labelled as organic, since such produce cannot be chemically treated (Heaton and Jones, 2007). The results show that a 30 second pulsification stage prior to disinfectant treatment improves the attacking ability of hypochlorite greatly since the cells are more available (either loosened or free in suspension) for attack by the sanitizer. Less than 0.5-log coliforms (and even 0 in individual results) are recovered if this procedure is followed, suggesting that almost all (or all) coliforms are killed or removed when using this protocol. From these results it is apparent that potential pathogens would have a much greater likelihood of being removed from fresh food surfaces, such as those of watercress, if a vibrational stage was to be incorporated into the whole treatment process. The potentially pathogenic cells are then more available for attack by the disinfectant. When a pulsification step is included in the treatment and is performed prior to hypochlorite disinfection, a further 2-log reduction in coliforms is seen. The cells may not have the opportunity or time to attach stronger to the matrix if pulsification is performed early on during the manufacturing process – one or two days post-harvest as shown by the results, since after this period the cells appear to be attaching more strongly. Although a slightly higher recovery from the phylloplane was evident after a second pulsification step, suggesting that some coliforms are very strongly attached to the watercress phylloplane, this difference was not significant. This implies that a single pulsification stage would be efficient for incorporation by industries.

As discussed previously, the watercress phylloplane and leaf quality is not altered to a great extent after pulsification, although improvements may be made by investigating the

mechanics of the Pulsifier. A further project in collaboration with Engineers is at present investigating ways in which the Pulsifier efficiency could be improved further, to maintain the leaf quality to a greater extent and to possibly improve the bacterial extraction efficiency. The project will also build this bacterial shearing machine to a size that can be fitted into industrial wash processes.

CHAPTER SIX

THE INFLUENCE OF THE EXOGENOUS MOLECULAR SIGNAL NITRIC OXIDE ON DETACHMENT OF COLIFORMS FROM WATERCRESS LEAVES, INCREASING SUSCEPTIBILITY TO DISINFECTION

6.1 Introduction

Although the *in situ* presentation of biofilms in the phyllosphere is very rare in the literature, in Chapters Three and Four some evidence was shown using the EDIC/EF microscope. The leaf surface is a hostile environment and the formation of biofilms would provide the answer to how the phyllosphere supports such a diversity of microorganisms and how these can then lead to infection by human pathogens.

Bacteria in biofilms often use quorum-sensing (or cell signalling) to develop three-dimensional structures known as microcolonies. The bacteria that constitute these microcolonies become highly differentiated from free-living planktonic cells. The cells have been shown to be highly tolerant to antimicrobial agents, being as much as 1000-times more resistant compared to planktonic cells (Lewis, 2001). The results produced in this study (Chapter Five) also show this in terms of the phyllosphere, in that cells that had formed aggregates were more resistant to removal by washing than those cells that were singular. Cells that were singular were washed into suspension more readily by any disinfectant and therefore, were prone to attack by the disinfectant, compared to cells that were as part of aggregates. The results showed that the aggregated bacteria had to be first broken up by the Pulsifier for the cells to be released into suspension.

Webb *et al.* (2006) have demonstrated that *P. aeruginosa* biofilm development and dispersal can be controlled by use of the molecular signal, nitric oxide (NO) at nanomolar concentrations (Mcdougald *et al.,* 2011). The final objective of this study was to determine the effect of nitric oxide on the dispersal of coliforms (potential biofilms or aggregates of cells), from the surface of watercress leaves.

6.2 Methodology

See Chapter Two.

6.3 Results

6.3.1 The effect of a 12 hour SNP treatment on coliforms recovered from watercress leaves, with pulsification and the effect of SNP on 50 ppm hypochlorite disinfection.

The aim of this Section was to determine the effect of a 12 hour treatment of the watercress phylloplane, with either 0 nM, 20 nM, 500 nM or 5 mM NO (or SNP), on coliform numbers recovered post-treatment combined with a 30 second pulsification. The phylloplane was then subsequently treated to 50 ppm hypochlorite to determine any effects of NO on the killing efficacy of this disinfectant.

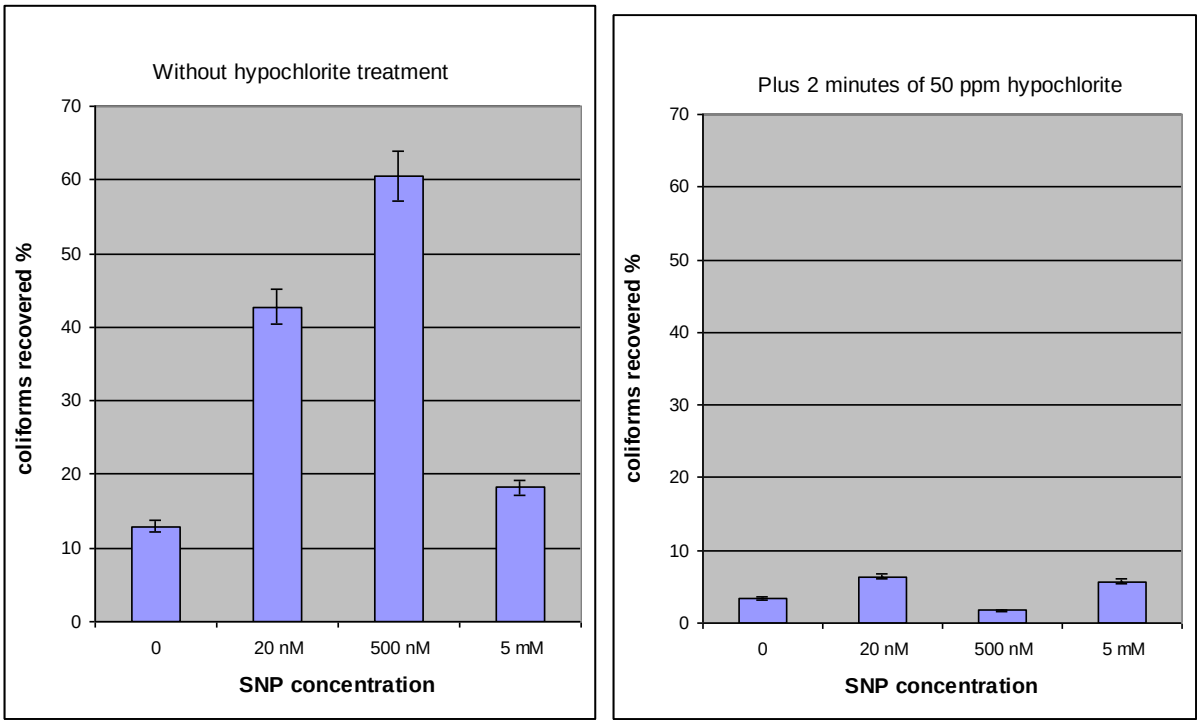


Figure 59: Graph to show the percentage coliforms recovered after pulsification for each treatment compared to the total that was recovered for each treatment. The results suggest that NO treatment combined with pulsification treatment results in a synergistic effect on coliform recovery. A distinct pattern is evident at the different NO concentrations. 500 nM NO leads to an enhanced recovery of coliforms; 500 nM NO also can be seen to lead a greater kill when the watercress is also treated with 50 ppm hypochlorite. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference overall in the coliforms recovered across the different NO concentrations, as shown using ANOVA ($p<0.05$). Using a t-test between each concentration showed that there was a significant difference between 0 nM and 20 nM, 0 nM and 500 nM, 20 nM and 500 nM , 500nM and 5 mM, 20 nM and 5 mM ($p<0.05$); both with and without hypochlorite. P values less than 0.05 were regarded as significant.

The data presented in Figure 59 shows that the 0 nM NO treated samples have the lowest numbers of coliforms recovered after a 12 hour treatment; 13% of coliforms were recovered after a pulsification step alone compared to the total that was recovered using the combined pulsification and SNP treatment.

Furthermore, the results show that NO treatment leads to a greater recovery of coliforms from the watercress surface. After 12-hour incubation with NO, up to a 50 % increase in coliform recovery is evident compared to the treatment without NO. All concentrations of NO appear to lead to an increase in recovery compared to the 0 nM NO; however, the greatest increase in recovery is evident when NO at a concentration of 500 nM is used (50 % increase in recovery), followed by 20 nM NO (30 % increase in recovery). Five nM NO can be seen to lead to increased recovery compared to 0 nM NO, although this effect is less apparent (<10 % increase compared to the 0 nM NO).

It is also evident that 500 nM NO treatment followed by a 50 ppm hypochlorite treatment leads to a greater kill than pre-treatment with 0 nM NO, and also a greater kill than hypochlorite treatment after 5 mM or 20 nM NO. After a 500 nM NO treatment, followed by hypochlorite treatment, on average only approximately 1 % of the total cells originally recovered post-pulsification were now evident. Five mM and 20 nM NO appear to reduce the susceptibility of coliforms on the phylloplane to chlorine treatment.

6.3.2 The effect of a 30 minute SNP treatment on recovery of coliforms from watercress leaves. Comparing different concentrations of SNP.

In this set of experiments the incubation period with SNP was reduced to 30 minutes and only 500 nM and 20 nM SNP were compared, since, from the data presented in Section 6.3.1 it was evident that these concentrations of SNP produced the greatest recovery of coliforms. An additional step was also included into the protocol to determine the effect of NO treatment without pulsification.

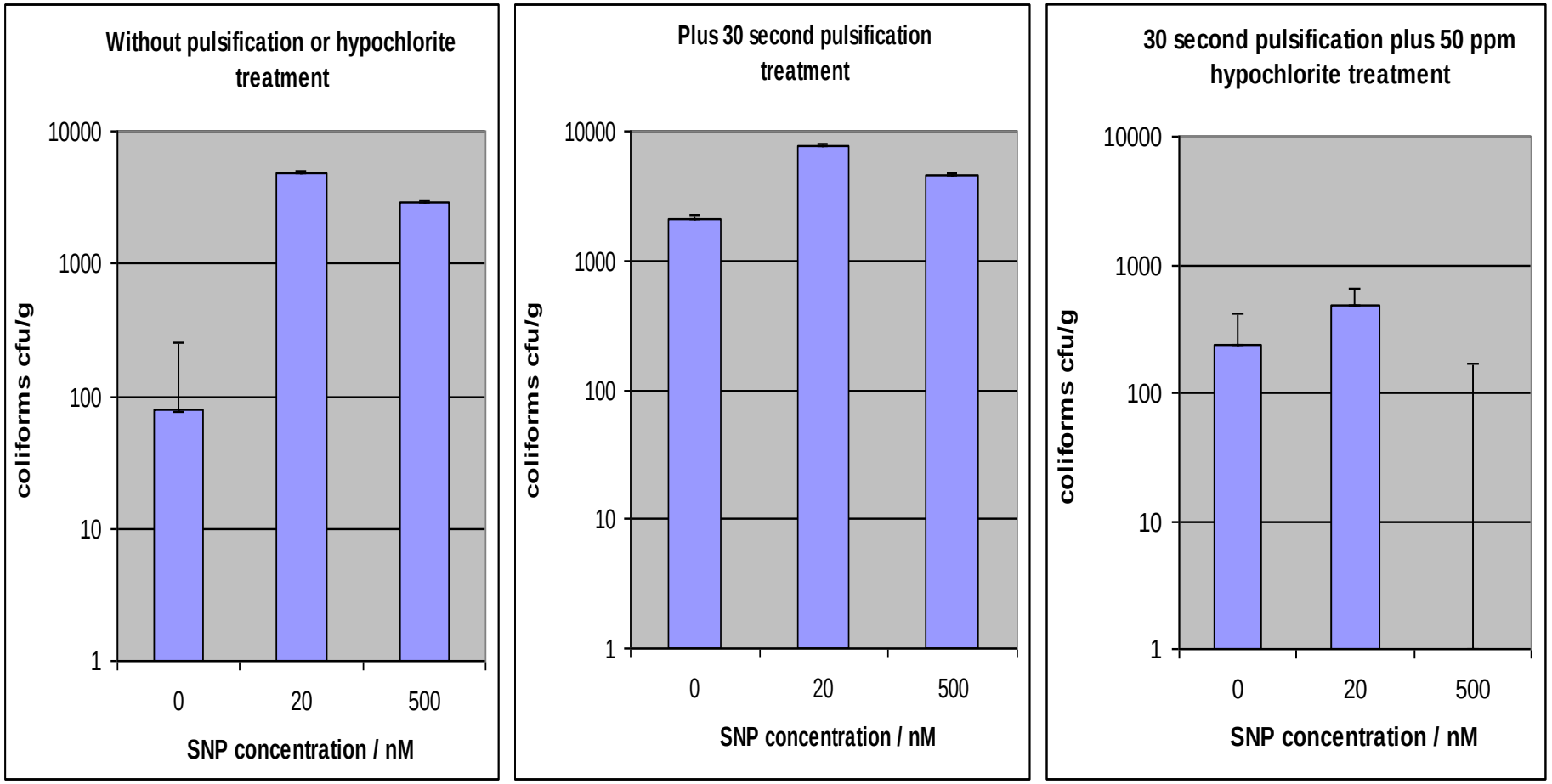


Figure 60: Coliforms recovered after a 30 minute 20 nM or 500 nM NO treatment alone; 30 minute NO treatment combined with 30 second pulsification; and 30 minute NO treated with 50 ppm hypochlorite treatment. 20 nM NO treatment appears to be optimal for recovery of coliforms off the watercress phylloplane; a synergistic effect is apparent when NO treatment is combined with pulsification and 500 nM NO pre-treatment results in the greatest kill of coliforms. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that in the data without pulsification or hypochlorite treatment there was a significant difference between the number of coliforms recovered between 0 nM and 20 nM NO ($p<0.05$) and between 0 nM and 500 nM NO ($p<0.05$) ; no significant difference could be seen between 20 nM and 500 nM NO ($p>0/05$). In the data post-pulsification, a significant difference was apparent between 0 nM and 20 nM NO ($p<0.05$) and 0nM and 500 nM NO ($p<0.05$); no significant difference was apparent between 20 nM and 500 nM NO ($p>0.05$). In the data post-pulsification and hypochlorite treatment, no significant difference was apparent between 0 nM and 20 nM NO ($p>0.05$) a significant difference was apparent between 0 nM and 500 nM NO ($p<0.05$) and 0 nM and 500 nM NO ($p>0.05$). These were shown by a t-test. P values less than 0.05 were regarded as significant.

The results presented in Figure 60 show that SNP causes greater release of coliforms from watercress leaves. NO treatment alone can be seen to lead to a 3-log increase in coliforms recovered after a 30 minute incubation time. With a treatment time of 30 minutes, 20 nM NO can be seen to lead to the greatest recovery, compared to the 12 hour incubation time during which 500 nM NO resulted in greater recovery.

Results from work in Chapter Four showed that pulsification leads to a greater recovery of cells from the leaf matrix, this effect is once again evident in the results presented here; a 3-log increase in recovery is evident after a 30 minute pulsification treatment. When pulsification treatment is performed after 30-minute SNP incubation, a synergistic effect can be seen; on average an approximate 6000 coliform increase is evident. When analysing the results individually, it is apparent that NO treatment alone has a greater recovery than pulsification alone. Although NO treatment leads to a 3-log increase in coliform recovery, as does pulsification treatment, the results show that on average 3000 more cells are recovered after NO treatment.

As for the results shown in the previous section, NO treatment combined with 50 ppm hypochlorite disinfection leads to a lower recovery of coliforms. This suggests that NO makes the cells more susceptible to the oxidising killing power of hypochlorite solution. A 500 nM NO treatment prior to hypochlorite disinfection can be seen to lead to a greater killing effect

than 20 nM hypochlorite. After pre-treatment of watercress leaves with 500 nM NO, hypochlorite disinfection was shown to lead to almost a 4-log reduction in coliforms.

6.3.3 The effect of a 10 minute SNP treatment on recovery of coliforms from the watercress phylloplane. Comparing different concentrations of SNP.

In this Section, the watercress phylloplane was treated to 0 nM, 20 nM or 500 nM NO, with a reduced treatment incubation period of 10 minutes. The phylloplane was then subsequently pulsified for 30 seconds and then treated with 50 ppm hypochlorite to determine the effect of these factors.

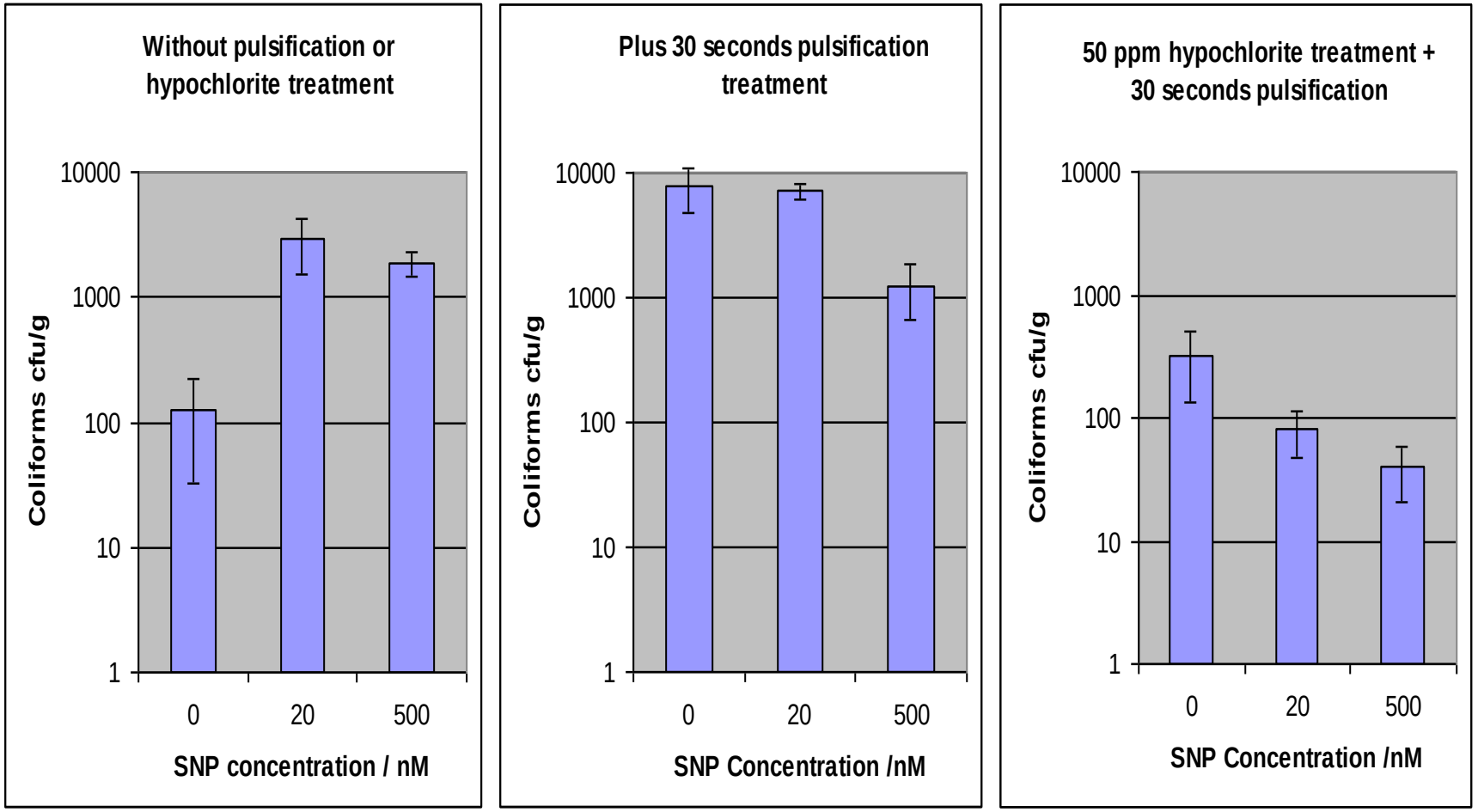


Figure 61: Coliforms recovered after 10 minute 20 nM or 500 nM NO treatment; 10 minute NO treatment combined with 30 second pulsification; and 10 minute NO treatment followed by 50 ppm hypochlorite treatment. 20 nM NO treatment results in the greatest recovery of coliforms from the watercress phylloplane; a synergistic effect is apparent after 20 nM NO treatment and pulsification, although pulsification alone for 10 minutes results in a similar recovery; 500 nM NO treatment enhances the hypochlorite killing effect the most. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was a significant difference between the number of coliforms recovered without pulsification between 0 nM and 20 nM NO ($p<0.05$) and 0 nM and 500 nM NO ($p<0.05$); no significant difference was apparent between 20 nM and 50 nM NO ($p>0.05$). After a 30 second pulsification treatment, no significant difference was apparent between 0 nM and 20 nM NO ($p>0.05$) a significant difference was apparent between 0 nM and 20 nM NO ($p<0.05$) and 0 nM and 500 nM NO ($p<0.05$); a significant difference was apparent between 0 nM and 20 nM NO ($p<0.05$) and 0 nM and 500 nM NO ($p<0.05$). In the results post-pulsification and hypochlorite treatment, no significant difference was apparent between 0 nM and 20 nM NO ($p>0.05$); and no significant difference was apparent between 20 nM and 500 nM NO ($p>0.05$); a significant difference was apparent between 0 nM and 500 nM NO ($p<0.05$). These were shown by a t-test. P values less than 0.05 were regarded as significant.

From the results presented in Figure 61, it is evident that after a 10 minute NO incubation, a 3-log recovery of coliforms occurs; 20 nM NO is shown to be more efficient at this, with approximately 1000 more cells being recovered.

Pulsification treatment alone results in a 3-log recovery of coliforms, as does NO treatment followed by pulsification. On average, an additional approximately 4000 cells are recovered when SNP treatment is combined with pulsification.

However, with a 10 minute incubation period with NO, the results show that it is more advantageous to use a 30 second pulsification step alone to release approximately 1000 more cells compared to when pulsification is combined with NO treatment. A 30 second pulsification step alone can also be seen to lead to approximately 5000 more cells being released compared to a 10 minute NO treatment alone.

NO treatment prior to hypochlorite disinfection results in 300 more coliforms being killed compared to disinfection treatment without a NO stage.

6.3.4 The effect of a 30 minute SNP treatment on the quality of watercress leaves.

The aim of this Section was to determine the treatment conditions that result in optimal watercress phylloplane integrity. Three panellists blindly evaluated produce for general appearance; including, colour, bruising, water-logging of the leaf tissue, leaf blackening, tearing, and slime production, to determine the overall quality. Sensory attributes were rated by assigning scores, a score of 1 being in a bad condition and that of 3 being in a good condition. All evaluations were carried out within 1 hour of removal of samples from storage, and on day 5 of storage at 4 °C. Samples had been treated with NO for 30 minutes prior to storage.

Table 7: Sensory scores of nine watercress samples after treatment with either 0 nM NO, 500 nM NO or 20 nM NO. A score of 1 being in a bad condition and that of 3 being in a good condition. It is evident that 20 nM NO treatment results in the highest integrity score.

	0 nM NO			**500 nM NO**			**20 nM NO**		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Panellist 1 Score	3	3	2	3	3	2	3	3	3
Panellist 2 Score	3	2	2	2	3	2	3	3	2
Panellist 3 Score	3	2	2	3	3	2	3	3	2
Average Total Score	2.44			2.56			2.78		

From the results presented in Table 7, it is apparent that on average the 20 nM NO treated samples resulted in the maintenance of a greater quality of leaves ("shelf life"), after 5 days of storage at 4°C, as judged by their physical appearance. 0 nM NO treatment had the lowest score, suggesting that the quality of the leaves was poorer without prior treatment with NO.

6.3.5 The characterization of coliforms recovered from the watercress phylloplane with different concentrations and contact times of SNP.

The aim of this section was to characterize the main coliforms (over 20 % of the total coliforms on the VRBA plate) that were recovered from the watercress phylloplane, after different concentrations of SNP and/or different treatment lengths. The Microgen GN-ID kit was used for this, after a series of steps the final result was a GN-ID profile number, which could subsequently be matched up to a final coliform type.

Table 8: The identification of the main coliforms recovered at different SNP concentrations and treatment lengths. The Table shows the phenotype of the coliforms released, the GN-ID profile number and the final coliform identification. *Acinetobacter haemolyticus, Enterobacter agglomerans* and *Serratia marcescens* are the main coliform types recovered.

SNP Treatment Length	**SNP Treatment Concentration/ nM**	**Description of coliforms**	**Microgen GN-ID Profile Number**	**Final Identification of main colonies recovered (plus 20 % of the total found on the plate)**
0 min	**0**	Faint colonies; Large; Small pinprick	4002; 0676; 0002;	*Acinetobacter haemolyticus; Enterobacter agglomerans; Acinetobacter haemolyticus,*
30 min	**500**	Sharp colonies; Large dark smudge; With halo;	6656; 0644; 2646;	*Enterobacter agglomerans; Serratia marcescens*
30 min	**20**	With halo; Large dark smudge; Large	2646; 0676; 6656	*Serratia marcescens; Serratia marcescens; Enterobacter agglomerans*
10 min	**500**	Sharp colonies	0644, 2646	*Enterobacter agglomerans, Serratia marcescens*
10 min	**20**	Sharp colonies	0644, 2646	*Enterobacter agglomerans, Serratia marcescens*
12 h	**500**	Large; With halo	0676; 6656	*Enterobacter agglomerans; Serratia marcescens*
12 h	**20**	Sharp colonies**;** Large dark smudge;	0644; 2646	*Enterobacter agglomerans; Serratia marcescens*

From the results presented in Table 8, it is evident that *Acinetobacter haemolyticus*, *Enterobacter agglomerans*, *Acinetobacter haemolyticus* and *Serratia marcescens* have all been identified as the microorganisms that are released whether SNP is present in the treatment of the samples or not. It can be seen that in the 0 nM SNP samples, all species are detached, except for *Serratia marcescens*; and in the SNP-treated samples *Acinetobacter haemolyticus* was not detected.

6.3.6 The effect of 20 nM and 500 nM SNP treatment on *Salmonella* recovered from spinach leaves.

Salmonella Thompson labelled with GFP was inoculated onto watercress leaves and then the samples were treated with NO for 30 minutes. The *Salmonella* that were recovered from the spinach leaves were plated onto Rambach agar and the colonies counted and recorded. The spinach leaves were viewed under the EDIC/EF microscope in order to obtain *in-situ* quantitative (if possible) and qualitative results on the affects of NO on *Salmonella* on the phylloplane.

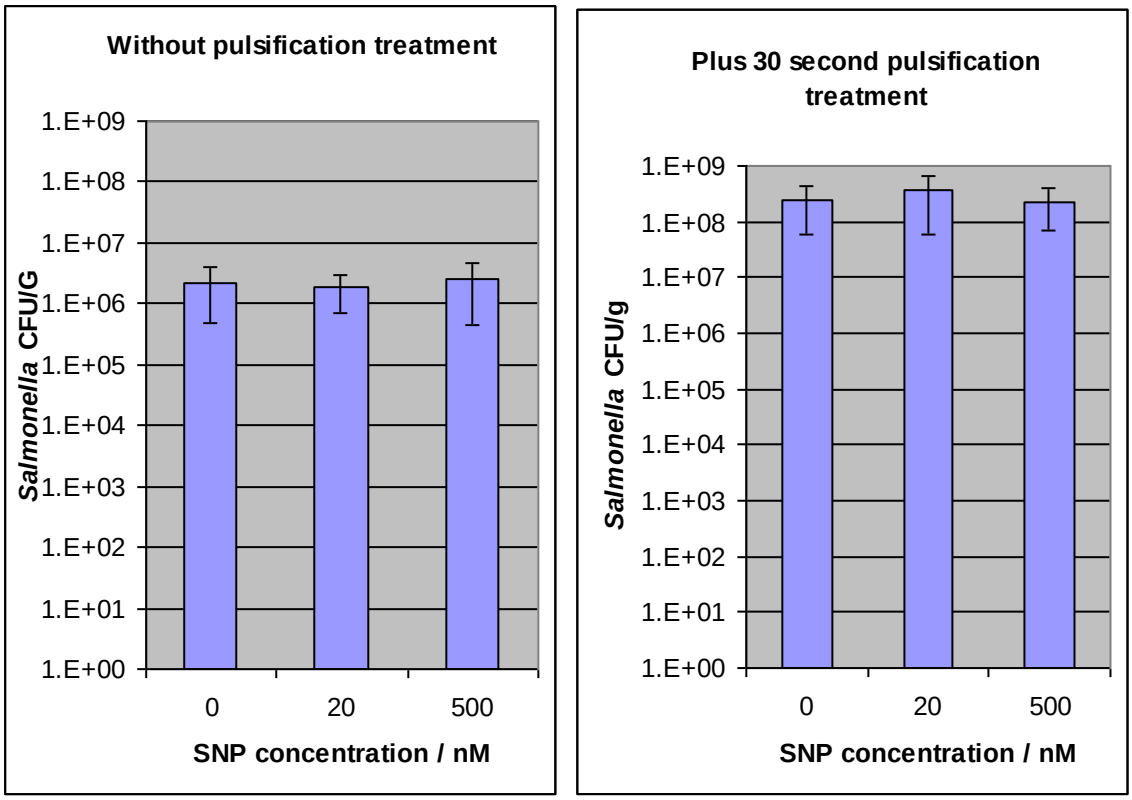


Figure 62: Coliforms recovered after 30 minute 20 nM or 500 nM NO treatment and 30 minute NO treatment (20 nM and 500 nM) combined with 30 second pulsification. An approximate 2-log increase in recovery is evident after pulsification, no significant differences are obtained when the phylloplane is treated with NO. Error bars are representative of the ± standard error of the mean of three experiments for each treatment.

Statistical analysis showed that there was no significant difference between the number of *Salmonella* recovered between either 0 nM NO, 20 nM NO and 500 nM NO ($p>0.05$). A significant difference was apparent between treatments without pulsification and with pulsification ($p<0.05$). No significant difference could be seen between the 0 nM, 20 nM and 500 nM treatments that were combined with a 30 second pulsification step ($p>0.05$). These were shown using a t-test. P values less than 0.05 were regarded as significant. The results suggest that 30 minute NO treatment has no effect on numbers of *Salmonella* recovered pre- and post-pulsification.

Unfortunately, it was difficult to quantify the *Salmonella*, using the EDIC/EF microscope since almost all of the *Salmonella* on the surface of the leaves were found in clusters or

groups of cells that were almost entirely located on the edges of the leaf cells, this was similar to that prior to treatment. The biological variation between different fields of view was very great, some fields of view contained no *Salmonella*, and others contained too many *Salmonella* to count.

Overall differences between inoculated levels before and after NO treatment were not apparent when the recovered *Salmonella* were plated onto Rambach agar, as shown in Figure 62; levels detached after 30 minute NO treatments being very similar to those detached after 30 minute treatments without NO (0 nM NO). An approximate 2-log increase in recovery of coliforms was evident when the phylloplane was pulsified for 30 seconds, regardless of NO treatment.

Chapter Six Discussion

Sodium nitroprusside acts by the release of NO, and it is a non-selective vasodilator; one of its uses is in the reduction of blood pressure during anaesthesia in order to reduce bleeding in surgical procedures. SNP is rapidly metabolized to cyanide and thiocyanate which is excreted via the kidneys and a total dose up to 16 mg can be used in adults during medical procedures (Testa and Tobias, 1995). NO is known to activate smooth muscle soluble guanylyl cyclase (GC) to form cGMP. Increased intracellular cGMP inhibits calcium entry into the cell, thereby decreasing intracellular calcium concentrations and causing smooth muscle relaxation. SNP has been shown to have a rapid-onset of action of 30 seconds, a peak hypertensive effect within 2 minutes and a return of blood pressure, to baseline values, within 3 minutes of its discontinuation (Testa and Tobias, 1995). NO has also been shown to act on K^+ channels which leads to hyperpolarization and relaxation. NO can act through cGMP and stimulate a cGMP-dependent protein kinase that activates myosin light chain phosphatase, which also leads to relaxation (Nakamura *et al.*, 2007). In this study, SNP was used to release NO and concentrations in the nanomolar range were investigated, although the actual level of NO causing the effects observed at the cellular and molecular levels would be expected to be lower than the nanomolar. NO has never before been investigated as an exogenous molecular means of microbial detachment from food or other biotic surfaces. Strategies such as that described in this study would have broad

application and huge implications in the medical, environmental and industrial settings. In the food industry, biofilm dispersal is also likely to lead to an increased shelf-life.

NO has been shown to lead to stomatal closure (Desikan *et al.*, 2002; Garcia-Mata and Lamattina, 2002), and this would be an added advantage in terms of disinfection processes since it should reduce any internalization; this may also be a factor that led to increased recovery in the experiments performed in this study, although these effects on *Salmonella* internalization would need to be investigated further. Conversely, if *Salmonella* are inside the leaf before NO treatment, then they would become trapped and less easily removed (as discussed later). NO functions include modulation of hormonal, defence and wound healing processes (Paris *et al.*, 2007), as well as regulation of cell death, and a factor delaying leaf senescence. Also, Tatiana *et al.* in 2007 showed that the expression of a NO degrading enzyme induced a senescence programme in *Arabidopsis*; nanomolar levels of NO had a similar effect in this present study on watercress leaves, this is advantageous in terms of increased shelf-life. Nitrate reductase has been recognized as a candidate for NO production during plant-pathogen interactions (Yamamoto *et al.*, 2003). Several studies have shown that after challenge with a pathogen, NO accumulates in resistant plants and establishes a direct correlation between disease resistance responses against biotrophic pathogens and NO. NO has been shown to promote germination, low levels promote leaf extension (Lesham and Haramaty, 1996) and root growth. All of these roles of NO as a developmental regulator would be beneficial to salads, fruit and vegetables. Furthermore, NO has been shown to act as a key signal in plant resistance to incompatible pathogens by triggering a resistance associated hypersensitive response (HR). Plants react to pathogen invasion by eliciting a HR at the site of infection and by establishing systemic acquired resistance (SAR), which is defined as a long-lasting systemic immunity that protects the entire plant from subsequent invasion of a broad range of pathogens (Ryals *et al.*, 1996; Shirasu *et al.*, 1997; Durner *et al.*, 1998). Durner *et al.* in 1998 showed that NO treatment induced SA accumulation in tobacco (which has been shown to result in the stimulation of oxidative burst and defence gene regulation) and therefore, these results indicate that NO plays an important role in the induction of signalling pathways leading to the establishment of SAR. NO production has been recorded in response to high temperature, osmotic, UV-

B stresses and drought (Beligni and Lamattinia, 2001; Garcia-Mata and Lamattina, 2002; Gould *et al.*, 2003). Furthermore, the study of the effect of nitric oxide on the dispersal of coliforms and its potential use in food processing or the field environment is supported by the fact that nitric oxide has been found to inhibit shiga-toxin (Stx) synthesis by enterohaemorrhagic *E. coli* (Vareille *et al.*, 2007).

The results in this study suggest that NO in the nanomolar range, leads to enhanced dispersal of Enterobacteriaceae in the phyllosphere, as for the other studies discussed here. It is likely that it is the bacteria within the phyllosphere biofilm that are undergoing coordinated dispersal events, in which attached biofilm cells are converting to free-swimming planktonic bacteria (Sauer *et al.*, 2004). This process is thought to benefit bacteria, since it allows for the single free-swimming cells to travel to new matrix areas and colonize new habitats. In this study it is useful as it acts as a decontamination procedure of salad leaves, and with the combined use of large quantities of water, replaced regularly (as is done in most food industries) it potentially allows for enhanced recovery of pathogens that would otherwise go on to cause infections. The recovery of Enterobacteriaceae from the watercress phyllosphere is 3-log after a 10 minute or 30 minute treatment with 20 nM SNP; this is likely to simultaneously have detached other microorganisms from the phyllosphere too, via a physical loosening of the general microflora. A 30 minute SNP treatment duration appears to be the most effective for recovery of coliforms from the phylloplane compared to 10 minutes. With a treatment time of 30 minutes or 10 minutes, 20 nM NO can be seen to lead to the greatest recovery compared to the 12 hour incubation time during which 500 nM NO resulted in greater recovery; it is unclear why this may be the case, since in the study done by Barraud *et al.* (2006), 500 nM NO also appeared to distinctly be the best concentration for increased detachment of *P. aeruginosa.* The reason for this difference may be because coliforms in general are being detected in this study, possibly with many different species being released, and it may be that each species reacts physiologically to the NO in different ways.

A mechanism by which bacteria could detach from the biofilm phenotype is suggested by the *P. aeruginosa* microarray studies, which previously revealed that upon exposure to NO,

genes involved in adherence were down-regulated (Firoved *et al.*, 2004). Further work should involve the study of the effects of NO on the adherence genes in Enterobacteriaceae and *Salmonella* within the phyllosphere.

When pulsification treatment is performed after 10 minute, 30 minute or 12 hour SNP incubation a synergistic effect can be seen; after 30 minute incubation, on average an approximate 6000 coliform increase is evident; this increase is significant considering that the infective dose of most pathogens is very low, typically 50 to 5000 cells can lead to illness. The reason for this synergistic effect may be that NO first leads to physiological biofilm dispersal, after which, the cells that still remain attached to the phylloplane may be more susceptible to the vibrational mechanical removing effects of the Pulsifier. Unpublished results (D. Caddy, personal communication) investigating the effect of NO on MRSA biofilms show that after treatment, biomass was considerably reduced, this would mean the remaining biofilm was thinner and therefore more prone to any further disinfection or mechanical attack; *P. aeruginosa* biomass was also shown to be reduced after NO treatment (Barraud *et al*., 2006). The results produced with pulsification after 30 minutes of NO treatment also show that 20 nM NO had a greater effect on coliform recovery; this may once again be due to reason described above for 30 minute NO treatment alone. The results show that 30 minute NO treatment alone has a greater recovery than pulsification alone, when analyzing the results individually. If only one of these procedures were to be used at the industrial scale for salad decontamination, NO would be more advantageous. Apart from the increased recovery of cells from the phylloplane, SNP is relatively cheap, very low nanomolar concentrations result in release from the complex matrix, and it also has other advantageous effects on the phylloplane, already reported in the literature, as described above and in Chapter One.

However, with a 10 minute incubation period with NO, the results show that it is more advantageous to use a 30 second pulsification step alone to release approximately 1000 more cells than when pulsification is combined with NO treatment. A 30 second pulsification step alone can also be seen to lead to approximately 5000 more cells being released compared to a 10 minute NO treatment alone.

When analyzing the incubation periods, it is apparent that the most productive in terms of recovery, is a 30 minute incubation with 20 nM NO; at this timing a 3-log increase in coliform recovery occurs. At present it is unclear why these timings are having such effects; however, if the longer treatment durations are required, then nitric oxide may be employed on the field before harvest of the salads. From the results it is also apparent that, on average, the 20 nM NO treated watercress resulted in the maintenance of a greater quality of leaves ("shelf life") after 5 days of storage at 4°C, as judged by their physical appearance. This is likely to be due to the fact that compared to the 0 nM NO treated samples, the 30 minute 20 nM NO treated phylloplanes had approximately 2-log fewer coliforms on the leaf surface, resulting in less spoilage. Another reason for the benefits of NO treatment, as reported in the literature, is its effect on reducing senescence (Belligni and Lamattini, 2001). The results here further suggest that 20 nM NO treatment for 30 minutes would be an ideal treatment step to incorporate for greater sanitation and improvement in the quality of leaves.

The results show that NO treatment renders the coliforms cells more susceptible to subsequent disinfectant treatment. After pre-treatment of watercress leaves with 500 nM NO, hypochlorite disinfection was shown to lead to almost a 4-log reduction in coliforms. In studies to date, typical published reductions show that chlorine only leads to an approximate 1-2 log reduction in coliforms from leaves (Li *et al.*, 2001; Foley *et al.*, 2002); also see Chapter Five and the 0 nM NO results produced here. Studies have shown that high concentrations of NO can have cytotoxic effects when combined with reactive oxygen species, where NO combines with O_2^- to form the peroxynitrite anion ($ONOO^-$), as described in Chapter One (Koppenol *et al.*, 1992). Hypochlorite is also an oxidant, and it is possible that when it is present, NO leads to this enhanced killing effect that is being observed in these results, but only once the cells have first been rendered more susceptible by release into suspension. It is apparent that 500 nM NO treatment prior to hypochlorite disinfection leads to a greater killing effect than 20 nM NO. It may be that 500 nM NO is the optimal concentration to illicit this effect on the leaf phylloplane.

Many of the pathogens that are known to cause human disease are suggested to be caused by integration of these pathogens into biofilms that are already established on the surface. It has been suggested in the literature that the inclusion of enteric pathogens in phyllosphere biofilms may augment their survival (Heaton and Jones, 2007). Therefore, if naturally occurring coliforms are being dispersed (which have been suggested to be more resilient to detachment compared to artificial or more recent contaminants) it is likely that pathogens that have integrated into the existing biofilms will be dispersed too, due to the physical effects described above, and the physiological effects of NO that are described further on.

The fact that NO has been shown to elicit its effect on biofilm bacteria gives a another strong indication for the presence of biofilms in the phyllosphere (as suggested in Chapters Three and Four); secondly, the fact that the chlorine killing effect is enhanced after the biofilm effect of NO - as for the studies by Barraud *et al.* (2006), further suggests that cells resist removal due to protection within existing biofilms. As results in Chapter Four and Five have shown, the loosening of cells from the phylloplane matrix lead to an enhanced killing effect by disinfectants such as hypochlorite, due to enhanced penetration of disinfectants to cells still in the matrix and those that are now loose, and reduced protection effects of singular cells. An additional two-fold logarithmic reduction in the number of coliforms recovered was observed when the phyllosphere was treated with 500 nM NO followed immediately by hypochlorite disinfection. Nitric oxide has been shown in this study to be the state of the art molecular tool for enhanced recovery from the phyllosphere matrix and although to date it is unclear how NO exposure leads to enhanced sensitivity to disinfectants, this study suggests that this is likely to do with the dispersal effects elicited by NO. Barraud *et al.* (2006) showed that the biomass of *P. aeruginosa* was decreased after NO exposure, unpublished data also show that NO has a similar effect on MRSA biomass, if the same is the case for coliform biomass in the phyllosphere, then it can be suggested that this effect also leads to increased sensitivity of the thinner biofilm cells to disinfection. Further work will have to be undertaken to look at the ratio of biofilm to planktonic cells in the phyllosphere to establish if NO is indeed causing Enterobacteriaceae biofilm cells to disperse as is strongly indicated.

Evolution may have led mammalian systems to utilize iNOS to generate NO at low concentrations that have been shown to modulate bacterial biofilms such as Enterobacteriaceae detected in this study, *P. aeruginosa* and MRSA (Barraud *et al.*, 2006; unpublished data, 2007). The spleen is an important part of the mammalian immune system and concentrations between 25 nM to 15 nM NO have been measured *in vivo* in spleen cells (Firoved *et al.*, 2004). As shown in this study nanomolar concentrations of NO induce dispersal of cells and enhanced recovery from difficult matrices such as those of the phyllosphere, it is likely that NO may be acting in this way in the spleen to enable the bacterial (or biofilm) cells to be less resistant. These concentrations of NO are also low enough to not compromise blood pressure and other homeostatic mechanisms in the host.

At higher exogenous SNP concentrations NO appeared to have either no effect or a reduced effect on the coliforms recovered from the phyllosphere. This is comparable to the results produced by Barraud *et al.* (2006) - which also established that higher exogenous concentrations in the millimolar range led to an increase in biofilm biomass. This may be the case in this study too, since high levels of NO have been shown to have toxic effects, in that it mediates nitration of protein tyrosine residues, peroxidation of lipids and deamination of DNA bases (Radi *et al.*, 1991; Wink *et al.*, 2003). Therefore, it may be expected that single cells under these conditions attempt to confine themselves to biofilms, where they would be safer from the high millimolar NO dose. The results produced showed that when the concentration of NO was increased to 20 nM the increased susceptibility of the coliform cells to hypochlorite disinfection appeared to be diminished. This effect may have been due to this principle in that the cells were still in the biofilm or attached phenotype, they had not been dispersed in the first instance, so less of the cells were killed by hypochlorite, and similar amounts were recovered to the 0 nM NO treated samples. Overall, the use of NO as a means for detachment of Enterobacteriaceae can be seen to be advantageous, at present industries, such as Vitacress Salads, are obtaining between 1- and 2-log reductions in coliforms levels post-hypochlorite treatment. The results in this study show that 3-log kills can be obtained when the treatment is combined with NO. Furthermore, chlorine treatment can be bypassed entirely due to the almost 4-log recovery of cells from the phyllosphere when NO treatment is combined with pulsification.

Acinetobacter haemolyticus, *Enterobacter agglomerans*, *Acinetobacter haemolyticus* and *Serratia marcescens* have all been identified as the microorganisms that are released regardless of whether SNP is present or not in the treatment. It is evident that in the 0 nM SNP samples all species are detached, except for *Serratia marcescens*; and in the SNP treated samples *Acinetobacter haemolyticus* was not detected.

Enterobacter agglomerans (more commonly known as *Pantoea agglomerans*) has been recognized as a clinically important plant pathogen, increasingly being isolated from hospital patients as a cause for infection, particularly in those patients that are immunocompromised, such as have cancer or have conditions that require immunosuppressive treatment (Cruz *et al.*, 2007). It is prevalent in the environment and is usually benign; however, it has the potential for nosocomial infection. The relationship between human infections by this organism and contact with plants is well recognized. Septic arthritis and joint infection has also been reported in patients caused by *Enterobacter agglomerans.* This organism can cause infections of the urinary tract and also blood stream infections related to the presence of medical devices such as urinary catheters and intravenous lines. A recent study investigated 53 paediatric cases due to infection with this organism associated with trauma associated with penetrating vegetative material and catheter-relate infection. Normally sterile sites that now were infected with the organism included 23 from the blood stream, 14 from abscesses, 10 from joints or bones, 4 from the urinary tract and 1 from the thorax (Cruz *et al.*, 2007). The fact that enhanced recovery from the watercress leaf matrix is evident after treatment with NO has the practical implication in that these detrimental microorganisms would be removed from the surface.

Acinetobacter haemolyticus was found to be present in the 0 nM SNP samples. *Acinetobacter* spp. are non-fastidious, Gram-negative cocco-bacilli that are also non-motile. These organisms grow in aerobic conditions and are the second most commonly isolated non-fermentors in human specimens, *Pseudomonas aeruginosa* being the most common. *A. haemolyticus* may be linked with clinical infections to immunocompromised individuals; however, are found as natural inhabitants of human skin, groin, toes, oral cavity, respiratory tract and intestine. Repeated isolation suggests that they are potential

pathogens. They are widely distributed in nature, and can be found in soil and water samples and have also been identified as spoiling factors in meat (Towner *et al.*, 1997). This species of bacteria is regarded as an opportunist due to the limited number of virulence factors, while no known cytotoxic are produced. Lipopolysaccharide is present in the cell wall, and features that enhance its survival include bacteriocin production, the presence of a capsule, and survival and viability under dry conditions. The capsule may inhibit phagocytosis. *Acinetobacter* nosocomial pneumonia is common in ICUs and large outbreaks have been described. An infrequent manifestation of *Acinetobacter* is nosocomial meningitis and these cases have been reported after neurosurgical procedures. *Acinetobacter* is highly resistant to antimicrobial agents and increasing antibiotic resistance has hindered therapeutic management. Although these bacteria were considered to be of low virulence in the past, the number of reports on nosocomial infections caused by *Acinetobacter* strains has increased in recent years (Towner *et al.*, 1997; Bergogne-BeAreAzin *et al.*, 1996). The fact that these *Acinetobacter* spp. are non-motile and strictly aerobic could imply that these microorganisms are not involved in biofilm formation and would give a strong indication as to why these were removed in greater quantities from the matrix without NO treatment. It could also be another reason to suggest NO is involved in biofilm dispersal and also for the presence of biofilms in the phyllosphere.

The results show that *Serratia marcescens* was recovered after all treatments; however, recovery was greatest in those samples that were treated with NO. *Serratia marcescens* is a species of Gram-negative bacteria in the family Enterobacteriaceae. It is a human pathogen and is involved in nosocomial infections, particularly urinary tract and wound infections. *S. marcescens* is a motile bacillus, and can grow in temperatures ranging from 5 to 40 °C. It is differentiated from other Gram-negative bacteria, as it is able to perform casein hydrolysis. This allows for it to produce extracellular metalloproteases that are believed to function in cell-to-extracellular matrix interactions. Due to its ubiquitous presence in the environment, once established, complete eradication of the organism is often difficult. Dispersal of this microorganism from the phylloplane would be advantageous considering all of the clinical implications of its presence. *S. marcescens* has been shown to form biofilm through a series of defined stages that result in a highly porous, filamentous biofilm

composed of cell chains, filaments and cell clusters and is dependent on quorum-sensing (Rice *et al.,* 2000, McDougal *et al.,* 2011). Several aspects of the biofilm life cycle including attachment, swarming and formation of the 3D structure are regulated by quorum sensing.

A recent study has described quorum sensing (cell-cell signalling) for the detachment of *Serratia marcescens* filamentous biofilms, results produced showed that nutrient conditions affected the biofilm morphotype. The effects of increasing NO concentrations were not studied; however, the study showed that under reduced nitrogen (or carbon) conditions *S. marcescens* formed a classic biofilm consisting of microcolonies. The results showed that quorum-sensing dependent behaviours, such as swarming motility, could be rendered quorum sensing independent by manipulating the growth medium. Quorum sensing was found to be involved in the sloughing or detachment of the biofilm from the substratum; *S. marcescens* biofilm was found to detach consistently after approximately 75 to 80 hours of development (Rice *et al,* 2000). According to the results presented here, this time period could be reduced down to even 10 minutes; in general, however, the variations in incubation timings did not result in large differences in this species being recovered from the phylloplane. The results suggested that as long as NO was present, certain species were recovered, although differences in quantities recovered varied as recorded previously.

Enterobacteria, as also shown for *Pseudomonas,* have been shown to contain GGDEF and EAL protein domains that are involved in the turn-over of cyclic di-GMP (a second messenger whose importance to microbial physiology is being increasingly recognised). Studies have shown that these domains regulate the transition of *P. aeruginosa* from the sessile to the motile state (Simm *et al.,* 2004). It has been discussed previously how bacterial growth on a surface often involves the production of a polysaccharide-rich extracellular matrix for structural support in the formation of biofilms. In many bacteria, including *Salmonella* and *E. coli*, cellulose is one of the main constituents of the biofilm matrix. A study by Re and Ghigo in 2006 showed that in *E. coli* cellulose production is regulated by involvement of a GGDEF domain protein. A similar process is common in other cellulose-producing Enterobacteriaceae, including *Citrobacter* spp. and *Enterobacter*

spp. (Zogaj *et al.*, 2003), suggesting that these domains are involved in biofilm formation. NO-sensing proteins called haem nitric oxide binding domain (HNOB) proteins have frequently been associated with GGDEF protein domains in diverse bacteria (Iyer *et al.*, 2003). These HNOB domains suggest a link between NO sensing and cyclic-di-GMP turnover and could be involved in the regulation of the production of cellulose which would have an effect on biofilm formation or dispersal.

Curli fimbriae are a major determinant of cell-cell interactions and cell adherence to hydrophilic and hydrophobic abiotic surfaces. They are extracellular surface fibres that are produced by many members of the Enterobacteriaceae and, together with cellulose, constitute the extracellular matrix components require for biofilm formation (Wang *et al.*, 2006). Curli fimbriae and cellulose have distinct roles in bacterial self-organization, virulence and transmission, adherence and invasion of epithelial cells (Sukupolvi *et al.*, 1997; Dibb-Fuller *et al.*, 1999; Bian *et al.*, 2001; Gophna *et al.*, 2001).

The data presented suggest that 30 minute NO treatment has no effect on numbers of *Salmonella* recovered pre- and post-pulsification. These results are quite unusual since according to the literature *Salmonella* produces both cellulose and curli fibres, and these are used for biofilm formation. *Salmonella* has also been shown to contain the GGDEF and EAL protein domains that are involved in the turn-over of cyclic di-GMP, as was discussed above, and these domains are thought to be linked to the effects of NO on biofilm dispersal. The results could suggest that *Salmonella* attaches more strongly to the phylloplane than coliforms and therefore, would require longer than the 30 minute NO incubation time that has so far been shown to be optimal for coliforms; this would need to be investigated further for watercress and spinach leaves. Some studies, such as that in 2002 by Barak *et al.*, show that the attachment of *Salmonella enterica* and *Pantoea agglomerans* to alfalfa sprouts is similar. One study has suggested that the *Salmonella* biofilm is not formed quickly after initial exposure or inoculation, and instead requires at least one week for attachment and formation (our study allowed one day for this), this could explain why *Salmonella* was not dispersed from the phyllosphere by NO (Lapidot *et al.*, 2006). Although the results need further investigation, the data suggest that after initial

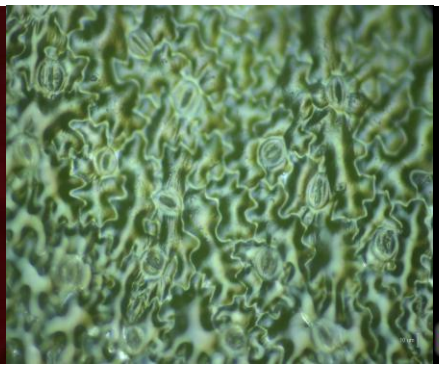
inoculation, other factors are more important in *Salmonella* survival in the phyllosphere than biofilm formation. The EDIC/EF images show that most of the *Salmonella* was located in clusters on the edges of epidermal cells, and around stomata; furthermore, results in Chapter Three showed *Salmonella* to be internalized into the leaf surface. *Salmonella* spp. may rely on these mechanisms for initial survival rather than that of biofilm formation. The results produced here also suggest that *Salmonella* recruit these other means for protection over integration into existing biofilms, since if they did integrate into existing biofilms it would have been expected for *Salmonella* to have been dispersed along with the coliforms.

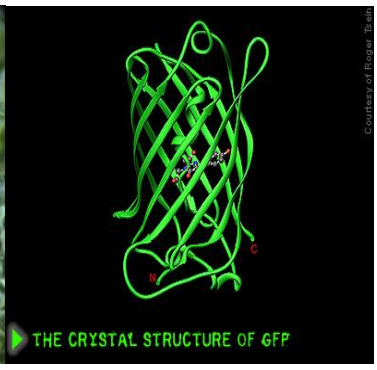
Food industries could utilize NO in their decontamination processes, nanomolar non-toxic concentrations of SNP added to the water-wash would lead to enhanced recovery of coliforms, but not *Salmonella*, from the leaf matrices. The rate of water turnover could be increased to allow for a constant supply of NO and for the constant removal of the pathogens that are in suspension. The results also show that NO treatment renders the cells more susceptible to subsequent disinfectant treatment; therefore, the used water could then be decontaminated separately (i.e. by use of hypochlorite), prior to re-contact with fresh leaf samples. NO could also be used as a molecular tool by diagnostic companies, in combination with the Pulsifier. Results have shown that SNP treatment leads to a synergistic effect when used with the Pulsifier on recovery of microorganisms in the phyllosphere; pulsification can be combined with SNP for enhanced recovery by this mechanical tool. SNP has the potential to be applied at the field, where along with the effects it has on dispersal it would be advantageous with the other effects it has on the phyllosphere as described through this study.

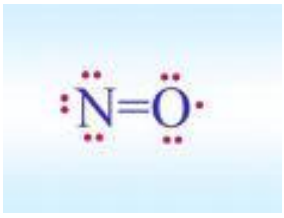
GENERAL CONCLUSIONS

AND

FUTURE WORK







The literature shows that due to the complex structure of the leaf surface little is known about the endogenous microbes or coliforms on leaves. It is also unclear how pathogens interact with the natural microflora on leaf surfaces and how they survive the harsh environmental conditions posed by the leaf matrix; therefore, one of the aims of this thesis was to attempt to begin establishing this. This was the first study to use the EDIC microscope to examine the complex phylloplane matrix and it could be concluded that this technique is the most reliable and efficient protocol available to date to evaluate the phylloplane using bright field microscopy. Minimal to no sample preparation or time was required; therefore, the *in-situ* images obtained were unaltered realistic views of the phylloplane, similar to work done with EDIC microscopy on other matrices (Walker and Keevil, 1994; Keevil, 2001; Keevil *et al.*, 2003).

Three different phylloplane types, lettuce, spinach and watercress were characterized. The images produced using EDIC microscopy show that bright field images enable clear and direct visualization of the structural environment that pathogens encounter when on the leaf matrix, without the use of fixation or dehydration. The three leaf types were structurally distinct, and this resulted in differences in the colonization patterns of the bacteria. All leaf types were found to contain numerous grooves, crevices, cell marginal areas and stomata, which could provide possible structural or physiological niches. Watercress epidermal cells were the smallest and most irregular in shape and therefore, had the most grooves per leaf; however, the leaf was thinner and thus had lower insulatory properties to bacteria. EDIC microscopy showed that stomata are located on both the underside and the topside of all the salad leaves; this would have implications on contamination. This directly shows that bacterial splash from the soil or irrigation water would result in an entrapment of bacteria on the underside stomata and animals and birds could easily contaminate the upper side stomata directly – both resulting in an increased likelihood of internalization of pathogens.

Areas of contamination could be visualised using EDIC/EF microscopy, this was particularly high on the thicker spinach leaves; although contamination could be found across the whole leaf surface, the majority was found around the epidermal cell margins.

3D areas of contamination were apparent, these may be indicative of biofilm material, particularly where slimy areas suggestive of biofilm exopolymer were visible. This secreted polysaccharide material forms a slime area around bacterial cells, and facilitates adhesion, each strain producing only one type of capsular polysaccharide (Leigh and Coplin, 1992). The polysaccharide of different bacterial species have been suggested in the literature to interact with each other; it is thought that the distinct compositions of these slime areas contribute to differences in attachment strengths shown by different bacteria after no wash, bore-hole water (hand-wash), pulsification or stomaching treatments. This is first study to show direct evidence of the slimy exopolymer of biofilm on the phylloplane. Although, published work has previously shown the evidence of biofilm on the phylloplane using LSCM (Carmichael *et al.,* 1999) and SEM (Gras *et al.,* 1994). Biofilms may assist in the survival of epiphytes and also pathogens on the difficult phylloplane matrix, protecting the bacteria from changing environmental stresses (Morris and Monier, 2003).

The natural microflora colonization patterns in relation to the leaf structure were also established using EDIC/EF microscopy. From the microscopy images obtained after BacLight™ staining, combined with EDIC/EF microscopy, it could be established that live bacteria tend to concentrate around the edges of the epidermal cells or in the regions of stomata, suggesting that this may be an active process. This is similar to other studies in the literature that have used other microscopy techniques, such as SEM or LSCM (Yadav *et al.,* 2005; Monier and Lindow, 2004). Many species of live bacteria are motile and therefore are able to migrate to niches to improve their chances of survival during stress or adverse conditions. Regions of stomata have higher nutrient availability, thereby providing a physiological niche; these regions also form a route for internalisation and consequently could protect microorganisms from disinfectants or environmental stresses. Cuts on the phylloplane have also been shown to result in increased colonization by bacteria, these regions exude nutrients; Takeuchi and Frank (2000) showed internalization in these areas by *E. coli* O157:H7, resulting in protection from the environment and chemical disinfectants. EDIC microscopy is useful for the rapid and direct assessment of damage to the phylloplane. The BacLight™ assay also enabled it to be established that dead bacteria

were mainly found singularly in the centre of leaf epidermal cells, whereas live bacteria tended to be in groups. Aggregation would enable greater protection from stresses compared to single cells, since this allows for interaction forces and quorum sensing between bacteria to stop them from being washed away from the protective areas on the leaf matrix. Research has shown that bacteria that are attached to surfaces are phenotypically different from their planktonic counterparts (Hinsa *et al*, 2003; Morris and Monier *et al*, 2003). Genes that are associated with the physical mechanism of attachment are upregulated. In general, possibly due to the reasons explained above, the total numbers of live bacteria on the phylloplane were shown to be greater than that of dead bacteria in the population. However, this may also be due to limitations in the use of propidium iodide (below).

Overall, the use of the BacLight™ assay for comparative studies of different pre- and post-treatments to leaves was shown to be inefficient. Although colonization patterns could be observed, it was difficult to observe differences in recovery between varying treatments. BacLight™ comparisons showed no difference to be apparent, in recovery whereas use of traditional cell culture established that a difference was actually evident when leaves were treated in varying methods. BacLight™ staining resulted in high background fluorescence, particularly when using propidium iodide to stain the dead bacteria. Propidium iodide fluoresces red, this being close to the fluorescence of chlorophyll; therefore it was difficult to distinguish areas of chlorophyll from dead bacteria. Although the individual bacterial cells were easier to visualize using SYTO 9; a high degree of background fluorescence was evident due to penetration of SYTO 9 into the leaf cuticle. This was particularly evident for the thinner watercress leaf. This may also be due to biological variation of the colonization of every individual leaf; it may be more efficient to refine the protocol used in this thesis by carrying out treatments on individual leaves, particularly when performing *in-situ* comparisons. Due to these reasons it was difficult to obtain quantitative results pre- and post-treatment of the phylloplane. BacLight™ has been evaluated as a potentially useful stain for the direct quantitative enumeration of viable and total bacteria for a simpler matrix, such as drinking water (Boulos *et al*, 1999). This live/dead staining protocol has also been previously used on alfalfa, clover and mung bean sprouts combined with LSCM

for qualitative examination of aggregates of native bacteria; however, this is the first study to evaluate its use combining EDIC/EF microscopy (Fett and Cooke, 2005). Aside from the difficulties presented in the use of this stain on the watercress phylloplane, recently BacLight™ has also been shown to possibly have other limitations and inaccuracies. For example, it is unable to distinguish intermediate states of damage incurred by bacterial cell membranes (Berney *et al*, 2007), resulting in inconclusive evaluations.

The cyan-fluorescent nucleic acid stain, DAPI, combined with EDIC/EF microscopy was also evaluated for use on the watercress phylloplane, for the qualitative analysis of the colonization patterns of natural epiphytes in comparison to niches and areas of contamination that were visible by EDIC microscopy. As for BacLight™, quantitative analysis was also attempted. DAPI staining proved difficult for the examination of thin watercress leaves; results were greatly variable according to the incubation period with DAPI. DAPI staining of the thicker spinach phylloplane allowed it to be established that areas visible on EDIC bright field images as contamination were not entirely bacterial in nature. Some areas of the contamination were not stained; these regions may be areas of exopolysaccharide matrix of biofilms. Bacteria were visible as aggregates and single cells, as also shown by the BacLight™ images, and colonization occurred in cell margins as well as centrally on the leaf. It proved difficult to obtain quantitative comparisons of leaves stained with DAPI before and after different treatments, this was mainly due to biological variations between leaves, in each field of view, future work refining the protocol used in this thesis to single leaves should improve this. Although staining of bacteria on the phylloplane, with stains such as DAPI, is useful due to obvious advantages gained for *in-situ* spatial comparison; the disadvantage is that this staining procedure does not differentiate viable from dead cells.

This thesis also compared LSCM to EDIC/EF microscopy. It could be concluded that the EDIC/EF microscope produced more informative images of the exact structural locations of inoculated *Salmonella* compared to those by LSCM. LSCM enabled the visualization of stomatal areas due to the red fluorescent properties of the leaf chlorophyll. However, variations in the exact physical nature of the leaf, crevices and grooves, damage to the

phylloplane or debris and 3D contamination could not be observed, for example see Brandl and Mandrell (2003). Another disadvantage of LSCM compared to EDIC/EF microscopy was the degree of training required and the time taken to obtain results, leading to further possible alterations in the sample. The main advantage of LSCM compared to EDIC/EF microscopy is that intracellular observations can be made. Internalization of *Salmonella* into the watercress phylloplane was shown for the first time in this thesis, the literature has previously shown the internalization of *Salmonella* into the cilantro phylloplane (Brandl and Mandrell, 2002). As Lapidot *et al.*, suggested in 2006, the data in the thesis indicates that the ability of *Salmonella* to penetrate leaves is an important factor in its protection (Lapidot *et al.*, 2006).

Overall, the use of EDIC microscopy is much more efficient and accurate than other forms of microscopy and it is more reliable, clearer and instantaneous for qualitative comparisons of the leaf structure. The microscopy techniques that are used in studies to date utilise techniques that fix, dehydrate or damage the phylloplane, resulting in extensive loss of spatial information. EDIC/EF microscopy combined with DAPI also enabled possible sites of biofilm formation to be visualised. However, the results in this thesis show that EDIC/EF microscopy combined with BacLight^{TM}/DAPI/GFP-labelled Salmonella is not efficient enough to obtain qualitative results when comparing different treatments. This was due to biological variations in each field of view and the large sample sizes (25 g) of phylloplane used for each treatment sample. Refinement of the methods used in this thesis may enable qualitative and quantitative comparisons before and after differing treatment of single leaves.

At present, the worldwide-adopted method for microorganism recovery is to use the Stomacher which uses mechanical waves to extract bacteria off matrices into suspension (Sharpe and Jackson, 1972). However, the Stomacher has many disadvantages; the main one being sample damage, due to the mechanical forces that are employed. Stomaching was confirmed not to be ideal for use when using filter methods such as that used for concentrating *E. coli,* and in general it has been shown to produce lower recovery of cells and greater sample damage. The aim in this thesis, was to determine if the Pulsifier (using

vibrational waves) is more efficient than the Stomacher in bacterial recovery off the watercress phylloplane and, if so, to establish the optimal working conditions of the Pulsifier, so that the same could be carried out throughout this thesis. To date, the working mechanics of the Pulsifier have not been refined for optimal recovery and sample integrity (Fung *et al*, 1998). Minimal sample damage is important for when the phylloplane is required to stay in-tact, for microscopy or filtration studies and also to minimise possible internalization of the microorganisms through cuts and abrasions (Seo *et al*, 1999; Garg *et al*, 1990; Brandl *et al*, 2002).

Sharpe *et al* (1972) stated that a general 15 second stomaching was suitable for most foods, although the data were not presented; therefore, in this thesis the optimal working conditions of the Stomacher were also investigated. For a sample size of 250 g the best treatment duration for bacterial recovery was found to be 30 seconds, for both the Stomacher and the Pulsifier. This study also allowed further characterisation of the microflora or pathogens on the surface of the leaf matrix, since we could establish how strongly/loosely these were attached depending on the length of pulsification treatment required for removal. The fact that coliforms were recovered after each of the treatment durations suggests that they are attached to the watercress phylloplane in differing strengths. This may be due to the different types of coliforms that can be found on the phyllosphere and the variable properties that these possess. If time were available it would be interesting to characterize the coliforms that are being detached and their temporal release pattern. The Pulsifier was also shown to be more efficient than the Stomacher in terms of cell recovery off the watercress phylloplane, possibly due to the fact that less debris is produced by pulsifying and therefore less of the bacteria remain on these debris and more become free in suspension. Previously published studies have shown the Pulsifier to detach microorganisms to a similar degree to that of the Stomacher, however, the studies in the thesis made these comparisons under optimal working conditions of both the Stomacher and the Pulsifier (Fung *et al.,* 1998). If the coliforms or *E. coli* have formed biofilms on the surface on the leaf structure, then it may be that the top cells in the biofilm are detached more easily than the lower layers. It may not be the biofilm areas on the leaf surface that are effected it might just be that some of the cells become attached or

trapped in crevices or grooves on the surface and require longer pulsification times; or it may be that some of the cells become attached to naturally occurring biofilms, possibly in a symbiotic relationship resisting detachment. The fact that the Pulsifier detached even a small number of cells shows that the bacteria do travel to or locate in niches to aid their survival, and they appear to attempt to attach more strongly to the matrix. The Pulsifier is able to overcome this mechanism of survival of the cells, by detaching them from the surface. It could be concluded that the Stomacher is out-dated for many types of analyses and should be replaced with the Pulsifier, particularly for salad leaf samples. From the results obtained it could also be concluded that it is more ideal if there is less air in the treatment bag when pulsifying or stomaching samples, since there is less noise, a lower likelihood of aerosol production and less damage to the matrix surface. Although this leads to a slightly lower bacterial detachment, the reduction is not significant. Either way, it is important to control the level of air between different tests so that variables are not changing in each experiment.

Five-log total heterotrophs were seen to be detached off the watercress phylloplane, after a hand-wash, and no significant difference was apparent with this compared to the control. This suggests that these cells were loosely attached to the phylloplane matrix. A further 2-log increase in recovery was evident after pulsification, detaching the more strongly attached cells. Although to date no conclusions have been established on how the Pulsifier elicits its effect, it is likely to be shaking bacteria into solution via its vibrational forces. Mutational studies with, for example, flagella or curli knockout strains, would be interesting to see if the Pulsifier is acting on a certain attachment factor of bacteria to the plant surface and/or to surrounding epiphytes. It would also be interesting to find out which specific heterotrophs are being detached after initial or prolonged pulsification or stomaching and correlate these with the presence of attachment factors or physiological pathways of biofilm formation such as quorum sensing pheromones.

EDIC images were examined of the phylloplane matrices before and after a hand-wash or pulsification; on average no differences could be seen. This may have been because the initial contamination levels on the phylloplane were very high, and the level of detachment

low, so that even though these treatments are recovering some epiphytes (as shown by cell culture), no overall difference can be seen directly. It may be that epiphytes are attached very strongly to the leaf matrix and therefore are not detached by a simple hand-wash; either way, this would have implications to potential consumers in terms of the shelf life of fresh produce or survival of potential pathogens. Subsequent investigations using cell culture showed that between approximately 1- and 2-log coliforms were recovered off the watercress phylloplane after a hand wash and approximately 5-log total heterotrophs. It is difficult to determine the percentage efficiency of a hand-wash on the naturally occurring microorganisms since we are uncertain of their levels on the watercress phylloplane initially; an extension to this study would be the use of generic and/or species specific PNA probes to investigate this. Nevertheless, it can be concluded that a 30 second hand-wash does remove microorganisms off the watercress phylloplane. As shown in the EDIC/EF bright-field and BacLight^{TM} stained images, hand-washing was shown not to result in any differences in the images obtained, again suggesting that the bacteria may be very strongly attached so that none are recovered or that the initial levels are very high; cell culture techniques again, subsequently suggesting that the 1- to 2-log reduction of cells after a hand-wash were camouflaged on the EDIC/EF images. Subsequent studies showed, using cell culture techniques, that up to 5-log epiphytes and up to approximately 2.5-log coliforms are removed off the phylloplane after pulsification. The fact that these levels are not clearly visible on the EDIC/EF images suggests that either BacLight^{TM} and DAPI are not staining the entire population of epiphytes or that the levels of epiphytes are very high and therefore on average the differences are not apparent.

In-situ visualization of the phylloplane resulted in no apparent differences in the EDIC microscopy images of the pre- and post-pulsified samples. This may be due to the large degree of contamination of the phylloplane camouflaging any differences after pulsification. However, BacLight^{TM} staining revealed lower levels of contamination post-pulsification, and those that remained on the phylloplane were aggregates of live bacteria that were mainly located in the grooves and crevices of the leaf. These bacteria are the natural epiphytes that were more strongly attached. A greater proportion of cells being in suspension is advantageous, since as this study shows, microorganisms that are attached to

matrices resist disinfection to a greater extent and are more difficult to kill. DAPI staining showed that areas of contamination that appeared to be biofilm on the phylloplane were not removed, suggesting that these were more strongly attached than single or smaller aggregates of bacteria. This agrees with previous research that shows that biofilm bacteria are difficult to detach from various matrices in general (Rogers *et al*, 1996; Carmichael *et al*, 1999; Lehtola *et al*, 2007).

The mechanism by which the Pulsifier asserts its recovery effects is at present unknown, however, the shearing forces may be the correct size that result in weakening of the attraction/attachment forces between bacteria/phylloplane or epiphytes/pathogens or the epiphytes/epiphytes. Studies have previously shown that fluid shear stress does play a role in the detachment of biofilm from leaves (Picioreanu *et al*, 2001). Some areas of biofilm or aggregates were shown to be removed by pulsification, this is advantageous since the exopolymer matrix of biofilms and the environment contained within biofilms has been suggested to buffer environmental changes and/or disinfection treatments (Gormon *et al*, 2001; Monier and Lindow, 2005).

The next objective of this thesis was to determine the effect of chlorine, ozone and pulsification treatments on the numbers of coliforms and *E. coli* extracted off the surface of the watercress matrix, using plate count methods and the Pulsifier for recovery. The results suggest that microorganisms are protected on the surface of the leaf and that this may be the main reason why even after disinfection procedures, pathogens are surviving and going on to infect consumers; this being similar to previous studies (Koivunen and Heinonen-Tanski *et al*, 2005). Pulsification was shown to produce a greater recovery of both *E. coli* and coliforms from watercress than stomaching in both control treatments and samples that had been treated with hypochlorite solution; hypochlorite solution (and ozone) appearing to preferentially attack more of those cells that were free in solution. Less than 1% of the *E. coli* in suspension was shown to survive disinfection; however, 82% of the original tightly attached *E. coli* was shown to be loosened off the phylloplane when pulsification and hypochlorite treatment were combined compared to pulsification treatment alone. A similar effect was apparent in the recovery of coliforms. An approximate 2-log coliform

kill was apparent after 90 ppm hypochlorite treatment; however, 1.4-log of the loosely attached cells had survived hypochlorite treatment, or were detached post-treatment. After ozone treatment, approximately 18 % *E. coli* that were in suspension survived treatment and 50 % that were shown to be strongly attached to the phylloplane were removed. This suggests that a combined treatment of ozone disinfection with pulsification may be optimal. This effect may be because the ozone may have killed a small number of microorganisms on the top layer of a cluster of cells, the ozone may have then subsequently have been quenched, but the cells may have become loosened for subsequent pulsification or stomaching and therefore more were detected on the chromagar. The increased contact produced due to more molecules in the treatment water (ozone or just oxygen) may also lead to increased removal of cells from the matrix. These results are not conclusive, since distinctions can not be made to ascertain if those cells that are not being recovered post-pulsification have been killed by the disinfectant or if they have attached more strongly to the phylloplane.

The results in this thesis also showed that coliforms are recovered in higher numbers than *E. coli* off the watercress phylloplane in general. However, the coliforms tended to be more tightly attached than the *E. coli*, a lower percentage being recovered initially with a hand-wash than for *E. coli*, whereas a higher percentage of coliforms were extracted off the watercress phylloplane after pulsification treatment. This may be because the *E. coli* is most likely from faecal contamination and therefore is more recent and has not yet established fully its defence mechanisms to any stress; also it may not have yet reached any niches for protection. It may also be that *E. coli* simply do not attach strongly to the phylloplane, for example, it may not produce curli which could be an important attachment factor. The coliforms appear to be more tightly attached and as discussed previously can also be natural soil bacteria rather than indicators of faecal contamination. As such, coliforms would be more environmentally adapted and robust; this would also explain why they might occur in higher numbers compared to *E. coli*.

Low ozone concentrations were shown to be more effective than high hypochlorite concentrations in initial experiments, 0.2 ppm ozone producing an 80% reduction of *E. coli*

in some samples, as also shown in previously published studies (Rice *et al*, 2000) whereas 90 ppm hypochlorite reducing *E. coli* levels by 77 %; however, subsequent experiments did not show this due to difficulties faced in maintaining constant concentrations of ozone. A similar effect was apparent on the levels of coliforms, due to variable ozone levels; compared to control treatment, ozone was shown to reduce coliforms by only 37 %, whereas hypochlorite was shown to reduce coliform levels by 90 %.

Ozone has been shown to attack numerous cellular constituents, including proteins, lipids, cell membrane enzymes, peptidoglycans and nucleic acids via oxidation (Murray *et al*, 1965; Dave, 1999; Chang, 1971). *Salmonella* has been shown to be killed by ozone by its action on the sulfhydryl groups on enzymes and the double bonds of unsaturated lipids, disrupting normal cellular activity (Dave, 1999). *E. coli* has been shown to be killed via ozone oxidising dehydrogenating enzymes in the respiratory system (Ingram and Haines, 1949). Although ozone has been established as a powerful broad spectrum antimicrobial, wide variations in the reported sensitivities of even one microorganism have been described in the literature (Finch *et al*, 1988; Broadwater *et al*, 1973; Kim and Yousef, 2000). One study has also suggested that hypochlorite treatment is the most effective disinfectant compared to ozone to use on tomato surfaces for the irradiation of *Salmonella* (Chaides *et al*, 2007). The results in this thesis suggest that ozone would be ideal for use, since the phylloplane was shown not to be damaged, and initial results showed reductions in levels of *E. coli*, coliforms and *Salmonella*; however, the experiments demonstrated that it would be difficult maintaining ozone concentrations at the industrial level. The ozone may also be rapidly quenched by the organic load once the leaves are immersed in the solution. Ozone concentrations must not exceed approximately 0.1 ppm (Buckley *et al*, 1975), however ozone has poor solubility so this is less of a problem.

The results in this thesis show that as watercress ages the number of *E. coli* recovered decreases, this may be because the cells are dying or that they are attaching more tightly, since even after pulsification the numbers recovered are still very low. Microscopy methods using GFP can be used in further work, to track the cells and clarify this. Of note, when the cells were allowed an extra twenty-four hour incubation time on the agar, more

cells were shown to grow; this indicates the presence of sublethally damaged cells. These results indicate that this may be a reason why foodborne pathogen outbreaks are still occurring, since some pathogens are undetected using conventional isolation procedures since they are sublethally damaged and subsequently grow at a slower rate. This suggests that current adopted methods used for cell recovery are out-dated, improvements would involve allowing longer incubation times and also use of the Pulsifier in replace of the Stomacher.

The results in this thesis suggest that a step involving the pulsification principle could be incorporated at the industrial scale for the detachment of epiphytes and pathogens from the phylloplane, followed by hypochlorite treatment; this resulted in a greater reduction of microorganisms off the phylloplane compared to when the majority of the cells were protected from hypochlorite disinfection by being attached to the surface of the leaf matrix. A pulsification treatment performed prior to hypochlorite disinfection was shown to result in less than 0.5-log coliform recovery with a subsequent pulsification step compared to an approximate 2.5-log recovery when hypochlorite disinfection was utilised without a prior pulsification. A single pulsification was shown to recover 3-log coliforms in total. The results show that chemical disinfection could also be completely replaced using only vibrational energy. The novel Pulsifier can be used effectively to remove potential pathogens off food surfaces with 2- to 3-log reductions in pathogen load; this is without sample damage and also without the production of harmful by-products. This reinforces the fact that pathogens are protected on the leaf surface, these can be loosened off the surface by use of the Pulsifier shearing forces; the results also suggest that microorganisms may respond to chemical disinfection using their intrinsic stress response mechanisms (e.g. attachment). Pulsification may disrupt biofilms and also detach bacteria that are trapped or wedged in grooves and crevices, large volumes of water are then capable of washing the bacteria away to very low or zero concentrations or a chemical could potentially be then subsequently used (since our results may suggest that chemicals preferentially attack those cells that are loose in solution).

The watercress phylloplane is a complex matrix, with grooves providing physical and physiological niches on both the underside and upper surfaces. Aggregates of epiphytes and inoculated pathogens can be seen to be formed. Studies have suggested that ozone is more effective on smooth surfaces compared to wrinkled (Achen *et al*, 2001). A simple hand-wash was shown not to result in a significant difference in the level of pathogens in suspension compared to no treatment. No treatments without pulsification resulted in approximately 2-3 log recovery of coliforms, a 5-log recovery of heterotrophs, a 10% recovery of inoculated *Salmonella*, and on average 9 *E. coli*. The results presented in this study suggest that the Pulsifier's shearing forces are required to detach an extra 3-log coliforms, 7-log heterotrophs, 68% inoculated *Salmonella* and an extra 7 *E. coli*.

This thesis also showed that samples of watercress from different farms across the world have varying degrees of contamination and numbers of coliforms, present in the original untreated samples. Watercress phylloplane samples from the USA were shown to contain higher background numbers of coliforms than those from Portugal; this may be due to a variety of factors, such as growing conditions, soil type, weather conditions etc., which would effect the potential growth of any bacteria. This may also be affected by the location of the farms, how much irrigation water is used, where this water comes from and if it is originally faecally contaminated.

An important question arising from this work is if bacteria in general, i.e. the coliforms, are protected on the leaf surface due to protection from the leaf environment, or if this is due to an altered physiological response, since the cells are clustering together or forming biofilms. This was investigated using a nitric oxide (NO) generating system. The NO donor, SNP, has recently been found to be a powerful effector of attached cell release. Webb *et al.* (2007) showed that reactive nitrogen intermediates (RNI) play a role in cell dispersal from aerobically grown *P. aeruginosa* biofilms. Dispersal was induced with low, sublethal concentrations (25-500 nM) of the donor sodium nitroprusside (SNP). Consequently, the final objective of this study was to determine the effect of the molecular signalling molecule, nitric oxide, on the numbers of natural coliforms recovered off the watercress phylloplane matrix and any subsequent effects on susceptibility to hypochlorite

disinfection; since the Pulsifier mechanical release data indicated that detached bacteria were more susceptible. NO in these small concentrations is safe for use and leads to no production of by-products that can be detrimental to health. The results in this thesis suggest that nanomolar concentrations of NO are able to disperse coliforms off the watercress phylloplane in 3-log amounts, and that NO treatment renders the cells more susceptible to subsequent disinfection treatment. The reason for the increased susceptibility may be that the cells are no longer in aggregates or in niches on the phylloplane, instead being single in suspension; this may increase the susceptibility of the coliforms to the oxidising killing power of hypochlorite. What is unclear at present is if the susceptibility is due to only physical release or also additional planktonic physiological changes induced by NO. Studies have shown that high concentrations of NO can have cytotoxic effects when combined with reactive oxygen species (Koppenol *et al*, 1992). Hypochlorite is also an oxidant; it may be that once the coliforms have been released into suspension, the 500 nM NO combines with the hypochlorite to result in this effect on the phylloplane matrix. The data presented in previous studies and those presented in this thesis, suggest that chlorine results in a 1-2 log kill of the coliforms on the leaf matrix (Li *et al*, 2001; Foley *et al*, 2002; Chapter Five; Chapter Six). However, after a pre-treatment with 500 nM NO, 4-log reductions of coliforms was apparent.

The window of optimal NO activity that was observed by Barraud *et al* (2006) is also apparent on the phylloplane. The greatest recovery of coliforms was evident when a NO concentration of 500 nM was used after 12 hour incubation and after 30 minute incubation the 20 nM to 500 nM NO range was optimal; 500 nM NO was also shown to have the greatest effect for enhancing hypochlorite treatment. This window suggests some sort of physiological process may be occurring. Five mM NO appeared to reduce detachment and susceptibility of coliforms on the phylloplane matrix to chlorine treatment. The optimal incubation time of NO was 30 minutes. A synergistic effect was apparent when a 30 second pulsification was combined with NO treatment for 30 minutes. Almost 4-log increase in recovery was apparent when both treatments were combined. This might suggest that bacteria in complex communities are attaching or detaching by NO-mediated and NO-independent mechanisms; an example of the latter might involve curli attachment

although there is no direct evidence yet to support this hypothesis. The reason for this synergistic effect may be due to the fact that initially the 30 minute incubation with NO caused coliform biofilm cells to be physiologically dispersed, the cells that remained on the phylloplane may subsequently have been more susceptible to the shearing effect of the Pulsifier. Unpublished results (D. Caddy, personal communication), investigating the effect of NO on MRSA biofilms showed that after treatment, biomass was considerably reduced, i.e. the remaining biofilm was thinner, this would enable the cells to be more prone to further disinfectant or mechanical attack. Studies by Barraud *et al* (2006) also showed the biomass to be considerably reduced after NO treatment. Although NO and Pulsification treatments were shown to have a synergistic effect, the recovery produced by NO alone was greater than that produced by pulsification alone; therefore, if only one procedure could be utilised it would be more advantageous to use NO. This means that NO-mediated attachment or detachment mechanisms for coliforms in the phyllosphere are more important than NO-independent systems. Other advantages of NO use compared to pulsification are that SNP is relatively cheap and no risks of mechanical damage would be entailed in a protocol. In contrast to a 30 minute incubation period, a 10 minute incubation period with NO was most effective in recovering high numbers of coliforms when combined with a 30 second pulsification, so both should be incorporated if this shorter duration is required. Also, a 30 second pulsification was shown to be more effective in cell recovery than the 10 minute NO treatment. Therefore, if NO is to be utilised it is more advantageous to incubate for 30 minutes.

The results in this thesis show that a treatment of watercress leaves with 20 nM NO for 30 minutes improved their shelf-life. This is likely to be because these samples had approximately 2-log fewer coliforms on the phylloplane, resulting in less spoilage. NO has been shown in the literature to reduce the senescence of leaves and establish system acquired resistance, thereby protecting plants from subsequent invasion from pathogens (Belligi and Lamattini, 2001; Ryals *et al*, 1996; Shirasu *et al*, 1997 and Durner *et al*, 1998); these factors may be playing a role in resulting in better quality leaves after NO treatment. NO has been shown to be a key signal in plant resistance to incompatible pathogens; Tatiana *et al* (2007) showed that the expression of a NO-degrading enzyme induced a

senescence programme in *Arabidopsis*. The results suggest that NO is advantageous in terms of sanitation of the phylloplane and the shelf life of watercress leaves. Other benefits of NO have been the promotion of germination, root growth and leaf extension; these factors would be important if the NO was to be used pre-harvest (Lesham and Haramaty, 1996). Furthermore, the use of NO to disperse microorganisms from the phylloplane before or after harvest is supported by the fact that very recently NO has been found to inhibit shiga-toxin synthesis by enterohaemorrhagic *E. coli* (Vareille *et al.*, 2007).

Characterization of the coliforms recovered off the watercress phylloplane with different concentrations and contact times of SNP showed that *Acinetobacter haemolyticus*, *Enterobacter agglomerans* and *Serratia marcescens* are released from the phylloplane by NO. Variations in the incubation period or concentrations of NO did not appear to affect the coliform type recovered, although quantities of each type recovered were seen to vary. *Acinetobacter* are largely non-motile microorganisms and are strictly aerobic, this may imply that they are not involved in biofilm formation; this may suggest why this species was recovered in high numbers in the control samples that had not been treated with NO, as well as in the samples that had been NO treated (Towner *et al*, 1997; Bergogne-BerAreAzin and Towner, 1996). *Serratia marcescens* was found after all treatments, however, greatest recovery of this microorganism occurred after NO treatment. Several studies have reported that *S. marcescens* forms biofilms in a series of defined stages, resulting in a biofilm that is highly porous and filamentous that relies on quorum sensing (cell-cell signalling) for attachment and swarming. Studies have shown that nutrient conditions affect the *S. marcescens* biofilm morphotype; although the effect of increasing NO concentration was not investigated, it was shown that under reduced nitrogen conditions this microorganism formed a classic biofilm consisting of microcolonies. The study showed that quorum sensing-dependent behaviours such as swarming motility could be rendered quorum sensing-independent by manipulating the growth medium. Quorum sensing was found to be involved in the sloughing or detachment of the biofilm from the substratum. (REF) *Enterobacter*, have been shown to contain GGDEF and EAL protein domains that are involved in the turn-over of cyclic di-GMP, as for *Pseudomonas*. The literature suggests that these domains are involved in turn to regulate the transition of *P.*

aeruginosa from the sessile to the motile state (Simm *et al.*, 2004). In many bacteria, including *Salmonella* and *E. coli*, cellulose is one of the main constituents of the biofilm matrix; studies have shown that GGDEF protein domains are involved in the regulation of cellulose production in *E. coli, Citrobacter spp. and Enterobacter spp.* (Da Re and Ghigo, 2006; Zogaj *et al.*, 2003). Haem nitric oxide binding domain proteins (HNOB) have frequently been associated with GGDEF protein domains in diverse bacteria; this suggests the link between NO sensing and cyclic-di-GMP turnover and thereby regulation of cellulose production and biofilm formation or dispersal (Iyer *et al*, 2003). Curli fimbriae are produced by many species of the Enterobacteriaceae and together with cellulose constitute the extracellular matrix components required for biofilm formation (Wang *et al*, 2006). Curli fimbriae and cellulose have distinct roles on bacterial cell organisation, virulence and transmission, adherence and invasion of epithelial cells (Bian *et al*, 2001; Dibb-Fuller *et al*, 1999; Gophna *et al*, 2001; Sukupolvi *et al*, 1997). A mechanism by which bacteria could detach from the biofilm phenotype has been suggested by *P. aeruginosa* microarray studies, which showed that after exposure to NO, genes involved in adherence were down-regulated (Firoved *et al*, 2004). It would be useful to perform microarray studies on the coliforms and *Salmonella* investigated in this present study.

In this thesis, watercress leaves were shown to contain no *Salmonella* naturally, and between 10-20 *E. coli* per g of watercress, however despite this, outbreaks of *Salmonella* suggest that this pathogen is an important cause of GI illness. The recall of watercress in the UK in January 2007, due to contamination by *Salmonella*, clarifies the importance of this pathogen on the phylloplane (Food Safety Authority of Ireland, FSAI Press release). Studies have shown that after colonization of plants, *Salmonella* becomes inseparable from the surface and attaches with a similar strength to *Pantoea agglomerans* (a plant associated bacteria), so the reason for a lower recovery of *Salmonella* may be due to its greater attachment strength. *Salmonella* has been shown to attach to alfalfa sprouts 10- to 1000-fold more than *E. coli* (Barak *et al.*, 2002). Or it may also be that irrigation water and soil, animal faeces etc. may be less contaminated with *Salmonella* initially. Studies have shown that unlike most plant associated bacteria, such as *E. coli, Salmonella enterica* is unable to assimilate sucrose (Lin *et al.*, 1996).

As described for the natural microorganisms on the phylloplane, inoculated GFP-labelled *Salmonella* Thompson was shown to locate in the grooves, crevices and stoma of the leaf matrix, these areas potentially providing structural or physiological niches. Stomata are more likely to exude nutrients so this may be a reason for the active migration of *Salmonella* to this region. A study conducted on senescent leaf tissue that were prone to increased leakage of nutrients, also showed this phenomenon. Large concentrations of individual and aggregated cells were found on the senescent leaf areas and lesions compared to intact leaf regions, the pathogen was also shown to grow more under these conditions (Brandl and Mandrell, 2002). In regions of epidermal cell damage or nutrient leakage, *Salmonella* spp. have been reported to interact with cuticle waxes or sterols; likewise, *Pseudomonas* spp. have been shown to utilize bacterial appendages such as flagella for leaf interactions when the cuticle membrane was intact (Singh *et al.*, 2004). In this study, *Salmonella* spp. could sometimes be seen to locate with the naturally occurring aggregates of bacteria on the phylloplane to enhance survival. The main advantage of using GFP-labelled pathogens is that *in-situ* observations can be deduced, and there is a lower likelihood of loss of information via culture techniques. Accordingly, quantitative analysis was attempted, however this was shown to be difficult and greatly variable in each microscopy field of view; ranging from no visible *Salmonella* to levels that were too high to count and also aggregates in which the individual bacteria were difficult to distinguish and count. Comparisons were difficult to make and results became inaccurate because the sample size was too large; the 25 g watercress samples with the 225 ml diluent resulted in a greater likelihood of leaves overlapping etc. Smaller size samples or investigations using single leaves could potentially improve this.

To date, the effects of pulsification or stomaching had only been investigated on natural epiphytes (Wu *et al*, 2003; Kang *et al*, 2001; Fung *et al*, 1998). In this study, the effect of a hand wash, pulsification or stomaching on numbers of recovered *Salmonella* Thompson was compared. When the initial inoculum concentration was 8-log, approximately 7.5-log, or 32 percent of the pathogen was established to attach to the phylloplane. Pulsification was shown to result in an approximate 2-log increase in recovery of the pathogen compared

to the control samples. However, a total recovery of pathogens from the phylloplane was not apparent; 1-log *Salmonella* still remained strongly attached. On average, the efficiency of the Pulsifier in detaching pathogens, such as *Salmonella* was shown to be 68 percent; 32 percent surviving and remaining very tightly attached to the phylloplane. Although the reduction in levels of the pathogen is great after pulsification, refinements in the mechanics of the Pulsifier may improve this. The 32 percent of the pathogen population that is strongly attaching to the phylloplane may be the population that is continuing to produce GI illness; refinements in the mechanics of the Pulsifier may improve this. Compared to the Stomacher, the increased efficiency of pathogen detachment using the Pulsifier suggests that this should be the preferred sample preparation method when assaying fresh produce for the presence and enumeration of pathogens. As such, it proves an ideal tool for both research needs and routine food microbiology analyses.

The results obtained using LSCM showed that after a 30 minute incubation period the majority of *Salmonella* that had been inoculated onto the surface of the watercress phylloplane had not had sufficient time to migrate to areas of stomata. It was apparent that those few *Salmonella* that had sufficient time to migrate to the stomal regions began to internalise during the 30 minute incubation, by 2 μm. Approximately 1 percent of the inoculated *Salmonella* was shown to be internalized in this way; if the incubation period was increased further, such as to 12 hours, 27 percent of the *Salmonella* may be internalized. As stated previously, 12 hours is modest when considering that salads may be growing in the field, in contact with *Salmonella* for many weeks. The results presented suggest that *Salmonella* internalization is a contributing factor to the spread of foodborne disease; and this is the first study to show this occurring within 30 minutes of incubation on the watercress phylloplane. Investigations by Lapidot *et al* (2006) have also suggested that the ability of *Salmonella* to penetrate plant tissue was possibly a more important factor in the protection of *Salmonella* from environmental stresses and disinfection, compared to processes such as biofilm formation. Biofilm formation was shown not to occur initially; at least a week was required for this. Recently, a study by Kroupitski *et al.*, in 2009 has shown that the internalization of *Salmonella enterica* in lettuce leaves is induced by light and it involves chemotaxis and penetration through open stomata (Kroupitski *et al.*, 2009).

When 10-log *Salmonella* were inoculated onto the phylloplane 9.5-log attachment was shown to occur. Post-pulsification, 32% remained on the phylloplane, suggesting that these cells were attached very strongly. Ozone treatment was shown to reduce levels of *Salmonella* by 2-log, whereas a 50 ppm hypochlorite treatment or a 20 ppm hypochlorite treatment reduced these levels by approximately 7-log. This suggests that hypochlorite is a more efficient disinfectant than ozone for *Salmonella* treatment compared to *E. coli* treatment; however, this may not be conclusive, since the concentration of ozone being incorporated into the bore-hole water was greatly variable. A similar effect to that described earlier for coliforms and *E. coli* was apparent for *Salmonella*; after ozone treatment, approximately 2-log of the strongly attached pathogens that initially required pulsification for detachment are not detectable; this may be due to a loosening of these cells by the disinfection; or it may be that these cells have become even more tightly attached and can no longer be removed by pulsification. In general, the main problem of disinfection with ozone is due to the difficulties presented in maintaining constant levels in solution. Inoculation studies performed using *Salmonella* Thompson show that the hypochlorite concentration used by the food industries could potentially be reduced from 90 ppm to 20 ppm, still having similar 2-3 log reductions in pathogen loads on the watercress phylloplane. This would be very useful since it would reduce the excess production of trihalomethanes which have been shown to have long- and short-term health effects.

NO was shown not to effect the release of inoculated *Salmonella* Thompson on the watercress phylloplane. This was quite unusual because *Salmonella* produces both cellulose and curli fibres and has also been shown to contain GGDEF and EAL domains linked with NO and biofilm dispersal. However, other studies have shown that *Salmonella* species, after initial inoculation, utilise other mechanisms for protection from the environment and stresses, rather than production of biofilm. It is likely that the biofilm is not formed after the 12 hour incubation time for *Salmonella* and, therefore, NO can not elicit its effects on biofilm dispersal; studies have reported that at least a one week incubation period is required for the formation of biofilm by *Salmonella* (Lapidot *et al*, 2006). The results suggest that after a 12 hour incubation period, other factors are more

important in the survival of *Salmonella* on the phylloplane than biofilm formation. The EDIC/EF images show that most of the *Salmonella* was located in clusters or grooves on the matrix after a 12 hour incubation, this physical entrapment may be more important in *Salmonella* survival compared to the natural coliforms that may have been present on the phylloplane for long periods of time leading to the formation of biofilm. The internalization of *Salmonella* into the phylloplane after 30 minute incubation suggests that this feature may be more important in initial survival of *Salmonella* on the phylloplane. The results presented after NO treatment suggest that after 12 hour incubation, *Salmonella* recruit these other mechanisms for survival rather than integrating into existing biofilms (at least biofilms containing coliforms) on the phylloplane, this is because if a major reason for survival was integration into existing biofilms that contain high quantities of coliforms, then the *Salmonella* would have been likely to have been dispersed after NO treatment at the same time as the coliforms.

The increase in dispersal of naturally occurring bacteria has major implications on possible pathogens that may become entrapped within or attached to these biofilms. Further work should include greater study of *Salmonella* to determine if a longer incubation period results in the NO effect observed for coliforms; also if *Salmonella* integrates with biofilms consisting of coliforms; and the importance of internalization into the phylloplane. NO has been shown to result in the closure of stomata (Desikan *et al*, 2002; Garcia-Mata and Lamattina, 2002), this would entrap the internalised *Salmonella* and could suggest why *Salmonella* are not recovered while coliforms are. However, this effect would be useful for those pathogens or epiphytes that do not internalise as rapidly, since this would result in reduced likelihood of future immediate internalization. Of note, NO has been shown to modulate plant hormonal, defence and wound healing processes (Paris *et al*, 2007) that may contribute to the elimination of pathogens on fresh produce phylloplanes.

Overall, this thesis suggests that EDIC/EF microscopy is the most efficient mechanism for observations of epiphytes and pathogens in relation to the phyllosphere. Refinements in the protocols followed for the observation of leaves post-treatment, such as the use of smaller samples of leaves for treatment, would result in lower biological variations and

direct qualitative and quantitative comparisons should be possible using the EDIC/EF microscope. These microorganisms, including coliforms, *E. coli* and *Salmonella*, locate to structural or physiological niches, such as cell margins or stoma for protection from the environment or disinfectant procedures. *Salmonella* internalization to 2 μm depth, through stomata on the phylloplane after 30 minute incubation can be visualised, as shown by LSCM, which would protect this pathogen. The Pulsifier is more efficient than the Stomacher in the recovery of coliforms and *Salmonella* off the watercress phylloplane and for the integrity of the phylloplane. The optimal working conditions of the Pulsifier and Stomacher for a 25 g watercress sample with 225 ml diluent is 30 seconds, this would need to be investigated for any specific food samples being utilized. Air should be manually removed prior to pulsification for optimal phylloplane integrity and no significant difference in numbers of coliforms recovered; either way the level of air incorporated into treatment should be the same for different samples.

Food industries could utilize NO in their decontamination processes; nanomolar non-toxic concentrations of SNP added to the water-wash would lead to enhanced recovery off the leaf matrices, the rate of water turnover could be increased to allow for a constant supply of NO and for the constant removal of the pathogens that are now in suspension. The used water could then be decontaminated separately (i.e. by use of hypochlorite or UV irradiation), prior to re-contact with fresh leaf samples. NO could also be used as a molecular tool in combination with the Pulsifier. Results here have shown that SNP treatment leads to a synergistic effect when used with the Pulsifier on recovery of microorganisms in the phyllosphere; pulsification can be combined with SNP for enhanced recovery by this mechanical tool. SNP has the potential to be applied at the field, where along with the effects it has on dispersal it would be advantageous with the other effects it has on the phyllosphere as described through this study.

Overall, the use of NO as a means for detachment of Enterobacteriaceae can be seen to be advantageous, since, at present food industries are obtaining only between 1- and 2-log reductions in coliform levels post-hypochlorite treatment. The results in this study show that 3-log kills can be obtained combined with NO. Furthermore, chlorine treatment can be

bypassed entirely due to the approximate 3.6-log recovery of microorganisms from the phylloplane. Pulsification could be utilized alone to result in a 3.2-log recovery, or it could be combined with NO treatment for a 3.9-log coliform recovery off the phylloplane.

If time were available, future work involving the *in-situ* use of GFP-labelled bacteria would be undertaken; this would enable visualisation of pathogens directly on the surface of leaves and would enable study of the effect of all of the processes discussed in this project to attempt to clarify how these pathogens may be surviving on these matrices. Another potentially important use of GFP-marked bacteria is in the detection of pathogenic strains that are viable but non-culturable, i.e. they remain infectious but can no longer be cultured by conventional methods. Bacteria may enter this state, due to environmental stresses imposed within the food industry production chain. However, strains that are marked with GFP will be detectable no matter what state they are in, since bacteria that express the plasmid stably would constitutively fluoresce (Park, 2002; Cho and Kim, 1999). We can establish if the pathogens are attaching to the leaf surface directly or if they are attaching to naturally occurring bacteria to aid survival. Disinfection studies can then be performed to determine the direct effect of treatment on the cells.

Future Work

Overall, further work can be split into four Sections:

The further development of molecular techniques for the *in-situ* detection and tracking of pathogens on salad leaves. This would eliminate the problem faced by not detecting any possible viable but non-culturable species. This work is being done in a PhD study specifically on the Development of Molecular Techniques for the in-situ detection of pathogens on salad leaves.

Refinement of the mechanical mechanisms of the Pulsifier principle to potentially reduce the volume of water required for washing each kilogram of leaf, for the microbial release agent to be enlarged to that at the industrial scale; and efficiency of bacterial extraction and/or phylloplane integrity improved. This process could then be used in place of

chlorine disinfection. This work is currently being done in a collaboration of the School of Engineering Sciences, Research Industry for Science and Vitacress Salads.

Further use of the Pulsifier to characterize the physical attachment forces of various species on the phylloplane, investigate duration of treatment and temporal release of different species. These can then be characterized; for example, do they produce curli or other attachment factors. Can an intervention step be introduced to nullify these attachment forces.

Further investigations and refinements of the physiological effect of NO on aggregates of a range of micro-organisms or biofilms on the phylloplane. This would improve understanding of biofilms on the phylloplane and could potentially be utilised as a molecular signalling molecule for the release of pathogens and spoilage bacteria pre and/or post-harvest.

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