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FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Investigating a role for nitric oxide in the control of biofilm and zoonotic pathogen colonisation of the spinach phylloplane

by

Nicola Gibbins

Thesis submitted for the degree of

Doctor of Philosophy

April 2013

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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INVESTIGATING A ROLE FOR NITRIC OXIDE IN THE CONTROL OF BIOFILM AND ZOONOTIC PATHOGEN COLONISATION OF THE SPINACH PHYLLOPLANE

by Nicola Gibbins

Outbreaks of food poisoning associated with zoonotic pathogen contamination of RTE fresh products such as leafy salads are on the increase and new strategies for the control of pathogen contamination are required. *Salmonella* spp. are of particular concern as they remain the main cause of fresh produce associated food poisoning outbreaks in the U.S. and Europe.

Efficient colonisation of spinach leaves by *S.* Thompson and *S.* Typhimurium, and the formation of bacterial biofilms, was demonstrated using EDIC/EF microscopy. The novel biofilm dispersal agent, nitric oxide, did not induce detachment of *Salmonella* spp from the phylloplane but did influence attachment and colonisation of spinach leaves by *Salmonella* spp.

Factors influencing the attachment to and colonisation of surfaces by *S.* Typhimurium, (temperature and flagellar expression) were investigated and found to influence, but not be essential for surface colonisation and biofilm formation. Nitric oxide treatment also did not induce dispersal of *S.* Typhimurium biofilms in an abiotic surface model system. However, a proteomic investigation demonstrated that nitric oxide signaling is active in *S.* Typhimurium and that proteins involved in signaling and in energy production consistent with existence outside of a biofilm state were up-regulated.

Nitric oxide treatment was also investigated for its potential to induce biofilm dispersal for indigenous phylloplane populations for the improvement of product quality and was not found to induce significant removal of indigenous bacteria from leaves.

Together these investigations demonstrate that there remains much more to be understood about the interactions of zoonotic pathogens and indigenous phylloplane populations with fresh produce surfaces for development of novel decontamination strategies such as the use of nitric oxide for biofilm dispersal.

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DECLARATION OF AUTHORSHIP

I, Nicola Jayne Gibbins, declare that the thesis entitled

INVESTIGATING A ROLE FOR NITRIC OXIDE IN THE CONTROL OF BIOFILM AND ZOONOTIC PATHOGEN COLONISATION OF THE SPINACH PHYLLOPLANE

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;

- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

- 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;

- none of this work has been published before submission

Signed: ……..

Dated: ……

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LIST OF ABBREVIATIONS

AI-2	Autoinducer 2
ATCC	American type culture collection
c-di-GMP	Bacterial cyclic di-GMP (bis-(3’ -5’)-cyclic dimeric guanosine monophosphate)
CAM	Calmodulin
carb	Carbenicillin
CFU	Colony forming units
cGMP	Cyclic guanosine monophosphate
CSLM	Confocal scanning laser microscopy
DAEC	Diffusely adherent *Escherichia coli*
DGC	Di guanylate cyclase
DIC	Differential Interference Contrast
EAL	The EAL domain amino acid sequence Glutamic acid-Alanine-Leucine
E. coli	*Escherichia coli*
EDIC	Episcopic differential interference contrast
EDRF	Endothelial derived relaxing factor
EF	Epifluorescence
EHEC	Enterohemorrhagic *Escherichia coli*
eNOS	Endothelial nitric oxide synthase
ESEM	Environmental scanning electron microscopy
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
GAP	Good agricultural practice
GFP	Green fluorescent protein
GGDEF	The GGDEF domain amino acid sequence Glycine-Glycine-Aspartic acid-Glutamic acid-Phenylalanine
GMP	Good manufacturing practice
GTP	Guanosine triphosphate
HC	Haemorrhagic colitis

HPA	Health Protection Agency
HUS	Haemolytic uremic syndrome
H-NOX	Haem nitric oxide/oxygen
iNOS	Macrophage inducible nitric oxide synthase
kan	Kanamycin
LB	Luria-Bertani
$LB_{1/5}$	1/5 strength Luria-Bertani broth
LOD	Limit of detection
LWD	Long working distance
MAP	Modified atmosphere packaging
NCTC	National collection of type cultures
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitric oxide reductase
OD	Optical density
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
Pfs	methylthioadenoside/s adenosylhomocysteine nucleosidase
R2A	Reasoner's 2A agar
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTE	Ready-to-eat
sGC	Soluble guanylate synthase
S. Typhimurium	*Salmonella enterica* subspecies *enterica,* serovar Typhimurium
S. Thompson	*Salmonella enterica* subspecies *enterica,* serovar Thompson
SEM	Scanning electron microscopy
sdH_2O	Sterile distilled water
SNP	Sodium nitroprusside
TEAB	Triethylammonium bicarbonate

TSB	Tryptone soya broth
VRBA	Violet red bile agar
WHO	World Health Organisation
WT	Wild type

GLOSSARY OF TERMS

Aggregate	Collection of bacterial cells next to one another
Biofilm	Surface attached polymicrobal community encased in a protective EPS matrix
Commensal	An organism that shares nutrients with another, without affecting the growth or fitness of the other organism
Enterobacteriaceae	Taxonomic description of a large family of rod-shaped Gram negative bacteria that reside in the intestines
Epiphyte	A bacterial inhabitant of the leaf surface
Episcopic	'From above'
Epidermal cell (leaf)	Plant cells at the surface layer of leaves
Exopolymeric substance	Components of the extracellular matrix secreted by bacteria within a biofilm, including polysaccharides, nucleic acids, enzymes, lectins and proteins.
Gastroenteritis	Medical description of inflammation of the small intestine caused by bacterial or viral infection characterised by diarrhoea, vomiting and fever
Listeriosis	Description of disease caused by infection with Listeria
Macrocolony	Description often used for large developed microcolonies within a bacterial biofilm
Microcolony	Structural feature of a bacterial biofilm containing a closely associated sub-population of biofilm cells encased in a protective EPS matrix
Pathogen	An organisms (usually microbial) capable of causing disease
Phylloplane	The leaf surface
Phyllosphere	The collective habitat of leaf surfaces

Planktonic	Bacteria existing as individual independent single cells, usually in solution
Ready-to-eat	Food products that are intended for consumption directly as purchased, with no further processing
Rhizosphere	The immediate area surrounding plant roots
Salmonellosis	Description of disease caused by infection with *Salmonella*
Sessile	Fixed in one place; immobile
Symbiont	An organism living in symbiosis with another
Symbiosis	The mutually beneficial interaction of two or more orgnaisms
Zoonosis	Description of disease caused by zoonotic pathogen infection (plural; zoonoses)
Zoonotic pathogen	An microorganism that can be transmitted between animals and humans

CHAPTER 1

General Introduction

1.1 Overview: The need for microbial control at fresh produce surfaces

The availability of year-round fresh fruits and vegetables on the supermarket shelves of the developed world has become an everyday expectation. In the UK, the US and Europe, the current emphasis on promoting a healthy diet includes recommended guidelines for consumption of a minimum of 5 portions of fresh fruit and vegetables per day (Heaton and Jones 2008).

In order for the public to fulfil these dietary requirements, it is important to ensure a sufficient supply of healthy, convenient, fresh fruits and vegetables in the marketplace. Fresh products are increasingly available as ready-to-eat (RTE) convenience foods, intended to make healthy eating an easy option in the busy lifestyles of the population (FAO/WHO 2007). RTE Fresh produce has recently been highlighted as a significant vector for the transmission of human pathogenic contaminants to the food chain. As RTE products are supplied minimally processed and ready to eat with no further processing, washing or cooking after purchase, it is essential to ensure they are free from pathogenic contaminants at the point of sale. As pathogens would not be removed or killed by washing or cooking in the kitchen, RTE products carry a high risk for transmission of pathogens to humans. Indeed, outbreaks of foodborne zoonoses attributed to fresh produce are on the increase (EFSA 2012) which is evidence that current control and decontamination strategies used by the fresh produce industry are insufficient.

A number of significant outbreaks of food poisoning in recent years have been caused by ready-to-eat leafy salads, with *Salmonella* species and shiga toxin producing *Escherichia coli* most often identified as the cause (see Table 1). *Salmonella* spp. and *E. coli* are zoonotic pathogens (pathogens that can be transferred between humans and animals) that can enter the food chain from a number of pre- or post-harvest routes of contamination (see Section 1.2.1.2), and it is their persistence throughout the food production process that makes these pathogens a public health risk (Brandl 2006a). It is thought that the adaption of pathogens such as *Salmonella* and *E. coli* for survival under environmental conditions, where mechanisms for protection from external environmental stresses are well established, confers protection from current

wash processes and decontamination strategies for RTE leafy salads allowing their persistence throughout food production (Carmichael et al 1999).

Leaf surfaces are routinely home to large and complex microbial communities often present in matrix enclosed surface attached structures, or biofilms (Morris and Monier 2003). Extensive studies of biofilms at abiotic surfaces have demonstrated their persistence in the environment (Costerton et al 1987), however, the majority of the study of bacteria at fresh produce surfaces such as salad leaves has been carried out for plant pathogens, or for the assessment of microbial ecology of indigenous bacterial populations, rather than for the investigation of human pathogens (Andrews and Harris 2000, Brandl 2006a). The discovery of the persistence of human pathogens at the leaf surface, or phylloplane, is a relatively recent one, and the significance of the biofilm mode of growth for human pathogens at phylloplanes of fresh leafy produce is only beginning to be appreciated. This project considers the biofilm mode of growth at the phylloplane as a mechanism for the survival and persistence of *Salmonella* spp. associated with leafy salads, and investigates a novel biofilm dispersal agent, nitric oxide, for the control of phylloplane biofilms during the production of leafy salads to improve both product safety and product quality.

1.2 Microbial contamination of leafy salads

1.2.1 Zoonotic pathogens

Fresh produce is a significant vector for zoonotic pathogen entry to the food chain, and an increasingly important cause of food poisoning outbreaks (EFSA 2012, Lynch et al 2009, Sivapalasingam et al 2004). As populations of the developed world are turning away from fatty fast foods and becoming more aware of the importance of a healthy and nutritious diet, the demand for food products that are healthy and nutritious, yet convenient enough to fit in with busy lifestyles, is high (Harris and Shiptsova 2007). Pre-prepared RTE fruit and vegetable products such as leafy salads are one such popular food product which helps to meet this market demand, and serious outbreaks of food poisoning have been associated with their consumption (see Table 1). Leafy salads alone were estimated to be worth £500m to the UK economy in the 52 weeks to

August 2012, and the sector is still growing (Neilsen market data, unpublished), therefore not only is product safety and quality important for human health, it is also important to the health of the UK economy.

RTE leafy salads are intended for consumption with no washing or cooking after purchase; steps that, for other non-RTE foods, ensure pathogens are removed or killed before eating. Zoonotic pathogen contamination of RTE leafy salads with bacteria such as *E. coli* and *Salmonella*, which can have an infective dose of as little as 10 cells (USFDA 2012b) (see Section 1.2.1.1), is therefore a high risk to the consumer as any contaminating pathogen would be ingested and have the potential to cause food poisoning. With the growing consumption of RTE products increasing the potential for human exposure to such contaminating pathogens, ensuring microbiological safety of RTE leafy salads is essential for reducing outbreaks of food poisoning and for protecting public health. In order to better understand the persistence of zoonotic pathogens throughout the food production process, and ultimately to aid the development of novel intervention strategies for use against pathogen contamination of leafy salads, it is necessary to consider how bacteria contaminate fresh produce and how they exist at the phylloplane.

1.2.1.1 Pathogenic contaminants of leafy salads

A variety of pathogens have been associated with outbreaks of food poisoning from fresh produce (Heaton and Jones 2008). Commonly encountered zoonotic pathogens associated with leafy salads include *Salmonella* spp. and *E. coli*. *Listeria monocytogenes*, *Campylobacter jejuni*, *Shigella sonnei* and various viruses have also been linked (see Table 1).

Salmonella are Gram-negative rod-shaped cocci of the family *Enterobacteriacae* with a low infective dose in humans (15-20 colony forming units) and an incubation period of 1 to 2 days (Blaser and Newman 1982, USFDA 2012b). When ingested they can invade mucosal cells of the small intestine and cause gastroententeritis characterised by diarrhoea, abdominal cramps, fever and vomiting. Illness is usually self-limiting, lasting up to one week, although complications can occur including bacteraemia, leading to

septic shock, and reactive arthritis in the long-term (USFDA 2012b). The genus *Salmonella* is divided into two species *S. enterica* (the type-species of the genus) and *S. bongori*. *S. enterica* is further divided into six subspecies; *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (denoted I, II, III, IV, V and VI, respectively) (Brenner et al 2000). Members of species *S. enterica* subsp. *enterica*, which cause the majority (99%) of *Salmonella* infections, are further classified into serotypes usually named after the place from which they were first isolated. *S. enterica* subsp. *enterica* contains 1,454 of the 2,463 *Salmonella* serotypes that exist according to the Kauffmann-White classification scheme (Brenner et al 2000, Le Minor and Popoff 1987). Serotypes linked to outbreaks of food poisoning associated with lettuce products include Typhimurium, Newport, Thompson and Senftenberg (see Table 1).

Members of the genus *Escherichia* are also Gram-negative rod-shaped cocci of the family *Enterobacteriaceae* and are ubiquitous in the gut microflora of animals and birds (Wells et al 1991). Nomenclature and classification of *E.coli* bacteria has developed as understanding of the many variants has grown and is complex for historical reasons, with strains often described on the basis of their pathogenesis. Classifications of *E.coli* are enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EAIC) and diffusely adherent *E.coli* (DAEC). Strains of *E. coli* are identified on the basis of their O (membrane) and H (flagellar) antigens. Of the many strains of *E. coli, E. coli* O157:H7 has been identified as the cause of a number of lettuce-associated food poisoning outbreaks, and *E. coli* 0145 has also been linked (see Table 1).

E.coli O157:H7 was first identified as a foodborne pathogen in 1982 from an outbreak involving contamination of hamburgers in the US (Riley et al 1983, Wells et al 1983) and is a member of the enterohemorrhagic *E.coli* (EHEC). It has a low infective dose (10-100 CFU) and causes gastroenteritis characterised by bloody diarrhoea, abdominal cramps and vomiting which can progress to haemorrhagic colitis (HC) and/or haemolytic uremic syndrome (HUS) in severe cases (WHO 2005). HC causes severe abdominal cramps, haemorrhage of the mucosa of the colon and bloody diarrhoea (Riley 1987), whilst HUS causes thrombocytopenia, haemolytic anaemia and renal failure which may be fatal (Karmali 1989). Approximately 10% of *E.coli* O157:H7 cases

progress to HUS and young children and the elderly are particularly at risk (WHO 2005). This progression is thought to be due in part to the production of a shiga-like toxin (SLT), also known as the verotoxins (VT), which act primarily at globotriasylceramide receptors of the kidney (Nataro and Kaper 1998). Strains that produce SLT or VT are known as STEC (shiga-toxin producing *E. coli*) or VTEC (verotoxin producing *E. coli*), respectively. *E. coli* O157:H7 and *E. coli* O145 associated with outbreaks of food poisoning from lettuce are examples of VTEC strains which can cause kidney failure and death, highlighting the significance of lettuce as a vector for potentially fatal infection in humans.

Other enteric bacteria that have been associated with outbreaks of food poisoning from lettuce include *Listeria monocytogenes* and *Campylobacter jejuni* (Heaton and Jones 2008, WHO 1998). Whilst these are not often attributed to outbreaks of food poisoning directly from lettuce, and are more often associated with cross contamination from infected meat products in the kitchen (Little et al 2007, Luber et al 2006), it is possible that they account for some lettuce-associated outbreaks of unknown cause (Danis et al 2009). In addition, *L. monocytogenes* and *C. jejuni* have been isolated from leafy produce in the marketplace and have led to the recall of lettuce products (Kumar et al 2001, USFDA 2012a, WHO 1998).

L. monocytogenes is a Gram-positive rod shaped bacterium ubiquitous in the environment which can cause meningitis, septicaemia and can affect the unborn foetus (Francis et al 1999). *C. jejuni* is a Gram-negative spirochete resident of the gut of wild animals and birds and is a common waterborne pathogen, which can cause symptoms ranging from mild gastroenteritis to bacteraemia, neurological complications and reactive arthritis (Francis et al 1999, Gardner et al 2011, WHO 2000). Recent fresh produce outbreaks associated with these pathogens have caused serious illness. In one such outbreak attributed to the contamination of celery with *L. monocytogenes*, 5 deaths resulted from just 10 reported cases in Texas, US (Falkenstein 2010). In a separate outbreak in 2011, attributed to the contamination of cantaloupe melon with *L. monocytogenes*, 147 people across the US were infected, of which 33 died and one miscarriage was reported (CDC 2011a), demonstrating the severity of illness associated with *Listeriosis*. For *C. jejuni*, an outbreak in 2008,

attributed to the contamination of locally grown fresh peas, infected 30 people in Alaska, of which 5 were hospitalised (Gardner et al 2011). These outbreaks demonstrate the importance of fresh produce as a vector for the entry of zoonotic pathogens to the food chain, and their serious implications for public health.

1.2.1.2 Routes of contamination for zoonotic pathogen entry to leafy salads

Contamination of leafy salads with zoonotic pathogens can occur pre- or post-harvest. Figure 1 provides a pictorial overview of an example production process for a bag of RTE leafy salad, from sowing seeds through to the final product on the supermarket shelves.

The majority of leafy salads are grown in open field environments (Figure 1 A & B). Contamination of field grown crops can occur from irrigation of soil and crops with contaminated ground water (Solomon et al 2002a), from fertilisation of soils with contaminated manures (Nicholson et al 2000), from the use of poorly composted organic wastes as fertilisers (Avery et al 2005), from contaminated surface water run-off from neighbouring fields (Solomon et al 2002b), from rain splash from the soil (Lindow 2006), from direct faecal contamination from wild animals and birds (Gardner et al 2011, Heaton and Jones 2008, Jay et al 2007) and even from contamination by vectors such as frogs and slugs (Sproston et al 2006) which may carry pathogens from the soil to the leaf surface. Exposure of crops to zoonotic pathogens in the field can be minimised through hazard identification and risk management, for example, by identifying where contaminated run-off from neighbouring fields could enter farmland and providing additional drainage channels to divert potentially contaminated ground water, but it is unlikely that exposure to zoonotic pathogens from all sources can be eliminated entirely (FAO/WHO 2005). Direct defecation by wild birds onto open fields, and rain splash from the soil, are examples of where controlling exposure to zoonotic pathogens may not be possible.

Figure 1 A pictorial overview of the leafy salad production process

Post-harvest routes of contamination also exist. These are usually well controlled with good manufacturing and hygiene practices (FAO/WHO 2007), however, as shown in Figure 1 (C to G) there are many points throughout the post-harvest production of leafy salads where pathogens may be introduced. At harvest, soil particles may be disturbed and transferred to leaves, and as leaves are often packed into crates by hand, contact with contaminated farm workers may introduce human pathogenic bacteria to leaves (Figure 1 C). In addition, when harvesting under dry conditions leaves may be sprayed with water to prevent wilting (Figure 1 D). This water is supplied from tanks in the harvester and may introduce pathogens if tanks are not sterilised properly or if water is contaminated with zoonotic pathogens prior to spraying. Vacuum cooling of leaves within 1 hour of harvest is carried out to preserve leaves and prevent microbial growth on leaves (Figure 1 E). Leaves are then stored in humidified, refrigerated storage to preserve leaves prior to washing and packing (Figure 1 F). Humidification of air in storage rooms is usually supplied using potable water, which is not sterile, and therefore may harbour waterborne pathogens such as *Legionella pneumophila* (Giao et

al 2011, Keevil 2002), and it is therefore recommended that water used for humidification and air handling systems are routinely monitored to minimise the risk of these introducing zoonotic pathogens to fresh produce (FAO/WHO 2007).

Under carefully controlled growing and production conditions, contamination with zoonotic pathogens is minimised, however, in the modern global production and distribution of food products, crop growing and food production conditions cannot be standardised, controlled and monitored worldwide (FAO/WHO 2008). Poor farming practices, poor storage conditions, poor worker hygiene and cross contamination from other infected produce can introduce zoonotic pathogens to leafy salad products for distribution around the world. Global distribution of food products means that foreign pathogens can be introduced to areas of little or no underlying immunity in the human population resulting in food poisoning outbreaks, and once an outbreak occurs it can be difficult to identify the source in time for successful intervention and control (Taban and Halkman 2011). The significant number of food poisoning outbreaks that have arisen from leafy salad products (Table 1) demonstrates that current control methods are unsuccessful in eliminating zoonotic pathogen contamination of leafy salads.

1.2.1.3 Lettuce associated food poisoning outbreaks

Leafy salads are a significant vector for the entry of zoonotic pathogens to the food chain, with *E. coli* and *Salmonella* spp. most often associated with lettuce products. Table 1 lists a number of outbreaks of foodborne zoonoses associated with lettuce products. Of particular note were the following incidences.

An outbreak of *E. coli* O157 associated with Californian spinach in September 2006, caused 199 cases, 102 hospitalisations and 3 deaths (CDC 2006). The source of this outbreak was found to be faecal contamination of surface water by cattle and wild pigs, and the subsequent use of this water for irrigation (Jay et al 2007). This outbreak highlights the difficulty in controlling pre-harvest contamination of field grown crops. In a separate outbreak in 2010, contamination of Romaine lettuce with *E. coli* O145 caused 30 cases and 13 hospitalisations (CDC 2010). This outbreak rapidly spread across the US and the source was identified as a single processing factory from which

produce was distributed across the United States, highlighting the difficulty in controlling food poisoning outbreaks with food distribution across large geographical areas. For *Salmonella*, an outbreak in 2003 associated with the consumption of lettuce caused 361 cases of infection, and may have contributed to the death of one patient. The cause of this outbreak was *Salmonella* Typhimurium DT104, a multi-drug resistant strain (Horby et al 2003), highlighting lettuce as a possible vector for the transfer of antibiotic resistant zoonotic pathogens into the food chain.

Whilst the most commonly reported food poisoning outbreaks associated with lettuce products are *Salmonella* spp. and *E. coli*, other pathogens including *Listeria monocytogenes*, *Yersinia* species, *Campylobacter jejuni, Shigella sonnei* and viruses such as norovirus have also been linked (Beuchat 2002, Heaton and Jones 2008, Kapperud et al 1995, Olaimat and Holley 2012, Szabo et al 2000). It has been suggested that contamination of fresh produce, including leafy salads, with bacteria such as *Listeria* and *Campylobacter* species may significantly contribute to the numbers of food poisoning cases for which lettuce has been suspected but precise causes have not been established (Danis et al 2009, Szabo et al 2000) . *Listeria monocytogenes* is considered one of the most severe reportable foodborne pathogens due to the high death rate associated with cases of infection whilst *Campylobacter jejuni* remains the most commonly reported zoonotic pathogen of food origin in the EU (EFSA 2012).

In order for a pathogen to cause a food poisoning outbreak once associated with the leaf, it must be able to persist in association with the leaf throughout the production process and remain viable until the point of sale. A number of researchers have specifically considered the persistence of human enteric pathogens on leafy salad crops. Solomon *et al*, studied the contamination of lettuce with *E. coli* O157 and found that, when introduced to the plant by spray irrigation, 90% of the crop became contaminated and persisted at the phylloplane for up to 20 days (Solomon et al 2002a). Beuchat found contamination of lettuce with *E. coli* O157 to remain for 15 days post inoculation (Beuchat 1999). These studies demonstrate the ability of human pathogens to survive and persist outside of their mammalian hosts in a phylloplane environment. It has also been shown that not only can pathogens remain in the

phyllosphere following a contamination event, they can also proliferate. Using culture independent techniques, Brandl and Mandrell showed that *Salmonella* Thompson, Newport, Derby and Enteritidis can proliferate in the cilantro phyllosphere (Brandl and Mandrell 2002). This study also considered the interaction of *S.* Thompson with indigenous phylloplane bacteria and found that this bacterium formed aggregates and microcolonies at the phylloplane. Therefore, understanding the mechanisms by which bacteria exist at the phylloplane, and their interactions with existing bacterial inhabitants of leaf surfaces is key to developing novel methods for their decontamination (Aruscavage et al 2006).

Pathogen	Date	Food source (country of origin)	Location	Number of cases	Number of hospitalisations	Number of Deaths	Reference
***Salmonella* spp.**							
***S.* Seftenberg**	Jan 2007	Fresh basil (Israel)	UK	33	*	*	(Pezzoli et al 2007)
***S.* Ajiobo**	Jun 2006	Sandwiches containing salad leaves	UK	153	11	*	(Gillespie et al 2006)
***S.* Typhimurium**	May 2005	Lettuce (Spain)	Finland	56	*	*	(Takkinen et al 2005)
***S.* Newport**	Sep 2004	Lettuce	UK and Ireland	368	*	*	(Irvine et al 2009)
***S.* Thompson**	Nov 2004	Rocket	Norway	20	*	*	(Nygard et al 2008)
***S.* Typhimurium**	Aug 2000	Lettuce	UK	361	*	[1]	(Horby et al 2003)
***S.* Thompson**	Mar 1999	Cilantro	USA	41	3	*	(Campbell et al 2001)
***Escherichia* coli**							
***E. coli* O157:H7**	Nov 2012	Spinach	USA	33	13	0	(CDC 2012)
***E. coli* O157:H7**	Nov 2011	Romaine Lettuce	USA	60	30	0	(CDC 2011b)
***E. coli* O145**	May 2010	Romaine Lettuce	USA	30	12	0	(CDC 2010)
***E. coli* O157**	Sep 2007	Bagged lettuce (Netherlands)	Iceland, Netherlands	50	*	*	(Friesema et al 2008)
***E. coli* O157**	Aug 2005	Lettuce (Sweden)	Sweden	120	*	*	(Soderstrom et al 2005)
***E. coli* O157:H7**	Sep 2006	Spinach (California)	USA	199	102	3	(CDC 2006)

Pathogen	Date	Food source (country of origin)	Location	Number of cases	Number of hospitalisations	Number of Deaths	Reference
***Escherichia coli* (continued)**							
***E. coli* O157**	Aug 2005	Lettuce (Sweden)	Sweden	120	*	*	(Soderstrom et al 2005)
***E. coli* O157:H7**	Oct 2003	Spinach (California)	USA	16	*	2	(CDC 2006)
***E. coli* O157:H7**	May 1996	Lettuce (US)	USA	61	21	*	(Hilborn et al 1999)
***E. coli* O157:H7**	Jul 1995	Lettuce (US)	USA	40	13	0	(Ackers et al 1998)
***E. coli* O157:H7**	Nov 1995	Iceberg lettuce	Canada	21	*	0	(CCDR 1997)
Other microbial contaminants							
Campylobacter jejuni	2000	Lettuce	*	*	*	*	(Heaton and Jones 2008)
Shigella sonnei	Jun 1994	Iceberg lettuce	Norway [also Sweden and UK]	110 [data for Norway only]	*	*	(Kapperud et al 1995)
Calicivirus	2006	RTE salad	Finland	450	1	1	(EFSA 2007)
Hepatitis A	Mar 1988	Green salad	USA	202	40	[2]	(Rosenblum et al 1990)

*** data not available** **[] Illness may have contributed to death but other factors also present**

Table 1 Lettuce associated outbreaks of foodborne zoonosis

1.2.2 Indigenous phylloplane bacteria

Whilst the contamination of leafy salads with zoonotic pathogens is of great importance to public health, zoonotic pathogens are not the only bacterial inhabitants of leaf surfaces. Phylloplanes are routinely host to large and diverse microbial communities (Hirano and Upper 2000, Lindow and Leveau 2002), the presence and species composition of which may have a strong influence on how zoonotic pathogens interact with minimally processed foods such as salad leaves (Carmichael et al 1999). Bacterial colonisers of plant surfaces are known as epiphytes, and populations of epiphytes at the phylloplane may exist as commensals, symbionts, or plant pathogens (Beattie and Lindow 1995). The phylloplane habitat as a whole is known as the phyllosphere.

Characterisation studies of phyllosphere microbiology have shown that indigenous bacterial communities differ greatly in population size and species diversity which can be influenced by numerous factors including temperature, humidity, UV radiation and water and nutrient availability, as well as plant genotype, growing season, and age of leaf (Brandl and Amundson 2008, Hirano and Upper 1989, Hirano and Upper 2000, Lindow and Leveau 2002, Lindow and Brandl 2003, Yaun et al 2002). Table 2 (Hirano and Upper 2000) demonstrates the large diversity of bacterial inhabitants of leaf surfaces, as isolated from the phylloplanes of olive leaves and perennial rye.

For spinach leaves, no specific identification of whole indigenous phylloplane bacterial communities has been carried out, although in one study by Lopez-Velasco *et al*., community analysis of bacteria associated with minimally processed spinach during refrigeration using denaturing gradient gel electrophoresis (DGGE) has demonstrated high diversity of microbial phylloplane populations on spinach leaves at the genus level (Lopez-Velasco et al 2011). These researchers also identified culturable isolates from the spinach phylloplane that had the ability to affect the survival and growth of *E. coli* O157:H7, identities of which are given in Table 3 (Lopez-Velasco et al 2012). However, these identified isolates only represent a small proportion of the bacteria present at the spinach phylloplane. There remains much more to be discovered for the microbial ecology of spinach leaves, how bacterial populations interact with the

phylloplane, and how indigenous bacterial communities may affect the attachment of human pathogens to phylloplanes, which will have important implications for the targeting of novel decontamination strategies for leafy food products.

Plant and bacterial component or species	Relative abundance[c] (%)
Perennial rye	
Pseudomonas fluorescens	20.12
Xanthomonas campestris	19.64
"Coryneform bacteria"	8.37
Yellow chromogens	4.83
Flexibacter spp.	4.66
Listeria spp.	4.02
Pink chromogens	3.86
Staphylococcus saprophyticus	1.77
Other gram-negative rods	1.61
Klebsiella spp.	0.96
Acinetobacter spp.	0.96
Erwinia herbicola	0.80
Pseudomonas spp.	0.64
Staphylococcus spp.	0.64
Bacillus spp.	0.32
Micrococcus luteus	0.32
Orange chromogens	0.32
Unidentified isolates	26.57
Olive	
Pseudomonas syringae	51.0
Xanthomonas campestris	6.7
Erwinia herbicola	6.0
Acetobacter aceti	4.7
Gluconobacter oxydans	4.3
Pseudomonas fluorescens	3.9
Bacillus megaterium	3.8
Leuconostoc mesenteroides subsp. *dextranicum*	3.1
Lactobacillus plantarum	2.8
Curtobacterium plantarum	2.2
Micrococcus luteus	2.2
Arthrobacter globiformis	1.4
Klebsiella planticola	1.2
Streptococcus faecium	1.2
Clavibacter sp.	0.98
Micrococcus sp.	0.82
Serratia marcescens	0.81
Bacillus subtilis	0.57
Cellulomonas flavigena	0.4
Erwinia sp.	0.37
Zymomonas mobilis	0.3
Bacillus sp.	0.29
Alcaligenes faecalis	0.27
Erwinia carotovora	0.08
Pseudomonas aeruginosa	0.04

Table 2 Indigenous phylloplane bacteria of perennial rye and olive

(table from (Hirano and Upper 2000))

Identified bacteria (number of isolates)[O]	Strain number[P]	GeneBank accession number of closest match[Q]	% of similarity
Epiphytes that elicited presumptive growth inhibition of with E. coli O157:H7[R]			
Acinetobacter calcoaceticus (4)	KFS200725	FJ867364	96
Aeromonas encheleia (1)	KFS2007916	AJ458416	97
Arthrobacter spp. (1)	KFSISO88	GQ332346	97
Bacillus cereus (5)	KFSNO1C1A[a]	FJ763650	97
Bacillus pumilus (3)	KFSNO2A1B[a]	FJ263042	98
Bacillus spp.(1)	KFS2007301[a]	EU781520	97
Brevundimonas vesicularis (1)	KFSNO2A1A[a]	FJ999941	98
Curtobacterium herbarum (3)	KFSISO138B	AM410692	97
Erwinia persicina (24)	KFSORG1C4[a]	AJ937838	98
Erwinia rhapontici (2)	KFSORG1A3A	EU340562	98
Flavobacterium spp.(1)[T]	KFSNO2C3A[a]	AM110987	98
Frigobacterium spp. (1)	KFSNO2A4[a]	AF157479	98
Kaistia spp. (1)	KFSISO103	FJ006913	99
Microbacterium oleivorans (2)	KFSNO1G1[a]	EU71438	99
Microbacterium phyllospherae (2)	KFSISO37	NR025405	97
Paenibacillus polymyxa. (1)	KFSNO1H1[a]	FJ940900	86
Pantoea agglomerans (5)	KFSNO1E4[a]	EF050808	99
Pseudomonas fluorescens (2)	KFS2007178[a]	CP000094	97
Pseudomonas koreensis (8)	KFSISO85[a]	GQ368179	96
Pseudomonas rhodesiae (1)	KFS20071822	FJ462694	96
Pseudomonas spp. (12)	KFS2C3	FN547413	100
Rhodococcus spp. (1)	KFSISO31A	AY188941	97
Stenotrophomonas maltophilia (3)	KFSNO2G3B[a]	GU385870	97
Unclassified *γ-Proteobacteria* (6)	–	–	–
Epiphytes that elicited presumptive growth stimulation of E. coli O157:H7[S]			
Acidovorax konjaci (1)	KFSISO54	GQ260128	97
Brevibacillus brevis (1)	KFSISO142	EU931557	96
Flavobacterium spp. (5)[T]	KFSISO87	EF601822	100
Patulibacter spp.(1)	KFSISO110	AJ871305	100
Rhizobium spp. (1)	KFSISO80	FJ006911	98
Sphingomonas spp. (3)	KFSISO97	AF395031	98

Table 3 The identities of culturable bacteria isolated from spinach leaves (table from (Lopez-Velasco et al 2012))

The phylloplane is a particularly difficult habitat for the study of bacteria due to the diversity of bacterial populations associated with leaves (Beattie and Lindow 1999), the variation in environmental conditions found there leading to variations in growth and distribution of associated microbial populations (Kinkel et al 2000, Obrien and Lindow 1989) and due to changes in microbial communities in response to changes in the leaf itself . Furthermore, the techniques available for the study of bacteria at the phylloplane are limited by the fragility of the leaf structure and leaf surface topography

for microscopy based methods, background interference from plant material with staining techniques for microscopy and molecular methods, and by the requirement to remove bacteria from the leaf surface for analysis for non-microscopy based detection methods. Methods for the study of bacteria at the phylloplane are described further in Section 1.3.3. Identification of phylloplane bacteria has traditionally relied on the removal of bacteria from leaves followed by culture based approaches for isolation and identification of individual species. However, it is well known that not all phylloplane bacteria are able to be isolated by culture based approaches (Andrews and Harris 2000, Yang et al 2001). In one study by Yang *et al.* using molecular based identification of bacteria from the phylloplane of *Citrus sinensis*, it was found that the diversity of phylloplane communities is far greater than previously discovered from culture based approaches (Yang et al 2001). These findings, coupled with new molecular and microscopy based techniques for the direct visualisation of bacteria at the phylloplane (Section 1.3.3), have led to new insights into the identities of indigenous phylloplane bacteria and how these populations exist at leaf surfaces (Whipps et al 2008). The application of advanced molecular and microscopy based techniques to biotic surfaces such as the phylloplane remains in its infancy and there is still much more to be discovered about how bacteria, particularly human pathogenic contaminants, exist in phylloplane environments.

1.2.3 Food spoilage bacteria

Total microbial load at fresh produce surfaces is an important determining factor for product shelf life (Tournas 2005). Both indigenous phylloplane bacteria and contaminating zoonotic pathogens contribute to total microbial load at the phylloplane. As demands on resources for food production grow, there is a pressing need to reduce food waste (IMechE 2013), and as budgets tighten in the current economic climate, minimising financial losses to the food industry from wasted products is an important consideration. It is estimated that the UK produces 1.7 million tonnes of food waste annually, and that the US produces 27 million tonnes at an estimated cost of $41.9 million (£25.9 million) (Weber et al 2011).

Part of this food waste is due to microbial food spoilage. Food spoilage bacteria at leaf surfaces cause a metabolic burden on the leaf, leading to degradation of the leaf tissue and a reduction in product shelf life (Tournas 2005). Therefore, in addition to the implications for food safety, as described in Section 1.2.1, reducing total microbial load for leafy salad products is also an important consideration for improvement of product quality.

Current decontamination methods for leafy salads are outlined in Section 1.2.4, however, the continuing incidence of food poisoning outbreaks associated with leafy salads (EFSA 2012) shows that current mechanisms are not effective at controlling bacteria at the phylloplane. A better understanding of how bacteria exist at the phylloplane, and the mechanisms by which they evade current decontamination strategies is therefore required to aid the development of new and effective decontamination strategies.

1.2.4 Current decontamination strategies for leafy salads

Methods for the post-harvest sanitisation of fresh produce are currently in place in the food industry in an attempt to reduce microbial spoilage and improve product safety. Guidance documents, such as the Code of Hygienic Practice for Fresh Fruits and Vegetables from the Codex Alimentarius Commission, available to producers of fresh fruits and vegetables, sets out Good Agricultural and Good Manufacturing Practices (GAP and GMP, respectively) to ensure hygienic production of RTE fresh produce (FAO/WHO 2007).

Post-harvest decontamination methods for leafy salads routinely involve the use of large scale mechanical wash procedures (as shown in Figure 1 G) which may be used to wash leaves in water alone, or in combination with chemical disinfectant solutions such as chlorine, a powerful oxidising agent (Camper and McFeters 1979). Sodium hypochlorite (NaOCl) for example, which breaks down in solution to release hypochlorous acid (HClO), is recommended for use as an antimicrobial agent for fresh produce and associated wash water at 50 to 200 ppm for a contact time of 1-2 minutes, which has been reported to decrease pathogen numbers on leaves by 2-3 log units

(Parish et al 2003). However, the use of such compounds presents a number of problems. Firstly, the negative environmental impact of introducing chlorine to wastewaters is well documented and there are toxicological hazards for personnel handling hypochlorite (Bull 2007, Olmez and Kretzschmar 2009). Secondly, the efficacy of chlorine washing as a bacterial decontamination strategy is questionable. For example, in wastewater systems, *E.* coli and *Salmonella* have been demonstrated to be tolerant to chlorination treatments via the induction of a viable but nonculturable (VBNC) state (Oliver et al 2005). Single and multi-species biofilms have also been shown to enter the VBNC state upon exposure to chlorine 'disinfection' treatment (Leriche and Carpentier 1995) and this has also been demonstrated at the phylloplane. In a study by Warner, a reduction in total numbers of *S.* Thompson recovered from salad leaves was observed when enumeration was carried out by culture, however, when total recovered cells were enumerated by non-culture based detection (direct viable cell counts by microscopy) no reduction in bacterial numbers was seen, suggesting that chlorine treatment of leafy salads can also induce the VBNC state for zoonotic pathogens at the phylloplane (Warner 2009). In the VBNC state, bacteria are undetectable by culture-based approaches, and therefore assumed dead, however, they remain alive (in a metabolically altered state), remain viable and therefore still present an infection hazard. The VBNC state is further described in Section 1.3.3.4

In addition to sodium hypochlorite, other compounds used for the decontamination of fresh produce include chlorine dioxide gas (ClO_2), peroxyacetic acid and hydrogen peroxide, organic acids and various plant extracts. Additional methods include the ozonation of wash water, heat treatment of leaves, and exposure to UV or ionising radiation (Gil et al 2009, Parish et al 2003). For post packing preservation of leafy salads, the food industry routinely uses microbial growth control measures including modified atmosphere packaging (MAP) and temperature controlled storage to maintain quality and safety of the finished product (Smyth et al 1998). However, as the increasing incidence of food poisoning associated with fresh produce shows, these control measures are not sufficient for the elimination of pathogenic contaminants. So how do bacteria exist at the phylloplane and how can this confer such effective resistance to current decontamination strategies?

1.3 Bacterial colonisation of leaf surfaces

1.3.1 The leaf as a surface for microbial colonisation

Leaf surfaces are adapted for optimisation of photosynthesis and minimisation of water loss by transpiration and are therefore exposed to high levels of UV light from the sun and covered in a thick waxy cuticle, consisting of cutin, suberin and waxes (Taiz and Zeiger 2002). UV light is well known for its anti-microbial action (Chang et al 1985) and the thick waxy cuticle serves to minimise water and nutrient loss from the leaf surface, therefore keeping these within the leaf and unavailable for use by bacteria at the phylloplane (Marcell and Beattie 2002). In addition, field grown leafy salad crops are subject to harsh and variable environmental conditions such as large fluctuations in temperature, drought stress, exposure to wind, and high shear stress from water run-off (Beattie 2011, Kinkel 1997). It is for these reasons that the phylloplane has historically been thought of as an unfavourable location for bacterial colonisation (Lindow and Brandl 2003). Despite this apparent unsuitability, the microbial communities of the phyllosphere are diverse (Lindow and Leveau 2002).

Leaf surface topography is variable across the surface of a single leaf, and provides niches for bacteria to shelter from environmental stresses such as shear stress and UV light, with bacterial aggregates often being found at the boundaries between leaf epidermal cells, leaf veins and around stomata, rather than being distributed evenly across the whole leaf surface (Beattie and Lindow 1999). Nutrient availability has been demonstrated to be variable across the leaf surface, with bacterial colonisation apparently at areas where nutrient availability is high, for example, Leveau and Lindow demonstrated the colonisation of bean leaves by *Erwinia herbicola* at sites where fructose was available, and found colonisation to be in epidermal cell margins, on the surface of leaf veins and around stomata (Leveau and Lindow 2001). It is thought that nutrient availability is a key determining factor in defining bacterial colonisation of leaf surfaces (Remus-Emsermann et al 2012).

Despite the phylloplane being a hostile surface for bacterial colonisation (Lindow and Brandl 2003), bacteria, including human enteric pathogens, can effectively colonise the

leaf surface (Warner et al 2008), persist and even grow there (Beuchat 1999, Brandl and Mandrell 2002), indicating that both indigenous bacteria and pathogenic contaminants have effective survival strategies in place that allow them to successfully exploit environmental niches found at the phylloplane. These strategies may allow pathogenic bacteria to remain closely associated with salad leaves throughout the production process, evading current methods of decontamination and posing a risk to the consumer at the point of sale (Carmichael et al 1999).

1.3.2 Mechanisms for bacterial surface attachment and for attachment to the phylloplane

The mechanisms by which bacteria colonise the phylloplane remain poorly understood. Bacterial epiphyte communities are present throughout the complete life cycle of a plant; at no point can a field grown crop be considered free from colonisation by bacteria. Whether these bacteria come from the soil, the air, the water used for irrigation (Kinkel 1997), or from any of the other contaminating sources described previously, they are present, and present in large numbers (Andrews and Harris 2000). Throughout this plant associated life cycle complex plant-microbe and microbe-microbe interactions have evolved at plant surfaces. For zoonotic pathogens, it is not yet clear whether attachment occurs directly to leaf surfaces (Berger et al 2009), by incorporation into phylloplane biofilms, and/or by internalisation within the leaf tissue (Kroupitski et al 2009a), or indeed, colonise plants using a combination of these strategies (Beattie and Lindow 1999).

Figure 2 provides a diagrammatic representation of bacterial cell surface appendages that may play a role in colonisation of phylloplanes by bacteria. Taking the zoonotic pathogen, and focus of this thesis, *Salmonella*, as an example, the following describes cell surface appendages that have been linked to the colonisation of RTE food plant surfaces. Barak *et al.* showed that genes involved in the production of aggregative fimbriae and cellulose are involved in colonisation of alfalfa sprouts by *Salmonella* Newport, with mutations in these genes resulting in a log reduction in numbers of colonising bacteria (Barak et al 2002, Barak et al 2008). They also found that mutation of the general stress response regulator RpoS, which is involved in the production of

cell surface appendages and biofilm formation (Battesti et al 2011), conferred a similar log reduction in attachment to alfalfa sprouts by *S.* Enteriditis (Barak et al 2002). Berger *et al*. showed variation in the ability of *Salmonella* strains to colonise basil leaves, and that colonisation was dependent on flagella mediating direct attachment to leaves for *S.* Senftenberg but not for *S.* Typhimurium (Berger et al 2009). Given that many different cell surface appendages have been demonstrated to be involved in colonisation of plant surfaces by *Salmonella* spp., but that compromising any one of these appendages in isolation does not abolish colonisation of plant surfaces completely, it is likely that there is no single cell surface appendage governing colonisation of the phylloplane by *Salmonella* spp.

In addition, bacteria regulate the expression of cell surface appendages in response to environmental conditions (Karatan and Watnick 2009), and phylloplanes are exposed to constantly varying environmental stresses, therefore it is likely that bacterial colonisation of the phylloplane uses a combination of cell surface appendages to interact with phylloplanes depending on environmental conditions, plant species and bacterial strain.

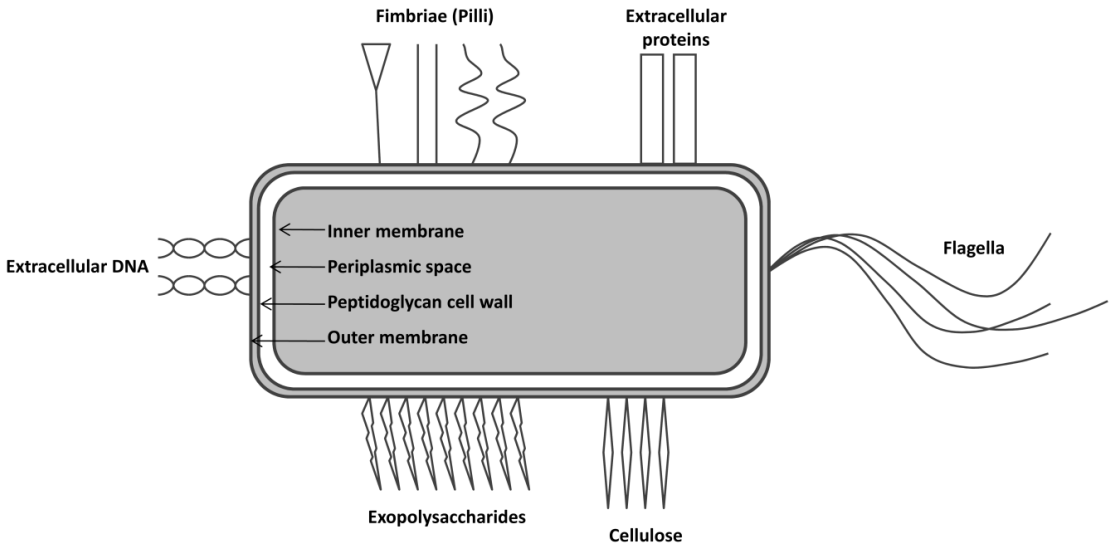


Figure 2 Bacterial cell surface appendages
(image adapted from (Alberts et al 2002, Warner 2009))

When considered in isolation, a single bacterium has a number of cell surface appendages available for initial contact with a surface. However, in nature, bacteria rarely reside as free-living single celled organisms, but rather exist in complex polymicrobial communities called biofilms (Hall-Stoodley et al 2004). Cell surface appendages contribute to the bacterial exopolymeric substance (EPS) matrix, which is an essential component of bacterial biofilms and key to the survival of bacteria in the environment (Flemming and Wingender 2010). Bacterial biofilm formation and the EPS matrix are discussed further in Section 1.4.4.

Bacterial biofilms are ubiquitous in the environment (Costerton et al 1995), and are notoriously difficult to decontaminate from surfaces (Romero and Kolter 2011). Bacteria have been demonstrated to exist in biofilm communities at the phylloplane (Monier and Lindow 2005a, Morris et al 1997, Morris and Monier 2003, Warner et al 2008) where they are an important consideration for zoonotic pathogen contamination of leafy salads. Existence in biofilm communities at the phylloplane may be the key to the persistence of zoonotic pathogens such as *Salmonella* throughout the food production process for RTE leafy salads

1.3.3 Methods for the study of bacteria at the phylloplane

1.3.3.1 Culture based approaches

The identification and enumeration of plant-associated bacteria has traditionally relied on culture based approaches. Culture based approaches usually require the recovery of bacteria from the plant surface into suspension for serial dilution and subsequent plating onto selective agars for identification, or agars such as Reasoner's agar (R2A) for isolation (Donegan et al 1991, Jacques and Morris 1995). This recovery step can be carried out in a number of different ways including sample homogenisation (which does not select for surface associated bacteria and may introduce bactericidal cellular components which may interfere with recovery), washing (which may not be a vigorous enough approach for the removal of bacteria strongly attached to the plant surface), sonication and other mechanical recovery procedures used by the food industry for the monitoring of food-associated pathogens, such as the Stomacher® and

the Pulsifier® (Fung et al 1998). Whilst our fundamental understanding of plant-associated bacteria has been discovered using culture based methods, as described in Sections 1.2.4 and 1.3.3.4, not all bacteria, particularly environmental bacteria, are detectable by culture. However, as the phylloplane is such a complex environment for the direct study of bacteria, and as culture is still used for monitoring food safety, removal of bacteria from plant surfaces for culture based detection remains an important component of investigations of bacteria at the phylloplane.

1.3.3.2 Microscopy and fluorescence techniques

In addition to the identification and enumeration of phylloplane bacteria, the importance of colonisation strategies and localisation of bacterial contaminants in relation to leaf surface structures is an important consideration for furthering our understanding of how bacteria survive in the phyllosphere so successfully and ultimately for the development of new decontamination strategies for fresh produce.

Scanning electron microscopy has been extensively used for the study of bacteria at the phylloplane (Hirano and Upper 2000). SEM is a powerful, high magnification technique that can give excellent resolution of bacteria in association with surfaces. However, traditional SEM requires extensive sample preparation, including sample dehydration, which can distort structures such as leaf tissue and the bacterial EPS matrix (Keevil and Walker 1992, Warner et al 2008). This can make SEM particularly unsuitable for the study of bacterial biofilms, where, for example, artefacts may be introduced through the condensation of the EPS matrix into structures resembling filaments (Keevil 2003). Alternatively, environmental SEM (ESEM), which does not require the same sample dehydration, can produce high magnification images of both bacterial biofilms and leaf surfaces (Hallet et al 2010). However, ESEM is highly labour intensive and therefore low throughput, and it is also very expensive.

Light microscopy techniques have also been used for the study of bacteria in association with the phylloplane. Whilst much lower magnification than SEM and ESEM, light microscopy is relatively low cost, involves less extensive sample preparation methods which are well suited to the study of bacteria in their natural

environments (Walker and Keevil 1994). Light microscopy techniques can also be a comparatively high throughput approach, and when coupled to fluorescence techniques, can be an excellent tool for observation of bacteria at the phylloplane (Morris et al 1997). Phase contrast and differential interference contrast (DIC) microscopy in particular can give excellent resolution of surfaces (Keevil and Walker 1992). DIC is a technique that uses a normarski prism to create a 3D-like image of surface topography from the differential refraction of two polarised light beams when applied simultaneously to the surface (Nikon 2009). Episcopic differential interference contrast microscopy, or EDIC, is a modification of this technique, developed at these laboratories, which couples the use of DIC microscopy with episcopic illumination (illumination of samples from above) allowing the detailed visualisation of opaque samples, and the use of long working distance (LWD) lenses to allow the visualisation of samples without the need for coverslips or oil. These combined modifications allow complex structures and associated bacteria to be visualised with little sample preparation and therefore minimal destruction from preparation. Additionally, epifluorescence filter blocks allow the exploitation of fluorescence techniques for the identification and visualisation of individual bacterial species in complex mixtures and when together with EDIC, in association with surfaces. EDIC/EF microscopy and its application for use at the phylloplane, is described extensively in Section 2.9.

Another high magnification fluorescence based microscopy technique which has provided valuable insights into the structure of bacterial biofilms in particular, is scanning confocal laser microscopy, or SCLM (Keevil 2003). This technique uses single or multi-photon excitation of fluorophores in a sample via a pinhole aperture to excite only a single area of a sample, which can then be scanned in the x, y and z plane, and multiple images processed electronically to re-build 3-D architecture of the sample (Claxton et al 2006). This approach has also been successfully applied to the study of bacteria at the phylloplane, however, again, sample processing techniques can lead to degradation of the leaf tissue leading to distortions in leaf surface topography (Warner 2009), additionally it is another expensive technique that limits high throughput screening of samples.

In summary, a wide range of microscopy techniques are available for the study of bacteria at the phylloplane, which all have their advantages and disadvantages. Therefore, careful consideration of microscopy techniques, with relevance to the aims, experimental design and within the scope of availability is necessary for selection of the best available microscopy technique for use in phylloplane investigations.

1.3.3.3 Molecular approaches

Molecular approaches for the study of bacteria at the phylloplane can be powerful techniques, for example, a study by Yang et al., demonstrated that diversity of microbial populations at the phylloplane are far greater than was previously realised from studies using microscopy methods and culture based approaches for the identification of phylloplane isolates (Yang et al 2001). In this study, the use of a combination of denaturing gradient gel electrophoresis (DGGE) by comparison to traditional culture based approaches demonstrated the existence of bacteria in the phyllosphere which had not previously been identified to exist in the phyllosphere. In addition, the emergence of genomic and proteomic approaches to identify not only gene expression, but how the expression of genes actually translate to proteins in a real-life environment are exciting new considerations for the study of bacteria in association with the phylloplane. In one study by Delmotte *et al*., for example, community analysis using functional proteomics demonstrated the consistent expression of a group of proteins of unknown function for bacteria at the phylloplane. It is possible that subsequent identification and analysis of this set of proteins could lead to new insights into how bacteria interact with leaves, and with eachother at leaf surfaces. The move towards such studies which classify bacteria based on what they 'do' functionally, rather than what they 'are' in terms of identification, is hoped to bring further understanding of bacterial interactions at the phylloplane (Delmotte et al 2009).

1.3.3.4 The viable but nonculturable (VBNC) state

The traditional view that a bacterium is dead if it does not grow on culture media is no longer recognised as correct, with bacteria entering a viable but nonculturable (VBNC)

state in which they have low metabolic activity and do not grown on culture media in order to survive when subjected to external environmental stresses (Oliver 2005). In environments where pathogen contamination may be problematic, for example for zoonotic pathogens at the phylloplane, or in drinking water supply systems, this VBNC state may allow bacteria to remain undetected and yet be able to be resuscitated under favourable conditions and remain a microbial hazard. Interestingly it is the opinion of some biofilm researchers that bacteria existing in a biofilm cannot be cultured (Donlan and Costerton 2002), and therefore these could also be described as VBNC. It is likely, however, that due to the diversity of bacteria within a biofilm, and the presence of microenvironments within the biofilm itself (see Section 1.4) there are populations of bacteria within a biofilm that can be cultured, and populations that cannot. Additionally the persister cell concept describes a sub-population of bacterial cells within a community that when challenged with an antibiotic are not killed and 'persist' to remain viable but undetectable (Stewart and Costerton 2001), these could also be described as VBNC, as could the traditional description of non-actively replicating cells as dormant (Kell and Young 2000). Whatever term is used for their description, the importance of VBNC pathogens is integral to food safety and for determining methods for the identification of pathogens in association with foods.

1.4 Bacterial biofilms

1.4.1 An introduction to bacterial biofilms

Biofilms are defined as "matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" (Costerton et al 1995). Prior to the definition and widespread use of the term "biofilm" and the recognition of these ecosystems as complex and highly regulated communities, structures of bacteria localised together have been historically referred to as aggregates, symplasmata, floccules, granules and microcolonies (Davey and O'Toole 2000, Morris and Monier 2003). These terms often group together bacterial biofilms that form in a specific location, such as floccules that form at air-liquid interfaces, or symplasmata that occur at plant surfaces (Achouak et al 1994, Duan et al 2007, Feng et al 2003) nevertheless, regardless of location, bacteria in

the environment are commonly found in matrix enclosed multi-species bacterial communities now recognised as biofilms.

Environmental microbiologists have long recognised the preference for bacteria to live in surface attached (sessile) communities over existence as single, free living (planktonic) cells (Ellwood et al 1982, Hall-Stoodley et al 2004, O'Toole et al 2000). The first description of bacteria existing in attached communities was by Henrici in 1933, who made microscopic observations of a "deposit of bacteria (which) becomes apparent in a few days and increases progressively, eventually becoming so thick that individual cells may be distinguished with difficulty…. (and) their occurrence in microcolonies of steadily increasing size" (Henrici 1933); a description now commonly associated with the development of bacterial biofilms (Watnick and Kolter 2000). As discussed below, existence within biofilm structures confers a number of benefits to a bacterium over existence as a single cell. A pictorial representation of the generalised structure of a bacterial biofilm is given in Figure 3 (Costerton et al 2003).

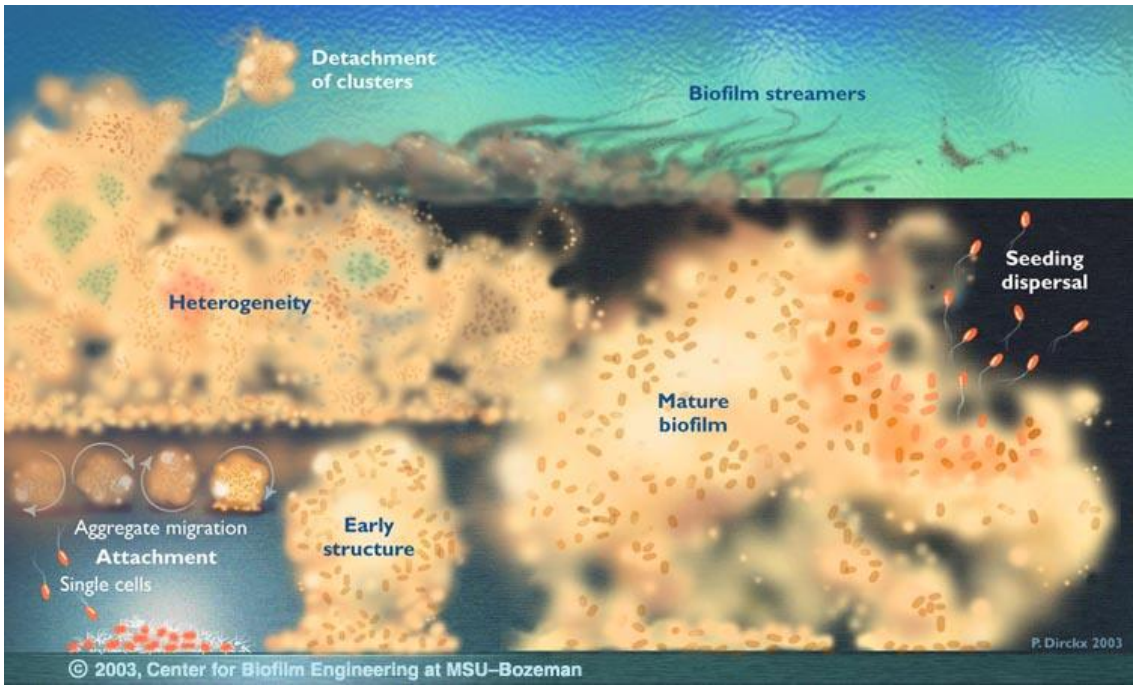


Figure 3 The structure of a bacterial biofilm

Image included with permission from the Montana State University Centre for Biofilm Engineering (Costerton et al 2003)

As modelled in Figure 3, bacterial biofilms are complex structures that begin with the attachment of single cells to a surface. These attached cells aggregate and migrate on the surface to form microcolonies that mature into complex structures composed of many different bacterial species which are highly differentiated from their planktonic equivalents (Stoodley et al 2002, Webb et al 2003a). Heterogeneity is a key characteristic of a bacterial biofilm. A biofilm is a dynamic structure that is constantly evolving and adapting to changes in environmental conditions (Stewart and Franklin 2008). Throughout the life cycle of a biofilm, when conditions outside the biofilm become favourable, or conditions within it become intolerable, sub-populations of bacterial cells leave the protection of the biofilm structure and disperse into the surrounding environment as planktonic cells, ready to exploit new niches in that environment and potentially colonise new surfaces (Kaplan 2010, McDougald et al 2012).

1.4.2 The benefits of surface attached life

The preference of bacteria to exist in surface attached communities, under favourable conditions, rather than as free living planktonic cells, in every environment inhabited by bacteria is evidence that surface attachment confers considerable benefits to bacterial biofilm populations over existence as planktonic cells (Costerton et al 1995, Davey and O'Toole 2000). Some bacterial species are prolific biofilm formers, whilst some do not form biofilms well, but are known to use existing biofilms for shelter and protection from external environmental stresses (Giao et al 2009, Keevil 2002, Wingender and Flemming 2011). Bacterial biofilms are encased in a protective matrix composed of a complex array of EPS, which include polysaccharides, lipids, lipopolysaccharides, glycolipids, nucleic acids and proteins (Davey and O'Toole 2000, Flemming et al 2007). This matrix protects the bacteria within it from external stresses such as shear stress (Rupp et al 2005), fluctuations in temperature, exposure to ultraviolet (UV) irradiation from the sun (Flemming and Wingender 2010), and predation by protozoa (Matz and Kjelleberg 2005), and has also been shown to facilitate the exchange of water and nutrients with the surrounding environment for use by cells within the biofilm (Flemming and Wingender 2010).

Biofilm architecture facilitates the transport of water, oxygen and other nutrients between bacteria in biofilms, and the close proximity of cells facilitates the sharing of nutrients, which is of particular benefit when external nutrient supply is limited (Flemming and Wingender 2010, Webb et al 2003a). Symbiotic relationships between bacteria within a biofilm exist where waste products from one species provide nutrients for another, resulting in populations of cells within multispecies biofilms that are highly dependent on each other for survival (Crespi 2001, Stewart 2003). Mutually beneficial relationships also exist between biofilm bacteria and plant surfaces, in which bacteria are protected from external environmental stresses by surface attachment and obtain nutrients from the plant, and the plant benefits from metabolites produced by the bacteria, as in the case of the nitrogen fixing bacteria of the rhizosphere (Ramey et al 2004). The presence of biofilms on plant surfaces may also serve to protect the aerial parts of the plant from fluctuating environmental conditions and from infection by plant pathogens. Indeed, the artificial introduction of *bacillus* species to crops has long been used on farms as a biological control agent in the fight against fungal plant pathogens (Chen et al 2012).

The close proximity of bacteria within a biofilm also facilitates the sharing of genetic information between bacteria (Madsen et al 2012). Conibear *et al.* for example demonstrated the high level of mutation inside microcolonies of *P. aeruginosa* biofilms which may allow the acquisition of beneficial traits such as antibiotic resistance (Conibear et al 2009, Macia et al 2005). In situations in which cells within a biofilm are exposed to sub-lethal concentrations of antibiotic, for example, in an environmental setting where antibiotics may be present in waste water or runoff from farm wastes (Watanabe et al 2010) or in a medical setting where penetration of biofilms by antibiotics is reduced (Anderl et al 2000, Stewart and Costerton 2001), selection pressures may give rise to antibiotic resistance within a bacterial biofilm. Biofilm bacteria have been demonstrated to be up to 1000 times more resistant to antibiotics than their planktonic equivalents (Ceri et al 1999) and given that, in a medical setting, antibiotic doses are designed to kill planktonic cells, the implications of the human impact of the acquisition of antibiotic resistance genes in a bacterial biofilm are clear (Hoiby et al 2010). In addition, the resistance of environmental biofilms to treatment with chemical biocides such as chlorine, is widely known (Bridier et al 2011). Chlorine

concentrations inside biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* for example have been shown to only reach 20% of the concentration applied to the outside of the biofilm (De Beer et al 1994). This sub-lethal exposure of bacterial cells to disinfectants is thought to contribute to tolerance of bacteria to biocides and the persistence of problematic biofilms (Stewart and Costerton 2001). In addition, as described in Section 1.2.4, the induction of the VBNC state in response to exposure of bacteria to environmental stresses, and to exposure to antimicrobial compounds, is emerging as a significant mechanism by which viable bacteria evade decontamination methods and detection methods to persist, and it is emerging that the biofilm mode of growth is central to this process (LeChevallier et al 1988, Trevors 2011).

Surface attached life in biofilm communities provides a multitude of benefits to a bacterium. Bacteria within a biofilm are highly specialised and differentiated from their planktonic equivalents, and biofilms as a whole are extremely dynamic, leading to their ability to persist in environments often thought to be unsuitable for sustaining bacterial life (Hall-Stoodley et al 2004). Therefore, bacterial biofilm formation is a sophisticated mechanism for bacterial survival, the stages of which are necessary considerations for understanding bacterial life at the phylloplane, and for identifying possible new targets for decontamination.

1.4.3 Biofilms at the phylloplane

Presently, few studies have observed bacterial biofilms directly at leaf surfaces. The observation of bacterial aggregates at leaf surfaces has historically been observed in studies using SEM, which had been the only high resolution microscopy method available for the observation of bacteria directly on leaf surfaces prior to the application of CSLM and EF to phylloplane investigations (Morris et al 1997). It is recognised that plant-associated bacteria exist predominantly in aggregates or biofilms at the leaf surface and that these are commonly found in association with leaf surface structures including, trichomes, leaf veins and the junctions between epidermal cells, as demonstrated from these early studies using SEM (Carmichael et al 1999, Monier 2006). However, due to the nature of sample preparation required for SEM, with extensive sample dehydration, whilst phylloplane biofilms had been suggested (Beattie

and Lindow 1995), the observation of these bacterial aggregates could not be conclusively defined as biofilms. An overview of microscopy methods available for the study of bacteria at the phylloplane is given in Section 1.3.3.2. Biofilms have now been described in association with the phylloplanes of cabbage, lettuce, spinach, celery, leeks, basil, parsley, beans, and broad-leaf endive using advanced microscopic methods, and it has become clear that bacteria at the phylloplane exist as both biofilm communities and as diffusely attached cells (Andrews and Harris 2000, Carmichael et al 1999, Monier and Lindow 2003, Morris et al 1997, Morris and Monier 2003, Warner et al 2008). Whilst biofilms have been demonstrated to exist at phylloplanes, there is remarkably little investigation of the interaction of zoonotic pathogens with these biofilms (Brandl 2006a), which is fundamental to our understanding of zoonotic pathogen persistence in association with leafy produce, leading to food poisoning outbreaks.

Warner *et al*. described the use of EDIC/EF microscopy to demonstrate the presence of indigenous phylloplane biofilms and to investigate the localisation of *S.* Thompson attachment at the spinach phylloplane (Warner et al 2008). Kroupitski *et al,* have demonstrated the existence of *S.* Typhimurium biofilms on romaine lettuce using CSLM (Kroupitski et al 2009b). In other, non-phylloplane environments, the incorporation of human pathogens in biofilm communities has been described (Donlan and Costerton 2002, Giao et al 2009, Keevil 2002, Wingender and Flemming 2011) but whilst it is likely that this occurs for zoonotic pathogens at leafy produce surfaces, it has not been demonstrated to date.

Given that biofilms are prevalent at leaf surfaces, and that zoonotic pathogens form biofilms at the phylloplane of leafy salad crops, it is hypothesised that phylloplane biofilms provide shelter and protection for zoonotic pathogens on leafy produce, allowing pathogens such as *Salmonella* to evade decontamination by washing and to persist in association with RTE leafy salads to the point of sale, thus posing a food safety risk. It is with this in mind that a novel decontamination strategy for leafy salads targeting bacterial biofilms is investigated in this work (see Section 1.5).

1.4.4 Bacterial biofilm development

Biofilms are ubiquitous in the environment (reviewed in (Costerton 1995, Hall-Stoodley et al 2004)). However, due to the complexities associated with biofilms in environmental situations, much of the study of the stages of biofilm formation has been carried out for single species biofilms under controlled experimental conditions at abiotic surfaces. A generalised developmental model for biofilm formation has been described, (for reviews see (Hall-Stoodley et al 2004, Monds and O'Toole 2009, O'Toole et al 2000, Stoodley et al 2002, Watnick and Kolter 2000), a diagrammatic representation of which is provided in Figure 4 (Monds and O'Toole 2009). Biofilms of *P. aeruginosa* (Sauer et al 2002), *E. coli* (Pratt and Kolter 1998, Reisner et al 2003, Tremoulet et al 2002) and *Vibrio cholerae* (Watnick and Kolter 1999a) have been shown to follow a developmental pattern of biofilm formation in the laboratory (reviewed in (Hall-Stoodley et al 2004). Much of the understanding for this developmental model has arisen from the study of single species biofilms at abiotic surfaces, although the life cycle of biofilms in the environment, encompassing many of the concepts described, has been known for many years prior to the definition of the 'developmental model for biofilm formation' ((Marshall et al 1971, Marshall 1976), for reviews see (Wimpenny et al 1993)). When considered outside of the laboratory, as discussed below, the induction of phenotypic changes in bacteria in response to environmental stimuli will undoubtedly alter the expression of extracellular appendages that mediate the developmental stages of biofilm formation described by these models. Therefore, as discussed in a review by Stoodley *et al.*, whilst these models summarise important concepts in biofilm research, it is important to exercise caution when applying them to biofilms of different species to those in which the discoveries have been made, to multi-species biofilms, and to biofilms in an environmental setting, as no model can encompass the variability found in the environment (Stoodley et al 1997). Nevertheless, these models provide a useful tool for understanding biofilm concepts, for understanding key events in the life cycle of a bacterial biofilm and for identifying potential targets for intervention, and are therefore described below.

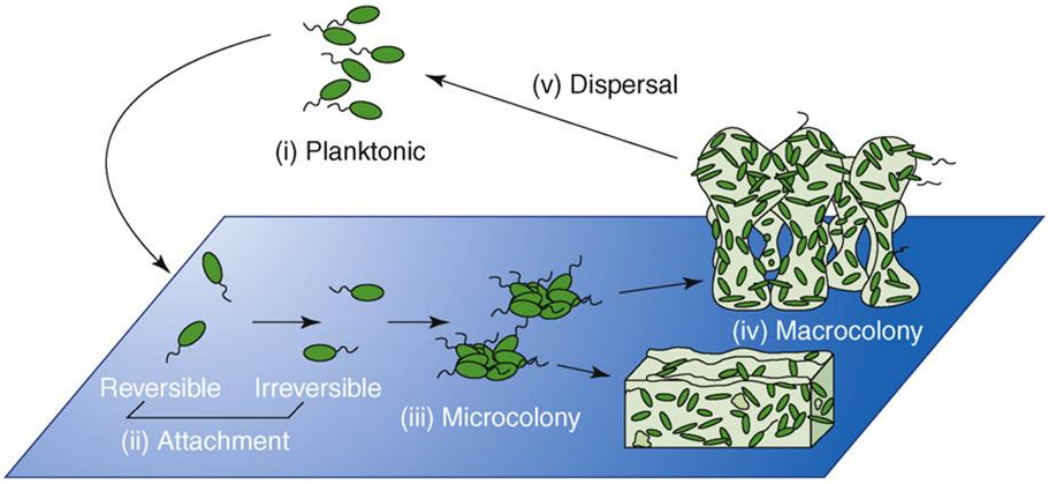


Figure 4 The five stage developmental model of biofilm formation (image from Monds and O'Toole 2002)

In this five stage model of biofilm formation, planktonic cells (Figure 4 i) travel to the surface where they attach (Figure 4 ii), before forming microcolonies (Figure 4 iii) and growing into a mature biofilm, often consisting of large microcolonies (referred to in some models as 'macrocolonies', as in Figure 4 iv). Sub-populations of cells are then periodically released from the mature biofilm in a process referred to as biofilm dispersal (Figure 4 v), which releases individual bacteria into the surrounding environment as planktonic cells (Figure 4 i) (Monds and O'Toole 2009). These five stages are described in more detail below.

1.4.4.1 Reversible and irreversible attachment

For the first stage of biofilm formation, bacteria loosely or 'reversibly' attach to a surface, before progression to a more permanent 'irreversible' attachment (Marshall et al 1971). Transport to a surface may occur by chance, for example by gravitational settling or movement in liquid flow, or by the directed movement of bacteria by chemotaxis, where, once in the proximity of a surface, molecular properties of both the bacterium and the surface, along with chemical properties of the surrounding environment govern whether a bacterium can attach (van Loosdrecht et al 1987). Electrostatic attractions, van der Waals forces and cell surface hydrophobicity are important for irreversible surface attachment (Hermansson 1999, Van Loosdrecht et al

1989) before more permanent 'irreversible' attachment occurs involving bacterial cell surface appendages and EPS (Pratt and Kolter 1999). Bacterial cell surface appendages are shown in Figure 2 all of which have been implicated as possible adhesins for irreversible surface attachment for various bacterial species (Jonas et al 2007, Klausen et al 2003b, Leigh and Coplin 1992, Ma and Wood 2009, O'Toole and Kolter 1998a, Pratt and Kolter 1998, Pratt and Kolter 1999, Ude et al 2006).

Flagellar-mediated motility for example has been shown to be essential for irreversible surface attachment by *E. coli, P. aeruginosa* and *P. fluorescens* and flagella themselves have been suggested to play a direct role in attachment of *S.* Senftenberg to the phylloplane (Berger et al 2009, Delmotte et al 2009, Genevaux et al 1996, O'Toole and Kolter 1998a, Pratt and Kolter 1998). However, different appendages have been shown to be essential for irreversible attachment for other species, for example the type I pilli of *E. coli* and the type IV pilli of *V. cholera*e (Pratt and Kolter 1998, Watnick and Kolter 1999b). In addition, it is apparent that in the absence of one mechanism another can restore the ability of a bacterium attach and form biofilm. The curli fimbriae of *E. coli* have been shown to confer irreversible surface attachment to a non-motile strain of *E. coli* (Vidal et al 1998) for which flagella were previously deemed essential, and biofilm formation restored to non-motile *P. fluorescens* upon the addition of nutrients (O'Toole and Kolter 1998b). It is therefore apparent that there exist a number of mechanisms for bacterial surface attachment and that these vary between species, strain and with environmental conditions.

When considering the formation of bacterial biofilms, rather than considering bacterial cell surface appendages as individual structures that may directly anchor a bacterium to a surface, it is necessary also to consider them as components of the overall exopolymeric substance (EPS) matrix of a bacterial biofilm. The EPS matrix plays an essential role in the establishment of bacterial associations with surfaces (Tsuneda et al 2003) and is a complex and highly dynamic functional structure (Flemming and Wingender 2010). Bacterial EPS surrounds cells within a biofilm, protecting them from environmental stresses and expression of its components is highly dependent on the microenvironment of individual bacteria within it (Flemming et al 2007). For example, the polysaccharide, alginate, of *P. aeruginosa* is expressed upon surface contact and is

responsible for the establishment of the distinct biofilm architecture observed for *P. aeuriginosa* (Davies and Geesey 1995, Hoyle et al 1993).

For *Salmonella* spp. multicellular behaviour has been well studied, with the EPS components curli and cellulose playing a key role in the adhesion of bacterial cells to abiotic surfaces and in biofilm formation (Gerstel and Romling 2003, Romling et al 1998). Romling *et al*. described the rdar (red dry and rough) morphotype in *Salmonella enterica*, which is characterised by the expression of curli and cellulose as part of the EPS matrix, corresponding to the ability of *Salmonella* to aggregate into multicellular structures (Romling 2005). *Salmonella*, like other bacterial species, are known to switch between adaption to conditions for pathogenesis inside their host species and existence outside of their host allowing survival in the environment. Romling *et al.*, have shown that this switching between pathogenesis ('unicellular life') and environmental survival ('multicellular life') for *Salmonella* spp., via the expression of the rdar morphotype, is linked to signalling via the global regulatory control molecule, bis – (3',5') – cyclic dimeric guanosine monophosphate (c-di-GMP) (Simm et al 2004). This regulatory signalling molecule (discussed further in Section 1.5.2) plays a key role in the bacterial response to environmental stimuli (Romling and Amikam 2006). It is known that factors including nutrient starvation, growth phase and temperature affect the expression of curli and cellulose by *Salmonella* spp. (Romling et al 1998). However, interestingly, whilst curli fimbriae are important for biofilm formation at the abiotic surface, when investigated for their role in colonisation of salad leaves by *S.* Typhimurium, a link could not be established between curli expression and leaf colonisation (Warner 2009). These findings suggest that the phylloplane environment provides a unique habitat for colonisation by *Salmonella* spp.

1.4.4.2 Microcolony formation and biofilm maturation

Once attached to a surface, bacteria form microcolonies by a combination of movement across the surface by twitching and swarming motility (O'Toole and Kolter 1998a), and clonal growth of attached cells (Klausen et al 2003a, Klausen et al 2003b). Bacterial swarming has been demonstrated to require flagella (Verstraeten et al 2008), whilst twitching has been shown to require Type IV pilli in the model species

P. aeruginosa (O'Toole and Kolter 1998a). Maturation of microcolonies into complex 3-dimensional biofilm structures, that include water channels and a protective EPS matrix, as modelled in Figure 3, then develops over time. As reviewed by Coggan and Wolfgang, bacterial phenotypes including motility are structural determinants of biofilm formation and are under the control of environmental stimuli (Coggan and Wolfgang 2012). This phenotypic plasticity results in bacterial biofilms taking on distinctly different structural and morphological characteristics in different environments. For example, *P. aeruginosa* forms biofilms with distinct mushroom shaped microcolonies when grown in glucose minimal medium, and biofilms with a distinctly flat monolayer appearance when grown in casamino acid or citrate minimal medium, demonstrating that biofilm development is strongly influenced by nutrient source (Klausen et al 2003b). Therefore, as biofilm microcolony formation, maturation and subsequent biofilm morphology vary in response to external nutrient conditions, in an environmental situation such as at the phylloplane where a biofilm is exposed to continually fluctuating environmental variables (Lindow and Brandl 2003), the structure of bacterial biofilms will also be variable and highly dynamic.

Bacteria within a mature biofilm are highly differentiated from those traditionally studied in the free living planktonic mode of growth (Stoodley et al 2002). Sauer *et al*. for example demonstrated using gene microarray and proteomic methodology, that gene and protein expression between biofilm derived and free-living planktonic *P. aeruginosa* PAO1 cells differed by 50% (Sauer et al 2002). When compared to planktonic cells of the same strain, this makes biofilm bacteria less similar to each other than to planktonic cells of a different strain (Sauer et al 2002). It is unsurprising then that the behaviour of biofilm bacteria is so different from those studied in the free living, planktonic mode of growth. Given that biofilms are so widespread in the environment, more work is required to better understand mechanisms governing biofilm lifestyles in the environment to develop novel anti-bacterial intervention strategies for practical applications such as the decontamination of leafy salads.

1.4.4.3 Biofilm dispersal

The final stage in the biofilm developmental model, and arguably the most important for the development of novel decontamination strategies, is biofilm dispersal. Dispersal events have been observed by microscopic observations of mature biofilm structures, which involve the death of cells at the centre of mature microcolonies, and the release of a surviving sub-population of planktonic bacteria into the surrounding environment (McDougald et al 2012). Webb *et al*. have described these dispersal events microscopically using the BacLight™ Live/Dead® stain (Invitrogen, UK), as shown in Figure 5 A. Cell death can be visualised within the centre of *P. aeruginosa* biofilm microcolonies, leading to the dispersal of the microcolony and the dispersal of surviving live cells from the centre of the biofilm structure, as indicated by an arrow (Figure 5 A) (Webb et al 2003b). A similar pattern of dispersal has also been observed for biofilms of the marine bacteria *Chromobacterium violaceum* and *Caulobacter crescentus* and shown in Figure 5 B and Figure 5 C, respectively (Mai-Prochnow et al 2008). Interestingly, this pattern of staining of dead cells within microcolonies, surrounded by live cells, has also been described for *Pseudomonas syringae* on leaf surfaces (Monier and Lindow 2003) which is direct evidence for the presence of microcolony structures at the phylloplane that may undergo similar dispersal events.

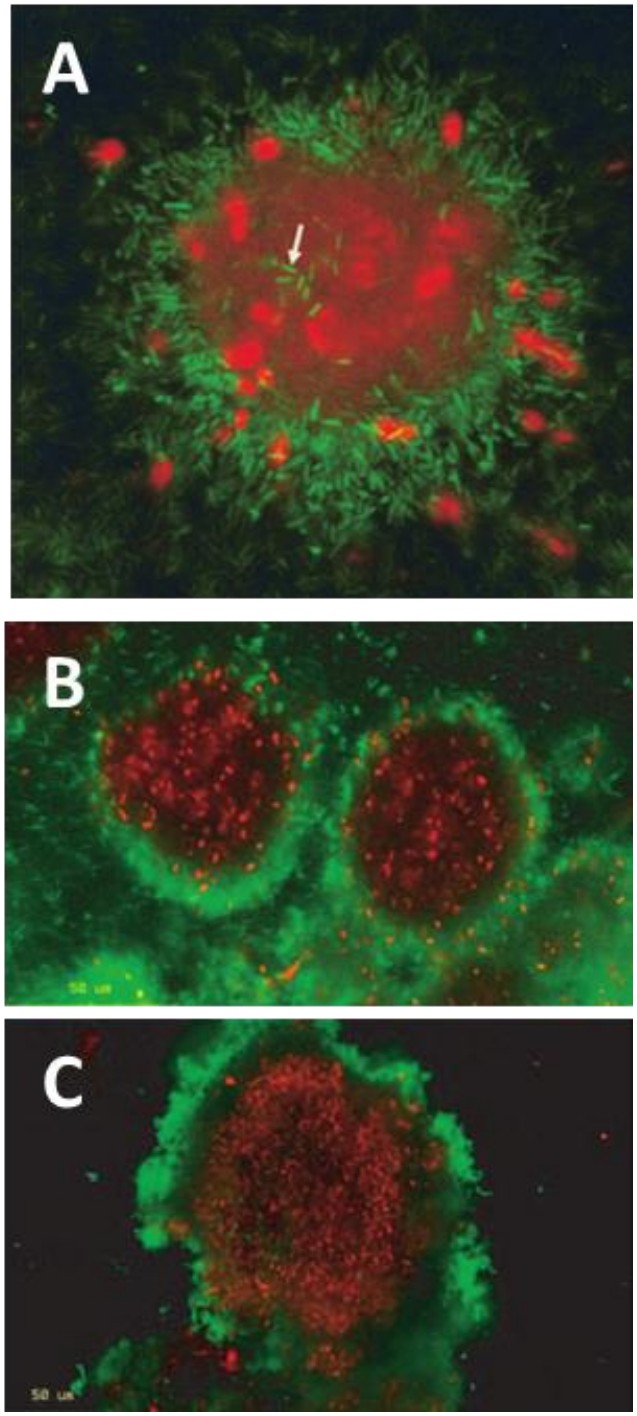


Figure 5 Cell death in biofilm microcolonies leading to biofilm dispersal

Cell death in microcolonies of leading to biofilm dispersal in *P. aeruginosa* (A) (image from (Webb et al 2003b)), *Chromobacterium violaceum* (B), and *Caulobacter crescentus* (C) biofilms (images from (Mai-Prochnow et al 2008))

Bacterial cells can also be detached from biofilms by physical mechanisms, for example by sloughing caused by shear stress in liquid flow (Stoodley et al 2001), but 'dispersal' of bacterial biofilm microcolonies is distinctly different from mechanical events as it is induced by molecular signalling mechanisms within the biofilm in response to quorum sensing signals, reactive oxygen species and intracellular signalling via bacterial c-di-GMP (reviewed by (Webb 2007)). The function of biofilm dispersal is thought to be to allow bacteria, which may have acquired beneficial traits during their time within the biofilm, to colonise new areas for the survival of the species as a whole, for example if conditions become too crowded within a biofilm and nutrient availability becomes limited (Kaplan 2010).

The discovery that molecular signalling by reactive oxygen and nitrogen species contributes to cell death and dispersal within bacterial biofilms (Webb et al 2003b), has led to a new target area for the induction of dispersal in bacterial biofilms as a biofilm removal strategy for surfaces. Of particular note is the small molecule signalling molecule, nitric oxide, which has been shown to disperse biofilms for a range of bacterial species (Barraud et al 2009b, McDougald et al 2012). Nitric oxide signalling is considered further in Section 1.5.

1.5 Nitric oxide: A novel strategy for the dispersal of bacterial biofilms

1.5.1 The nitric oxide story

Nitric oxide has been recognised as a signalling molecule since the 1970's. It was originally recognised that vasodilation in human endothelial cells was caused by a signalling molecule termed endothelial derived relaxing factor (EDRF) and later discovered that EDRF is the small molecule, free radical, gasous, nitric oxide (NO) (Ignarro et al 1987). These findings led to the award of the 1998 Nobel Prize in Physiology or Medicine to Robert Furchgott, Louis Ignarro and Ferid Murad for "their discoveries concerning the nitric oxide as a signalling molecule in the human cardiovascular system"(Zetterstrom 2009). Since this discovery, demonstrating that a gaseous molecule with a short half-life can act as a molecular signalling molecule, an enormous amount of research has been carried out to determine the signalling roles of nitric oxide in mammalian cells, plants, and more recently, in bacteria (Martinez-Ruiz et al 2011).

NO is a multi-functional signalling molecule in mammalian cells, involved in processes from olfactory vision to the cardiovascular system and the central nervous system (Alberts et al 2002, Martinez-Ruiz and Lamas 2009) and is used therapeutically in the treatment of a range of illness including angina and pulmonary oedema (Chen et al 2002, Ichinose et al 2004). NO is a small, lipid soluble gaseous free radical that can diffuse across cell membranes and is unstable with a short half-life in the body (1-5 seconds) (Ricciardolo et al 2004). The production of NO is therefore required close to its site of action for efficient signal transduction (Oess et al 2006). In mammalian cells, nitric oxide for use in signalling processes is produced by the nitric oxide synthases (NOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (Knowles and Moncada 1994). A third NOS, macrophage inducible NOS (iNOS) also exists, although this is more commonly associated with the generation of cytotoxic levels of nitric oxide for use in the defence against pathogens (Knowles and Moncada 1994). The NOSs are haem-containing flavoproteins, similar to the cytochrome P450s, which produce NO in the conversion of arginine to citrulline (Lamas et al 1992, Lyons et al 1992). This

conversion is in turn activated in response to an increase in intracellular calcium via a calmodulin (CAM) dependent pathway (Alberts et al 2002, Crawford 2006).

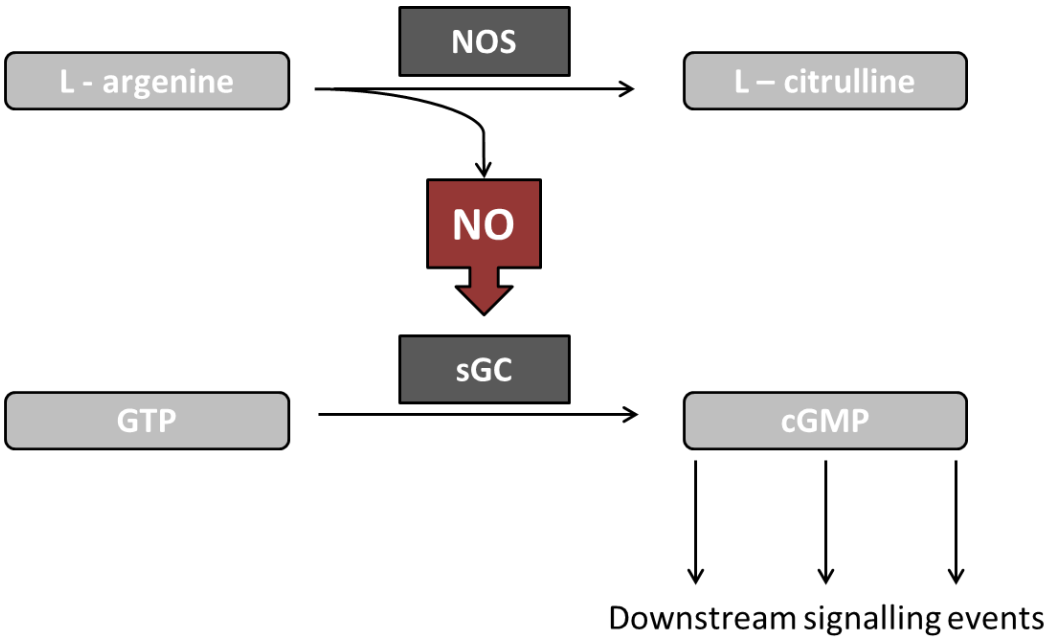


Figure 6 A schematic representation of the role of nitric oxide in cyclic GMP production in mammalian cells

The enzyme target for NO in mammalian cells, of interest for the investigations presented in this thesis, is the haem-containing flavoprotein receptor soluble guanylyl cyclase (sGC) (Wendehenne et al 2001). A schematic representation of nitric signalling via sGC in mammalian cells is provided in Figure 6. NO, produced by NOS, is small and lipid soluble and readily diffuses to sGC in the cytosol where it binds to the haem moiety (known as the H-NOX domain) leading to a conformational change in the enzyme causing its activation (Cary et al 2006, Derbyshire and Marletta 2009). Once activated sGC catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanisine monophosphate (cGMP), a second messenger involved in the control of a wide range of cellular signalling functions, including the control of gated ion channels, the activation of protein kinases and the regulation of phosphodiesterase enzymes (Lucas et al 2000).

In plants, whilst there is evidence to show that cGMP synthesis is activated by NO, a sGC analogue has not been identified to confirm the specific mechanism of nitric oxide signalling via the sGC/cGMP pathway in plants (Besson-Bard et al 2008). Similarly, a plant nitric oxide synthase has not yet been discovered (Frohlich and Durner 2011). Nevertheless, signalling roles for NO in plants are diverse and include the initiation of stomatal closure (Mata and Lamattina 2001), pollen tube germination and growth (Reichler et al 2009), stimulation of seed germination and host defence against pathogens (Bethke et al 2006a, Bethke et al 2006b, Gas et al 2009), and it is thought that NO signalling pathways are highly conserved between mammalian and plant cells (Wendehenne et al 2001). It is suggested that nitrosylation of metal containing proteins such as haemoglobin, post-translational S-nitrosylation of signalling proteins, and post-translational nitrosylation of tyrosine residues are also mechanisms of nitric oxide signalling in plants (Besson-Bard et al 2008).

In bacteria, nitric oxide is generated by nitric oxide reductase (NR) during the reduction of nitrate in the denitrification pathway and by bacterial nitric oxide synthase (NOS) similar to the NOSs found in mammalian cells, which produces NO in the conversion of arginine to citrulline (Crane 2008, Crane et al 2010). The molecular targets for NO in bacterial signalling are not yet fully understood, although it is apparent that there are many (Crane et al 2010). It is known that proteins containing H-NOX domains are present in bacteria, in a system with parallels to cGMP signalling in mammalian cells and it is emerging that that nitric oxide signalling in bacteria involves modulation of the levels of the signalling molecule c-di-GMP (Barraud et al 2009a), as discussed further in Section 1.5.2.

Additionally, it has emerged that the intracellular level of c-di-GMP controls the switch between sessility and motility in bacteria, leading to an interest in exploiting the modulation of intracellular c-di-GMP levels for biofilm control (Romling et al 2013), as discussed further in Section 1.5.2. The investigation of nitric oxide as a control strategy for bacterial biofilms of various species, via the presumed modulation of intracellular c-di-GMP levels, has therefore been explored with some success supporting the belief that this nitric oxide signalling mechanism may be conserved across bacterial species (Barraud et al 2009b).

1.5.2 Cyclic di-GMP signalling in bacteria

The bacterial second messenger signalling molecule bis-(3' -5')-cyclic dimeric guanosine monophosphate (c-di-GMP) was discovered in the 1980's for its role in the control of cellulose synthesis in the plant symbiont, *Gluconacetobacter xylinus* (Ross et al 1987, Tal et al 1998). The subsequent recognition of the role of c-di-GMP in coordinating the switch between sessility and motility in bacteria, has led to the investigation of the signalling molecule for its potential to control bacterial biofilms (Jenal 2004).

A careful balance of synthesis and degradation controls the intracellular level of c-di-GMP, which in turn coordinates an array of downstream signalling events leading to phenotypic changes in bacteria (see Figure 7) including motility, virulence and biofilm formation (Hengge 2009, Romling et al 2005, Tamayo et al 2007). High intracellular c-di-GMP leads to the increased expression of an attached, sessile phenotype whilst low intracellular ci-di-GMP leads to the increased expression of a free living, planktonic phenotype (Simm et al 2004).

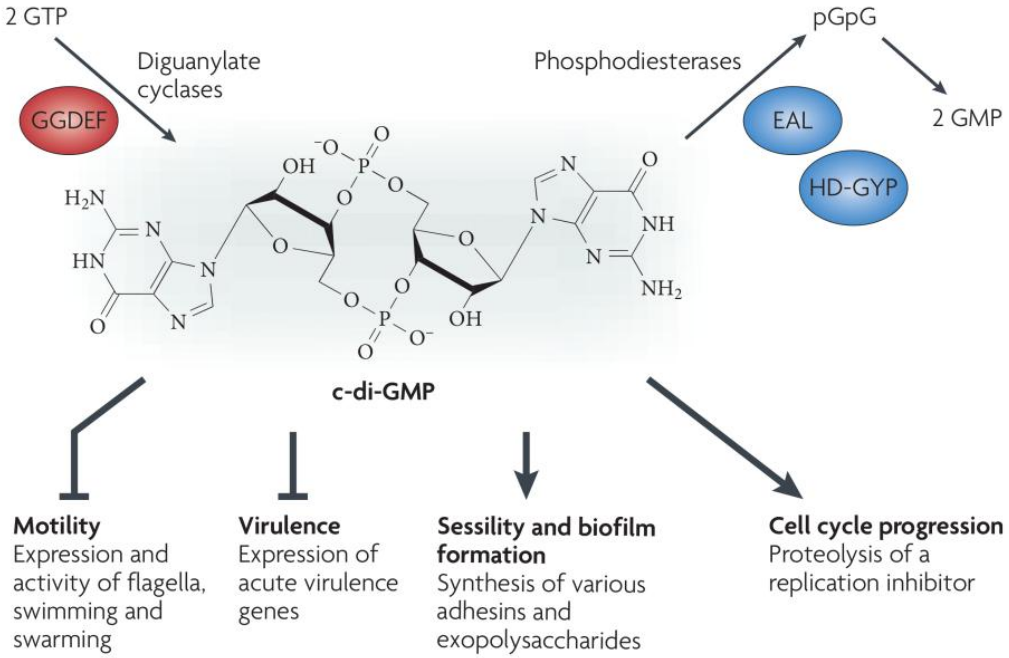


Figure 7 Structure and physiological functions of c-di-GMP signalling (image from (Hengge 2009))

Bacterial c-di-GMP is synthesised from guanosine triphosphate (GTP) by di-guanylate cyclases (DGCs), and broken down by hydrolysis by phosphodiesterases (PDEs) as shown by the schematic representation in Figure 7. A set of amino acid sequences that are conserved across bacterial genera control the synthesis and hydrolysis of c-di-GMP, with GGDEF domain proteins having DGC activity, and EAL (and HD-GYP) domain proteins having PDE activity (Simm et al 2004). GGDEF and EAL domain containing proteins are present across bacterial species, leading to the conclusion that turnover of c-di-GMP is a central regulatory control mechanism that is conserved across bacterial species (Simm et al 2004).

As shown in Figure 8, c-di-GMP is central to the coordination of the response to environmental signals, including nutrients, light and gaseous molecules including nitric oxide (Romling et al 2005). In response to environmental cues, modulation of c-di-GMP levels leads to phenotypic changes largely involved in the switch between a biofilm associated, sessile lifestyle, and a planktonic lifestyle associated with pathogenesis (Simm et al 2004). In *S.* Typhimurium for example, environmental signals received via the LuxR quorum sensing system signal via the transcriptional promotor csgD lead to high intracellular c-di-GMP levels and the subsequent expression of a biofilm phenotype known as the rdar morphotype, characterised by the increased production of EPS matrix components including curli and cellulose, in a process involving GGDEF and EAL domain containing proteins (Romling 2005, Simm 2007, Walters and Sperandio 2006).

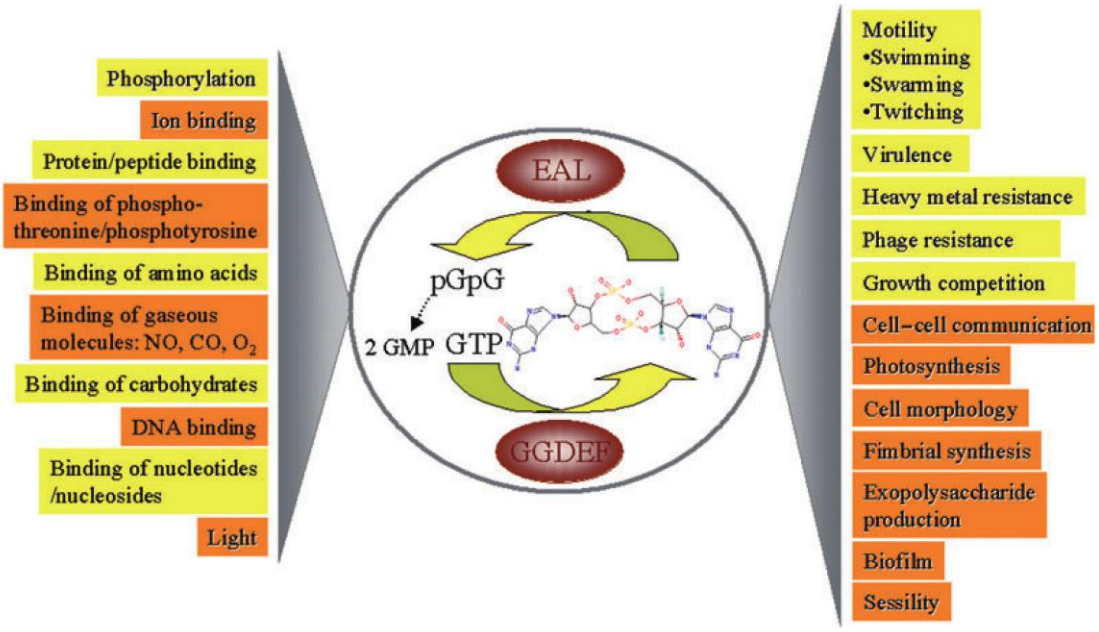


Figure 8 Known inputs and outputs of c-di-GMP metabolism in bacteria (image from (Romling et al, 2005))

The manipulation of intracellular di-GMP levels has received much interest for its potential to control problematic bacterial biofilms (Romling et al 2013, Simm et al 2004). It is however emerging that regulation of c-di-GMP levels in bacteria is a complex process, and further understanding of the signalling mechanisms involved is required before we can fully understand the role of c-di-GMP in coordinating phenotypic changes in response to environmental signals (Romling et al 2013).

1.5.3 The interaction of nitric oxide with c-di-GMP in bacteria

As described above, the intracellular level of c-di-GMP has emerged as a key determinant of bacterial lifestyles, particularly important in the decision between surface attached and planktonic existence (Hengge 2009). Despite the many mechanistic details that remain undiscovered, there are some promising results demonstrating the potential for modulation of intracellular c-di-GMP to be used to control bacterial biofilms (Romling et al 2013). One such discovery is that biofilm dispersal events for the model organism *P. aeruginosa* can be induced by exogenous application of nitric oxide, in a mechanisms involving the modulation of c-di-GMP (Barraud et al 2009a).

This discovery arose from the investigation into the mechanism of action for biofilm dispersal in *P. aeruginosa*, as described in Section 1.4.4.3 which occurs in response to the production of reactive oxygen and nitrogen species in biofilm microcolonies (Webb et al 2003b). It was subsequently found that exogenous application of nitric oxide to *P. aeruginosa* biofilms was able to induce these biofilm dispersal events (Barraud et al 2006). Critically, the signalling ability of nitric oxide in biofilm dispersal was demonstrated to occur at low, non-toxic, concentrations. Single planktonic cells were described leaving the biofilm following the death of the surrounding microcolonies in response to nitric oxide treatment, via the exogenous application of the nitric oxide donor SNP (Barraud et al 2006). A mechanism of action for nitric oxide-mediated biofilm dispersal has been proposed based on studies of *P. aeruginosa*, whereby nitric oxide induces the action of phosphodiesterases which break down c-di-GMP (Figure 7), leading to lower intracellular levels of c-di-GMP corresponding to a non-biofilm phenotype (Barraud et al 2009a). Figure 9 shows a schematic representation of the action of nitric oxide in the turnover of cyclic di-GMP in a bacterial cell.

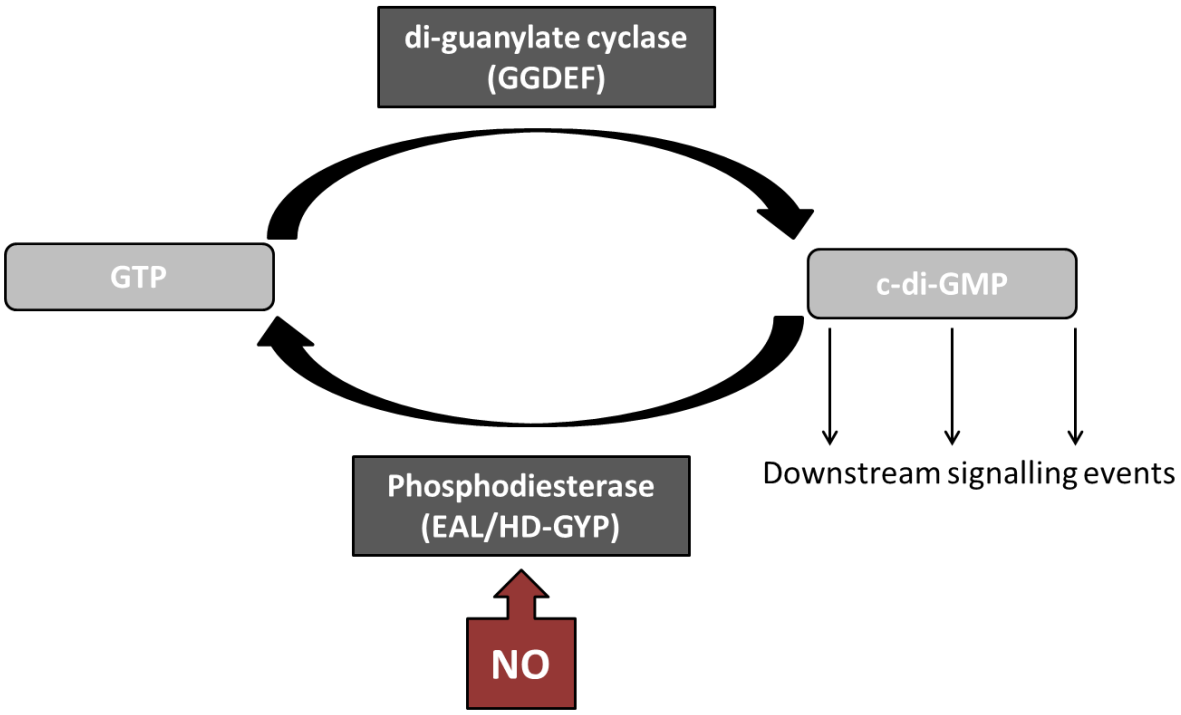


Figure 9 Schematic representation of nitric oxide signalling via bacterial c-di-GMP

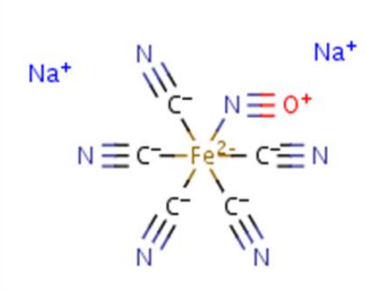
It has now emerged that signalling via bacterial c-di-GMP is a conserved mechanism, with parallels to cGMP signalling in humans, which is present across bacterial species (Romling et al 2013). Therefore, if nitric oxide signalling via c-di-GMP is also conserved across bacterial species, it may be able to be exploited for biofilm dispersal for a wide range of bacterial species. Indeed, nitric oxide treatment of *E. coli*, *Vibrio cholerae*, *Serratia marcescens*, *Fusobacterium nucleatum, Bacillus licheniformis* and *Staphylococcus epidermidis* biofilms has been shown to induce biofilm dispersal, and to reduce surface coverage for environmentally grown, mature multi-species drinking water biofilms (Barraud et al 2009b). Whether nitric oxide signalling can cause the dispersal of biofilms in a phylloplane environment has not yet been investigated. As previously described, the phylloplane is a complex environment for the study of bacterial biofilms, and the microbial ecology of the phylloplane is diverse (Hirano and Upper 2000, Lindow and Leveau 2002). It is an exciting possibility that a signalling molecule such as nitric oxide may be able to induce biofilm dispersal events in a diverse range of bacterial species, and it is hoped that the investigation of this at the phylloplane may lead to the development of novel decontamination strategies for use in a food production environment for the control of biofilms and zoonotic pathogen contaminants for fresh produce such as leafy salads.

1.5.4 Experimental nitric oxide donors

For delivery of nitric oxide to experimental systems for the results presented in this thesis the nitric oxide donor sodium nitroprusside (SNP) was used (see Figure 10). SNP is used as a therapeutic nitric oxide donor for the treatment of hypertension in humans (Yamamoto and Bing 2000), is used in toxicology tests to identify recreational drugs containing secondary amines such as MDMA (ecstasy) and methamphetamine (UN 2005), and is commonly used as a nitric oxide donor in experimental assay systems. SNP has been used successfully for the delivery of nitric oxide to biofilm assay systems for the induction of biofilm dispersal (Barraud et al 2006, Barraud et al 2009b).

A key characteristic for the selection of SNP as a nitric oxide donor for use in these investigations is its ability to induce a signalling response in bacterial biofilms at low,

non-cytotoxic concentrations leading to biofilm dispersal (Barraud et al 2006). Concentrations of SNP used for these investigations were selected based on findings by Barraud *et al*., who demonstrated that as little as 25 nM SNP is sufficient to cause dispersal of *P. aeruginosa* biofilms, whilst 500 nM SNP was optimal for reduction of biofilm biomass (Barraud et al 2006). These researchers also found that concentrations of SNP ranging from 25 nM to 10 µM were optimal for the induction of biofilm dispersal in *S. marcescens, V. cholera, E. coli*, *F. nucleatum, B. licheniformis, and S. epidermidis* (Barraud et al 2009b). These researchers also demonstrated, using a nitric oxide probe for the measurement of nitric oxide in solution, that the release of nitric oxide from SNP is approximately 1000-fold less than the concentration of SNP introduced into the assay system (Barraud et al 2009b), and therefore the concentration of NO sufficient to induce biofilm dispersal is very low, in the picomolar range. As shown in Figure 10 the primary mechanism for release of nitric oxide from SNP in solution is photodegradation, whereas in biological systems, release of SNP can occur by enzymatic or non-enzymatic one electron reduction which depends on the reducing capabilities of the surrounding environment (Vyas et al 2003, Yamamoto and Bing 2000).



$$3\ (Na_2[Fe(CN)_5NO]) + 2\ H_2O \xrightarrow{h\nu} 2\ (Na_3[Fe(CN)_5H_2O]) + NO^{\bullet}$$

Figure 10 The chemical structure and of sodium nitroprusside and the release of nitric oxide

In addition to sodium nitroprusside, a wide variety of experimental nitric oxide donors are available, including *S*-nitroso-L-glutathione (GSNO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) that have been used for the investigation of the effects of nitric oxide on biofilm dispersal (Barraud et al 2006). SNP was selected for use in these

experiments, in preference to other available nitric oxide donors, for its potential suitability for application in the food production process, in terms of cost, stability during storage, ease of preparation of SNP solutions, and stability in solution when stored protected from light. In addition nitric oxide is released from SNP steadily over an extended period of time (Barraud et al 2009b) which, due to the short half-life of nitric oxide in biological systems (Ignarro et al 1993), is required for the delivery of nitric oxide to experimental systems for the contact treatment timings used in these investigations (up to 24 hours). SNP has also already been demonstrated to lead to biofilm dispersal events, and has therefore been used as the experimental nitric oxide donor in these investigations for proof of concept for use at the phylloplane and for the dispersal of *Salmonella* biofilms.

1.6 Aims and objectives of this work

Due to the increasing number of food poisoning outbreaks associated with the consumption of ready-to-eat products, and the high incidence of outbreaks associated with RTE leafy salad products (EFSA 2012), novel decontamination strategies for fresh produce surfaces are required in order to ensure product safety. Additionally, the re-discovery of the importance of matrix enclosed, surface attached, polymicrobial mode of growth, or biofilms, which are the preferred mode of growth for bacteria in nearly every environment known to man (Hall-Stoodley et al 2004), has led to new theories as to how zoonotic pathogens persist through the leafy salad production process.

As the understanding of the importance of bacterial biofilms has grown, so has the interest in the identification of biofilm dispersal agents which may be able to be used to remove bacteria from surfaces where other anti-microbial agents are currently failing. The molecular signalling molecule nitric oxide is one such molecule that has been shown to induce the dispersal of bacterial biofilms from surfaces (Barraud et al 2009b). The main aim of this project was therefore to investigate the use of nitric oxide for the dispersal of zoonotic pathogen contaminants from the phylloplane of leafy salads.

Using *Salmonella* spp. at the spinach phylloplane as a model, Chapter 3 aimed to use EDIC/EF microscopy to investigate the attachment and colonisation of the salad leaves by zoonotic pathogens, their interaction with existing microbial populations at the phylloplane, whether *Salmonella* spp. form biofilms in association with salad leaves, and whether nitric oxide can be used to induce the dispersal of *Salmonella* biofilms from the phylloplane.

Following the outcomes of Chapter 3, it was determined that further investigation was required to understand the interactions of *Salmonella* at the phylloplane and the influence of nitric oxide. Chapter 4 aimed to investigate surface colonisation strategies used by *S.* Typhimurium at the phylloplane, and at the abiotic surface, and to investigate the influence of nitric oxide on the formation and dispersal of *Salmonella* biofilms, whilst Chapter 5 aimed to further characterise the effect of nitric oxide treatment on *S.* Typhimurium using a powerful proteomics approach.

Additionally, it is recognised that existing microbial populations present on salad leaves will have a strong influence the interactions of zoonotic pathogens such as *Salmonella* spp. with salad leaves. Therefore finally, Chapter 6 aimed to investigate whether nitric oxide treatment causes the dispersal of indigenous phylloplane populations from spinach leaves.

Throughout these investigations, the future possibility of the application of nitric oxide treatment for use in an industrial food production environment was considered for experimental design for relevance to the production of leafy salads.

CHAPTER 2

Materials and Methods

2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 4. Stocks were routinely prepared by streak plating bacterial strains onto TSA (containing antibiotic supplement where appropriate, see Table 5) and growing overnight at 37°C before harvesting bacteria into vials containing Protect™ glycerol beads (Fisher, UK) for storage at -80°C, in accordance with manufacturer's instructions.

Strain(s)	Description	Reference
S. Typhimurium		
NCTC 12023	*S.* Typhimurium 12023	National Collection of Type Cultures
NCTC 12023 GFP	*S.* Typhimurium 12023 containing plasmid pFPV25.1 for GFP expression	(Beuzon et al 2000)
ATCC 14028	Wild-type *S.* Typhimurium, ATCC 14028	American Type Culture Collection
ATCC 14028 *flh*C	*S.* Typhimurium 14028, deficient in flagellar production	(Simon et al 2007)
S. Thompson		
RM2311 GFP	*S.* Thompson clinical isolate strain RM1987 containing plasmid pWM1007 for GFP expression	(Brandl and Mandrell 2002) (Miller et al 2000)

Table 4 Bacterial strains

2.1.1 *Salmonella* Typhimurium

Salmonella enterica subspecies *enterica*, serovar Typhimurium (hereafter *S.* Typhimurium) strain NCTC 12023 was obtained from the national collection of type cultures held by the UK health protection agency (HPA). *S.* Typhimurium strain NCTC 12023 containing plasmid pFPV25.1 was kindly provided by Professor D. Holden from the Centre for Molecular Microbiology and Infection at Imperial College, London.

Plasmid pFPV25.1 contains genes for the green fluorescent protein (GFP) originally isolated from the jellyfish *Aequorea Victoria* (Shimomura et al 1962) and for carbenicillin (carb) resistance to allow for phenotypic selection of *gfp* mutants. This strain is denoted *S.* Typhimurium 12023 GFP.

A flagellar deficient (*flh*C) mutant of *S.* Typhimurium strain ATCC 14028 and the corresponding wild-type parent strain were kindly provided by Professor Michael Mahan from the Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California. The flagellar deficient mutant contains a *lac*Z transcriptional fusion to the *flh*C gene which includes a kanamycin resistance cassette to allow for phenotypic selection of *flh*C mutants. These strains are denoted *S.* Typhimurium 14028 *flh*C and *S.* Typhimurium 14028 WT, respectively.

2.1.2 *Salmonella* Thompson

Salmonella enterica subspecies *enterica*, serovar Thompson (hereafter *S.* Thompson) clinical isolate strain RM1987 (Brandl and Mandrell 2002) containing plasmid pWM1007 (Miller et al 2000), denoted strain RM2311, was kindly provided by Professor R. Mandrell from the US Department of Agriculture. Plasmid pWM1007 contains genes for GFP and for kanamycin (kan) resistance to allow phenotypic selection of *gfp* mutants. This strain is denoted *S.* Thompson RM2311 GFP.

2.2 Preparation of inocula and culture media

2.2.1 Preparation of standard inocula

Salmonella strains were routinely cultured in Tryptone Soya Broth (TSB, Oxoid UK) supplemented with antibiotics for the phenotypic selection of mutants where necessary, as detailed in Table 5. A single bead was transferred from stock vials into 10 mL TSB and incubated at 37°C for approximately 20 hours. Overnight bacterial cultures were then removed from incubation and diluted in sterile distilled water (sdH_2O) to an optical density (OD) of 0.1 as determined by spectrophotometric measurement at 620 nM (Jenway 6300 Spectrophotometer) to give a concentration of

approximately $1x10^8$ CFU mL^{-1} bacteria. Confirmation of concentrations was routinely carried out by performing serial 1 in 10 dilutions of these cultures in phosphate buffered saline (PBS) and plating 50 µL aliquots onto Tryptone Soya Agar (TSA, Oxoid UK). TSA plates were incubated at 37°C for 24 hours before enumeration of colonies. Cultures were further diluted 1:100 into experimental assay media to give final inocula containing approximately $1x10^6$ CFU mL^{-1}.

Strain(s)	Antibiotic	Concentration
S. Typhimurium		
NCTC 12023 GFP	Carbenecillin (carb)	100 mg mL^{-1}
ATCC 14028 *flh*C	Kanamycin (kan)	50 mg mL^{-1}
S. Thompson		
RM2311 GFP	Kanamycin (kan)	50 mg mL^{-1}

Table 5 Antibiotic supplements

2.2.2 Preparation of culture media

2.2.2.1 Nutrient broths

Tryptone Soya Broth (TSB)

TSB (Oxoid, UK) was routinely used for the growth of bacterial cultures, as described in Section 2.2.1. TSA is a high nutrient broth containing casein digest (17 g L^{-1}), soya bean digest (3 g L^{-1}), sodium chloride (5 g L^{-1}), dipotassium hydrogen phosphate (2.5 g L^{-1}) and glucose (2.5 g L^{-1}), and is a general purpose broth suitable for the growth of a range of bacteria (Oxoid Microbiology Products, UK). TSB was supplied as dehydrated culture media, made up from powder into distilled water (dH_2O) and sterilised by autoclaving at 121°C for 15 minutes.

Luria-Bertani Broth (LB)

LB broth (ForMedium™, UK) was routinely used for the growth of *Salmonella* biofilms in 6-well plates, as described in Section 2.7. LB broth is a high nutrient broth containing tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}) and sodium chloride (10 g L^{-1}) originally formulated for the maintenance of *E. coli* strains (ForMedium™, UK). LB broth was supplied as dehydrated culture media, made up from powder to 1/5 of the recommended strength in dH_2O for growth of abiotic surface biofilms (Barraud et al 2006) to give final concentrations of 2 g L^{-1} tryptone, 1 g L^{-1} yeast extract and 2 g L^{-1} sodium chloride, and sterilised by autoclaving at 121°C for 15 minutes.

2.2.2.2 Agars

Tryptone Soya agar (TSA)

TSA (Oxoid, UK) was routinely used for confirmation of inoculum concentrations, as described in Section 2.2.1. TSA is a high nutrient agar containing casein digest (15 g L^{-1}), soya bean digest (5 g L^{-1}), sodium chloride (5 g L^{-1}) and agar (15 g L^{-1}), suitable for the culture of a range of bacteria (Oxoid, UK). TSA was supplied as dehydrated culture media, made up from powder into dH_2O and sterilised by autoclaving at 121°C for 15 minutes. Molten agar was cooled to approximately 45°C in an incubator before pouring plates, which were stored refrigerated for up to 14 days before use. 50 µL aliquots of bacterial suspensions were spread evenly over 5.5 cm diameter plates (VWR, UK), dried under sterile air, and grown at 37°C for 24 hours before enumeration of colonies.

Rambach® agar

Rambach® agar (M-Tech Diagnostics, UK) was routinely used for culture-based enumeration of *Salmonella* colonies following recovery of bacteria from the phylloplane by pulsification (see Section 2.5). Rambach® agar is a chromogenic media suitable for the detection of *Salmonella* spp. in environmental samples (Rambach 1990). Rambach® agar exploits the novel ability of *Salmonella* spp. to metabolise propylene glycol in the media leading to an acidification and precipitation of the dye, Neutral Red, which causes *Salmonella* colonies to turn a distinctive red-pink colour (Rambach 1990), as shown in Figure 11 A.

Rambach® agar was supplied in pre-weighed portions of dehydrated culture media with accompanying aliquots of liquid supplement, and made up according to manufacturer's instructions by dissolving powder into pre-sterilised dH_2O, heating in a microwave until dissolved but not boiling, and adding the liquid supplement after the heating step. Molten agar was cooled to approximately 45°C in an incubator before pouring plates, which were stored refrigerated for up to 14 days before use. 50 µL aliquots of bacterial suspensions were spread evenly over 5.5 cm diameter plates (VWR, UK), dried under sterile air, and grown at 37°C for 24 hours before enumeration of *Salmonella* colonies.

R2A Agar (R2A)

R2A (Oxoid, UK) was routinely used for culture-based enumeration of indigenous phylloplane bacteria following recovery from the phylloplane by pulsification (see Section 2.5). R2A is a low nutrient medium containing yeast extract (0.5 g L^{-1}), proteose peptone (0.5 g L^{-1}), casein hydrolysate (0.5 g L^{-1}), glucose (0.5 g L^{-1}), starch (0.5 g L^{-1}), di-potassium phosphate (0.3 g L^{-1}), magnesium sulphate (0.024 g L^{-1}), sodium pyruvate (0.3 g L^{-1}) and agar (15 g L^{-1}), designed for the recovery of sub-lethally stressed cells from environmental samples (Reasoner and Geldreich 1985). Mixed phylloplane isolates growing on R2A are shown in Figure 11 B. R2A was supplied as dehydrated culture media, made up from powder into dH_2O and sterilised by autoclaving at 121°C for 15 minutes. Molten agar was cooled to approximately 45°C in an incubator before pouring plates, which were stored refrigerated for up to 14 days before use. 100 µL or 50 µL aliquots of bacterial suspensions were spread evenly over 9 cm or 5.5 cm diameter plates, respectively (VWR, UK), dried under sterile air, and bacteria grown at 22°C for 4 days before enumeration of colonies.

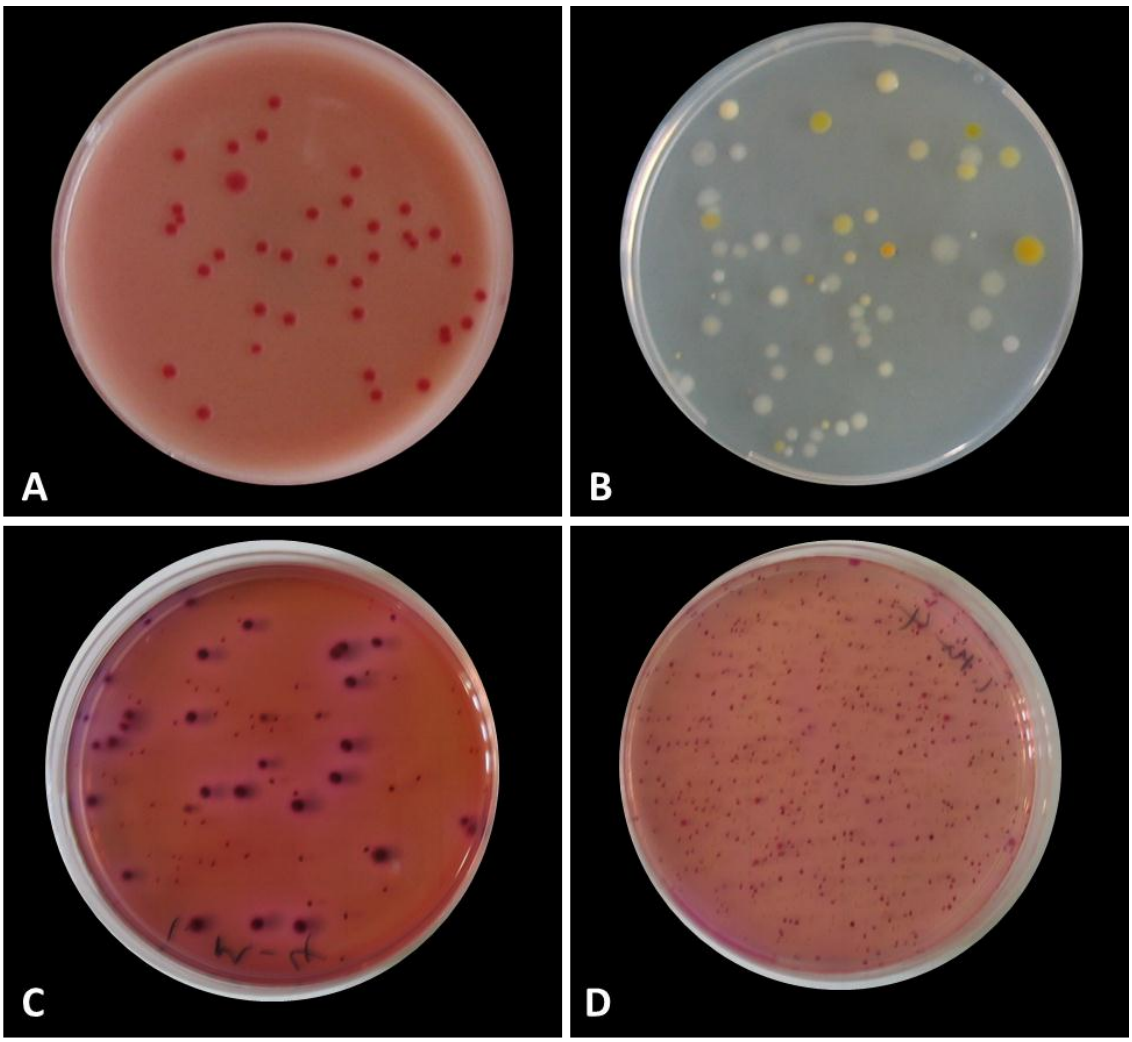


Figure 11 Nutrient agars used for these investigations

The distinct red/pink colonies produced by *Salmonella* colonies growing on Rambach agar are shown in Figure 11 A, mixed phylloplane isolates growing on R2A are shown in Figure 11 B, and mixed bacterial isolates growing on VRBA are shown in Figure 11 C (VRBA only) and D (TSA with VRBA overlay)

Violet Red Bile Agar (VRBA)

VRBA was investigated for its suitability for the assessment of coliforms in environmental samples isolated from field grown spinach leaves (see Section 6.3.4.2). VRBA is a colorimetric agar containing yeast extract (3 g L^{-1}), peptone (7 g L^{-1}), sodium chloride (5 g L^{-1}), bile salts (1.5 g L^{-1}), lactose (10 g L^{-1}), neutral red (0.03 g L^{-1}), crystal violet (0.002 g L^{-1}), and agar (12 g L^{-1}), designed for the detection of coliforms in complex mixtures such as milk (Oxoid, UK). Coliform bacteria growing on VRBA form large colonies of puple/pink colour surrounded by purple halo's, as shown in Figure 11 C. VRBA was supplied as dehydrated culture media, made up from powder into dH2O

and sterilised by autoclaving at 121°C for 15 minutes. Molten agar was cooled to approximately 45°C in an incubator before use.

VRBA pour plates were prepared by mixing 500 µL aliquots of bacterial suspensions (following serial dilution in PBS) with 19 mL VRBA. Pour plates were allowed to solidify for 2 hours +/- 30 minutes, before adding a 10 mL covering layer of VRBA, allowing to solidify, and incubating at 37°C for up to 72 hours before enumeration of coliform colonies. VRBA was also used in combination with TSA, as shown in Figure 11 D, to improve recovery of sub-lethally stressed cells in environmental samples from field grown spinach leaves (Feng et al 2002). Pour plates were prepared by mixing 500 µL aliquots of serially diluted samples with cooled molten 20 mL TSA (prepared as described above) which was allowed to solidify for 2 hours +/- 30 minutes before adding a 10 mL covering layer of VRBA for the selective detection of coliforms. Plates were incubated at 37°C for up to 72 hours before enumeration of coliform colonies.

Water Agar

Technical agar (Agar Technical (Agar no. 3), Oxoid, UK), was used for the fixation of leaf discs to microscopy slides and to maintain hydration of leaf samples during microscopy, as described in Section 2.9.3. Agar Technical (Agar no. 3) was supplied as dehydrated culture media, made up from powder to approximately 2% w/v in sdH_2O, and heated in the microwave until dissolved. This was denoted Water agar, and cooled to approximately 45°C on the bench before use.

2.3 Leaf samples

2.3.1 Laboratory samples

Samples of fresh, unwashed spinach leaves for use in laboratory experiments were obtained directly from Vitacress Salads ltd. St Mary Bourne, UK. All samples are of market specification, and sourced from Vitacress farms across the UK and Europe from plants not more than six weeks of age. Undamaged, flat, whole leaves were selected from each sample for analysis. Samples were routinely stored at 4°C for not more than 48 hours from the time of collection prior to use.

2.3.2 Field samples

Field sites in the south of England were selected for the field studies described in Chapter 6. Preliminary field investigations were carried out at Pinglestone Farm, Arlesford, Hampshire in August 2011 (see Section 6.3.4.1) whilst the main field investigations described in Section 6.3.4.3 were carried out at Mullins Farm, Pewsey, Wiltshire in September 2012.

An area of a commercial spinach field was allocated to the study and spinach was grown using standard agricultural practices by Vitacress Salads Ltd. Treatment and sampling plots were randomised within the defined area as described in Figure 12. For the main field trial (see Section 6.3.4.3) spinach plants were treated with nitric oxide, via exogenous application of SNP, over three consecutive days, with triplicate samples harvested at 1 and 24 hours post-treatment from each of three spinach beds containing one sampling block per treatment group to give a total of nine replicate samples per treatment group. For preliminary field investigations, triplicate samples were harvested from one spinach bed containing one sampling block per treatment group to give a total of three replicate samples per treatment group (see Section 6.3.4.1). A buffer area was maintained around the treatment plots to separate the trial area from the commercial spinach crop. Following completion of sampling, spinach crops within the trial area were destroyed.

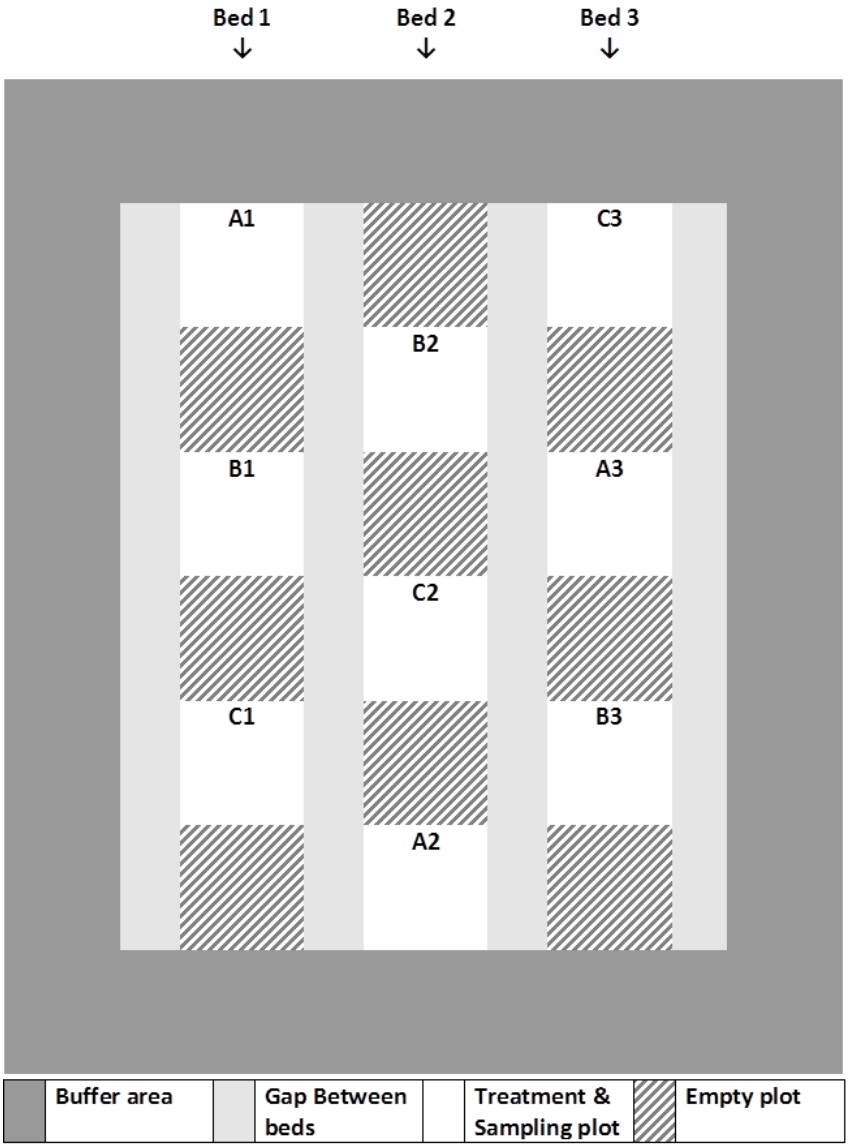


Figure 12 Treatment and sampling plot layout for field samples

Spinach samples were harvested from randomised treatment plots within a defined area of the spinach crop field. Samples in Bed 1 were harvested on Day 1, Bed 2 on Day 2, and Bed 3 on Day 3. Sampling plots labelled A, B and C correspond to Control (sdH_2O), 500 nM SNP and 1 µM treatment plots, respectively.

Leaves were selected at random from a 50 cm^2 area in the centre of each designated sampling plot and harvested by hand from plants within this area. Plants were not more than six weeks of age at the time of harvest. Sampling was carried out as aseptically as possible using a knife which was sterilised using 70% ethanol in between samples. Care was taken to minimise contamination from the soil during harvest. Samples were collected into sterile stomacher bags and placed immediately into a cold box containing cool packs for transport back to the laboratory.

Field leaf samples were transported back to the laboratory within 4 hours of harvest and bacteria recovered from leaves by pulsification, as described in section 2.7 on the day of harvest before transferring resulting suspensions into refrigerated storage pending enumeration of culturable cells. Samples for analysis by microscopy (see Section 2.9.3) were also analysed on the day of harvest.

2.4 Inoculation of spinach leaves with *Salmonella* spp.

For experiments in which *Salmonella* spp. were artificially introduced to the phylloplane (see Chapters 3 and 4), individual, whole, flat, undamaged spinach leaves were secured to the lid of sterile straight containers using Blu Tack® such that leaves were suspended in 100 mL sdH_2O inocula containing $1x10^6$ CFU mL^{-1} bacteria, prepared as previously described (Section 2.2.1). Leaves were suspended in the centre of each straight container to allow bacteria equal access to both the upper and lower leaf surface for attachment (see Figure 13). Leaves were incubated at 22, 30 or 37°C for between 1 hour and 48 hours, as detailed in individual results sections.

Figure 13 Method for the artificial inoculation of leaves with *Salmonella* spp., and for the treatment of individual spinach leaves with SNP

2.5 Recovery of bacteria from the phylloplane

Where it was necessary to remove bacteria from the phylloplane prior to analysis by culture based methods (Section 2.2.2.2) or for enumeration by microscopy (Section 2.9.1) recovery was carried out using a Pulsifier® (Microgen Bioproducts, UK). The Pulsifier® causes the release of surface attached debris, including bacteria, from the phylloplane using a combination of vigorous sample mixing and the application of shock waves, which are delivered by the oscillation of a metal ring moving backwards and forwards inside the chamber (see Figure 14). Leaf samples were processed in sdH_2O solution inside a Stomacher® bag (Seward, UK) which sit inside the metal ring for agitation of leaves and removal of surface attached bacteria whilst the leaf sample remains intact (Fung et al 1998).

Figure 14 The Pulsifier® for recovery of bacteria from leaf samples (MicrogenBioproducts 2003)

For *Salmonella* investigations (Chapters 3 and 4) individual leaf samples were removed from inoculation containers, rinsed by dipping three times in sdH_2O, transferred to Stomacher® 80 bags (Seward, UK) containing 50 mL sdH_2O and Pulsified for 30 s each. 10 - 20 mL aliquots of resulting suspensions were transferred to individual sterile universal tubes and placed in refrigerated storage prior to enumeration by either

culture on Rambach agar (see Section 2.2.2.2) or by microscopy (see Section 2.9.1) within 24 hours of recovery from the phylloplane.

For investigations of indigenous phylloplane bacteria (Chapter 6) 25 g leaf samples were transferred to Stomacher® 400 bags (Seward, UK) containing 225 mL sdH_2O and rinsed on a gyratory rocker (Stuart™ Scientific Gyro rocker STR9) at 60 rpm for 5 minutes. The rinse procedure was repeated twice to remove planktonic and loosely adhered debris (including bacteria) from the phylloplane. Leaf samples were then transferred to fresh Stomacher® 400 bags containing 225 mL sdH_2O and Pulsified for 30 s each. 10 - 20 mL aliquots of resulting suspensions were transferred to individual sterile universal tubes and placed in refrigerated storage pending enumeration by either culture on R2A (see Section 2.2.2.2) or by microscopy (see Section 2.9.1).

2.6 Preparation and delivery of sodium nitroprusside

A 5 mM working stock solution of sodium nitroprusside (SNP) was made in sdH_2O on each day of use, and stored protected from light. Dilutions of working stock were prepared in sdH_2O as required for addition to treatment assays at final concentrations ranging from 50 nM to 10 µM as detailed in individual results sections.
Delivery of SNP was achieved using the methods described below.

For laboratory experiments to assess the effect of SNP on attachment, early colonisation and detachment of *Salmonella* spp. from spinach leaves (Chapter 3), individual leaves were suspended in SNP solutions in straight containers as shown in Figure 13. To assess the effect of SNP on attachment and early colonisation, SNP was added to surrounding media (sdH_2O) during inoculation of leaves with *Salmonella* spp. (see Section 2.4). For detachment experiments, leaves were inoculated with *Salmonella* spp. for 24 hours to allow for establishment of bacteria, before transferring leaves to SNP solutions for 1 and 24 hours for the assessment of its effect on detachment of *Salmonella* from the phylloplane. Control leaves were suspended in sdH_2O only with no SNP for comparison.

For initial laboratory experiments to assess the effect of SNP on detachment of indigenous bacteria from spinach leaves (Section 6.3.1 and 6.3.2), 10 g samples of spinach leaves were immersed in 225 mL sdH_2O in sterile in Stomacher® 400 bags (Seward, UK) in presence of SNP. Control samples were immersed in 225 mL sdH2O only with no SNP for comparison. For development of this method for application for use in the field (Section 6.3.3), individual spinach leaves from a 25g samples were dip inoculated in SNP solution to mimic spray inoculation in the field. Samples were then pooled and transferred into sterile Stomacher® 400 bags for the required incubation period. Control leaves were subjected to the same procedure but dipped in sdH_2O only with no SNP for comparison.

For field experiments 5 mM stock SNP solutions were prepared in the laboratory on each day of the experiment, and stored protected from light until use. Spray bottles were sterilised by washing with 70% ethanol, followed by thorough rinsing in filter sterilised water from a Milli-Q® filtration unit fitted with a 0.1 µM filter, and filled with sdH_2O in the laboratory. SNP spray solutions of 500 nM to 10 µM were made up as required, immediately prior to delivery to leaves. Triplicate plots of spinach plants, as described in Section 2.3.2, were sprayed by hand with 1 L SNP solution each to ensure complete coverage of leaves. Control plots were subjected to the same procedure but sprayed with sdH2O only with no SNP for comparison.

For investigations into the effect of nitric oxide on *Salmonella* biofilms at the abiotic surface (Chapter 4), SNP was added to biofilm growth media at final concentrations ranging from 50 nM to 1 µM. For the assessment of the effect of SNP on colonisation of the abiotic surface, biofilms were grown for 24 hours in the presence of SNP. Control biofilms were grown in growth media only (with no SNP) for comparison. For the assessment of SNP on detachment and dispersal of biofilms from the abiotic surface, biofilms were grown for 24 hours, or for 7 days, in growth media only to allow for biofilms to establish, before the replacement of media with media containing SNP. Media for control biofilms was replaced with new media only (with no SNP) for comparison.

For investigations into the effect of nitric oxide on protein expression by *S.* Typhimurium 12023 (Chapter 5), SNP was included in bacterial culture media (TSB) at a final concentration of 500 nM SNP, and bacteria cultured overnight (16 hours) as described in Section 2.2.1.

2.7 Growth of abiotic surface *S.* Typhimurium biofilms

For growth of *Salmonella* biofilms at the abiotic surface (Chapter 4), standard inocula of *S.* Typhimurium 12023 GFP, *S.* Typhimurium 14028 WT, and *S.* Typhimurium 14028 *flh*C were prepared as described in Section 2.2.1. Biofilms were grown in static cultures at 22, 30 or 37°C in 6-well plates (Griener Bio-One, UK) containing 5 mL 1/5 strength LB broth ($LB_{1/5}$), or 5 mL sdH_2O. Low nutrient conditions were selected to mimic biofilm growth in an environmental situation such as the phylloplane. Biofilms were grown for between 1 hour and 7 days, as detailed in Chapter 4. EDIC/EF microscopy was used for the visualisation of biofilm morphology and for the assessment of percentage surface coverage, as detailed in Section 2.9.

2.8 Proteomic Investigations

S. Typhimurium NCTC 12023 was cultured as described in Section 2.2.1 in the presence or absence of 500 nM SNP, delivered as described in Section 2.6. Bacterial cells were harvested by centrifugation at 4000 rpm for 10 minutes and resuspended in 1.6 mL 0.1 M Triethylammonium bicarbonate buffer (Sigma-Aldrich, UK). Resuspended samples were transferred to Lysing matrix B tubes containing 0.1 mm silica spheres (MP biomedicals, UK). Cell lysis was performed by homogenisation using a Thermo Savant FastPrep® 120 cell distrupter system. Samples were run for 6 cycles of 30 seconds each with 30 seconds cooling on ice in between each cycle. Sample tubes were left on ice to settle for 10 minutes before carefully pipetting off the supernatant into sterile microcentrifuge tubes. Samples were spun at 5000 rpm for 5 minutes to remove any remaining insoluble material and the resulting supernatant filtered through a 0.22 µM low protein binding filter (Millipore, UK). Protein quantification was carried out using the BCA (bicinchoninic acid) protein assay (Thermo Fisher Scientific, UK).

For gel electrophoresis, 2.5% mercaptoethnaol (Sigma-Aldrich, UK) was added to each sample before the addition of NuPage® LDS sample buffer (Invitrogen life technologies, UK) and NuPage® Antioxidant (Invitrogen life technologies, UK), as per manufacturer's instructions, and heated at 70°C in a water bath for 10 minutes, before transferring to storage on ice. Samples were run on NuPage® Novex® 4-12% polyacrylamide mini-gels (Invitrogen life technologies, UK) for 55 minutes at 200 volts using NuPage® MOPS SDS running buffer (Invitrogen life technologies, UK). Gels were stained with the coomassie blue-based SimplyBlue™ SafeStain (Invitrogen life technologies, UK) for approximately 1 hour followed by two 1 hour rinses, and imaged using a transilluminator (Syngene G Box, Syngene UK).

Proteomic analysis was carried out using label free mass spectrometry (Waters SYNAPT®G2-S HDMS™) by the Centre for Proteomic Research, University of Southampton, UK. Data was returned and analysed using Microsoft Excel and the Gene Ontology tool Blast 2GO® provided by BioBam® Bioinformatics, Spain. Blast 2GO® performs BLAST searches on sequence data using the universal protein accession numbers provided for resulting proteins identified in the analysis by the Centre for Proteomic Research, as described above. The bioinformatics tool then tags each sequence with relevant descriptions of the molecular function performed by that protein (or peptide within a protein), the biological process that protein or peptide is involved in, as well as the cellular location of the protein. These gene ontology descriptions, or GO terms, are universal descriptions as agreed by the gene ontology consortium. As displayed in Section 5.3.2, these descriptions can then be used to group protein data for interpretation in graphical format. Full tables of protein identities and GO terms are provided in Appendix 3.

2.9 Episcopic differential interference contrast microscopy coupled with epifluorescence

Episcopic differential interference contrast microscopy, or EDIC, is an advanced light microscopy technique that has been used in these investigations for the enumeration of total bacteria following recovery of cells from the phylloplane (Section 2.9.1), for the

visualisation of biofilm morphology and quantification of percentage surface coverage for *Salmonella* biofilms using the abiotic 6-well plate assay (Section 2.9.2), and for the direct visualisation of bacteria on spinach leaves (Section 2.9.3). EDIC can be coupled with fluorescence techniques to aid the visualisation of fluorescently stained bacteria in complex mixtures, or at complex biotic surfaces making it an excellent tool for the study of phylloplane bacteria (Warner et al 2008).

The EDIC microscope is a modification of the Nomarski DIC (differential interference contrast) light microscope that provides a cost effective, minimal sample preparation alternative to techniques such as Environmental Scanning Electron Microscopy (ESEM) and Scanning Confocal Laser Microscopy (SCLM) (Keevil 2003). The EDIC microscope (Nikon Eclipse LV100, custom modified by Best Scientific, UK), contains a Nomarski prism to create a pseudo-3D image of the sample surface, as for conventional DIC microscopy, allowing high image contrast and excellent resolution of samples in the z-plane. However, in preference to the use of transmitted light used for conventional DIC microscopy, light is delivered to the sample surface episcopically ('from above') to provide detailed visualisation of surface topography for opaque samples that cannot otherwise be viewed using transmitted light alone. A diagrammatic representation of the light path of an EDIC microscope is shown in Figure 15 (Keevil and Walker 1992).

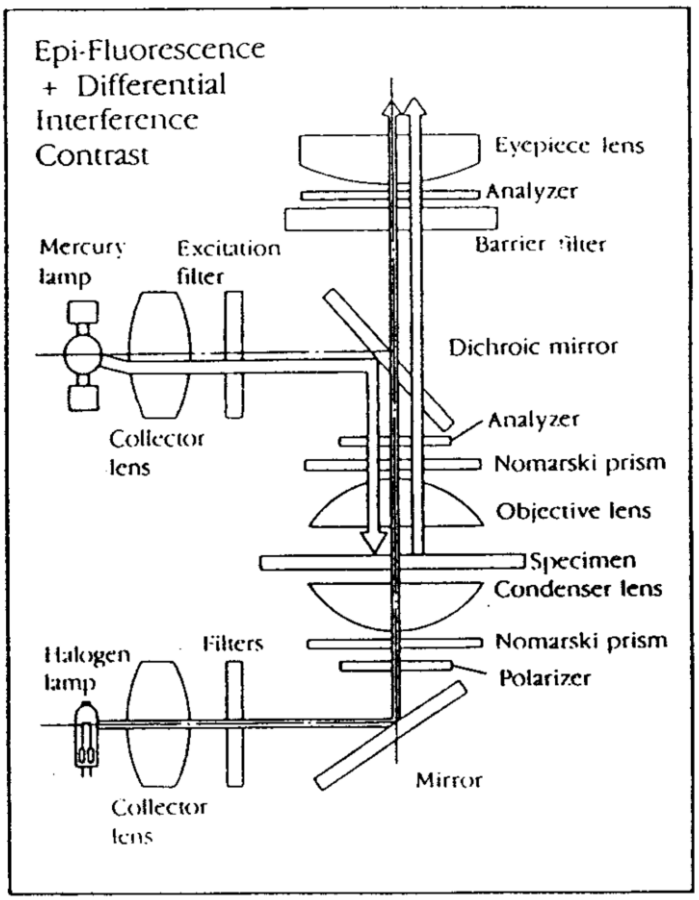


Figure 15 The light path of a DIC microscope

(image from (Keevil and Walker 1992))

For EDIC microscopy, light is delivered to samples from a metal halide (mercury) lamp via a dichroic mirror, polariser (analyser), Nomarski prism and objective lens. The use of long working distance (LWD) lenses with EDIC microscopy allows samples to be visualised in their natural, unaltered state, with minimal sample preparation and without the need for coverslips or oil (Keevil and Walker 1992). The extensive sample preparation required for alternative, high resolution microscopy methods such as Scanning Electron Microscopy (SEM) can lead to the degradation of sample integrity and introduce artefacts, particularly to samples with high water content such as biofilms, but this is not the case for EDIC microscopy, making EDIC microscopy an ideal tool for the study of both biofilms (Keevil 2003) and phylloplanes (Warner et al 2008). An EDIC image of the spinach phylloplane, showing clear 3-dimensional visualisation of leaf surface topography, leaf epidermal cells, leaf surface debris and presumptive bacterial rods residing in the margins between epidermal cells (white arrows), is provided in Figure 16.

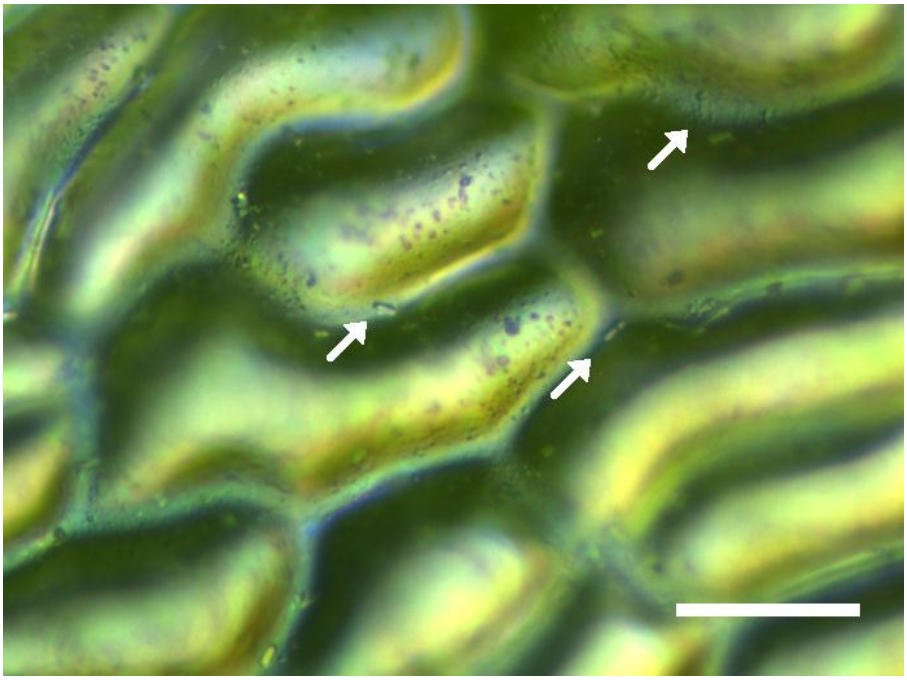


Figure 16 Visualisation of the spinach phylloplane by EDIC microscopy

Representative EDIC micrograph of a minimally processed spinach leaf clearly showing the topography of the leaf surface, epidermal cells and leaf surface debris, including presumptive bacterial rods as indicated by white arrows. Bar = 50 microns

In addition to the visualisation of opaque samples using EDIC microscopy alone, this technique can be coupled with epifluorescence (EF) to allow the visualisation of bacteria on surfaces *in situ* to show, for example, localisation of bacteria in relation to leaf surface structures (Warner et al 2008), as described in Section 2.9.3. For EF, the EDIC microscope is fitted with filter blocks (Figure 17) containing dichroic mirror, excitation filter and emission filter for the selection of specific wavelengths of light to excite and detect specific fluorophores. For these investigations the nucleic acid stains Syto®-9 (excitation max 485 nm, emission max 498 nm) and 4',6-diamidino-2-phenylindole (DAPI, excitation max 358 nm, emission max 461 nm), as well as *Salmonella* strains consituitively expressing GFP (excitation max 395 nm, emission max 509 nm) were used to visualise and quantify bacterial biofilms at both the abiotic surface and at the phylloplane (see Sections 2.9.1 – 2.9.3).

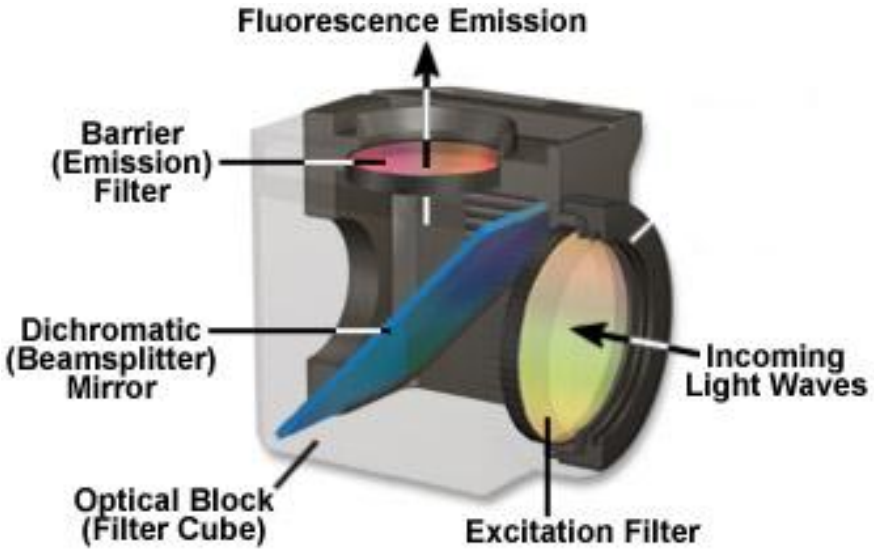


Figure 17 A schematic representation of the fluorescence filter blocks used to select specific excitation and emission wavelengths for fluorescence imaging (image from Nikon MicroscopyU)

Microscopy images were captured using a computer controlled digital (CCD) camera (Roper Industries, UK) mounted on the EDIC microscope. ImagePro® plus software (MediaCybernetics) was used for image capture. ImageJ software (MacBiotronics) was used for further processing of microscopy images where required. Further processing of images included the decreasing of background fluorescence for EF phylloplane micrographs to remove interference from leaves to facilitate visualisation of bacteria at the phylloplane, for the creation of image overlays (see Figure 18), for the false colouration of EDIC phylloplane micrographs (red) to enhance the visualisation of bacterial cells expressing GFP (Chapter 3), and for the quantification of percentage surface coverage for abiotic surface biofilms (see Section 2.9.2).

2.9.1 Enumeration of total bacteria following recovery from the phylloplane

Total numbers of indigenous phylloplane bacteria following recovery from spinach leaves by pulsification (see Section 2.5) were quantified using EDIC/EF microscopy. Suspensions of recovered samples were serially diluted and triplicate 1 mL aliquots from each sample were separated into individual sterile 1.5 mL microcentrifuge tubes (Fisher Scientific, UK) and stained with DAPI (Sigma-Aldrich, UK). 100 µL DAPI solution (28.5 µM) was added to each tube and bacteria stained at room temperature in the dark for 20 minutes before being filtered onto 0.22 µM black nucleopore® membrane filters (Fisher Scientific, UK). Filters were observed at x1000 magnification using oil immersion and a minimum 10 fields of view containing at least 200 cells were analysed for each filter.

2.9.2 Visualisation and quantification of percentage surface coverage for abiotic surface *S.* Typhimurium biofilms

Salmonella biofilms were grown at the abiotic surface as described in Section 2.7. EDIC/EF microscopy was used for the visualisation of biofilm morphology (Figure 18) and for the quantification of percentage surface coverage.

For *S.* Typhimurium 12023 GFP biofilms, media was carefully pipetted from each well and biofilms gently rinsed three times with 1 mL sdH_2O before drying under sterile air and observing by EDIC/EF microscopy at x500 or x1000 magnification using long working distance lenses. For *S.* Typhimurium 14028 WT and *S.* Typhimurium 14028 *flh*C biofilms, media was carefully pipetted from each well and biofilms gently rinsed twice with 1 mL sdH_2O before staining biofilms with Styo®-9 (Invitrogen, UK). 5 µM working stock solutions of Syto®-9 were made up on each day of use and stored protected from light. Biofilms were stained with 100 µL working stock per well for 1 hour in the dark. Stained biofilms were rinsed twice in 1 mL sdH_2O before drying under sterile air and observing by EDIC/EF microscopy at x500 or x1000 magnification using long working distance lenses.

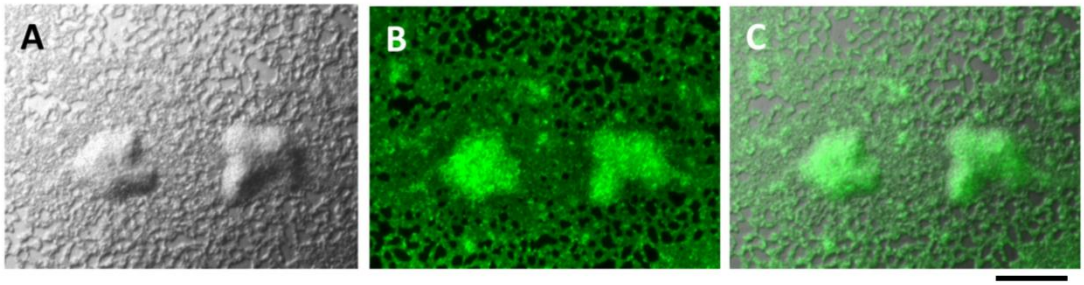


Figure 18 EDIC/EF microscopy for the visualisation of *Salmonella* biofilms at the abiotic surface

Representative micrographs of an *S.* Typhimurium 12023 GFP biofilm grown in $LB_{1/5}$ are shown. Three-dimensional morphology of biofilm microcolonies can clearly be seen using EDIC (A) and fluorescence imaging confirms the biofilm to consist of GFP expressing *S.* Typhimurium (B). An EDIC/EF image overlay, created using ImageJ software (MacBiotronics), shown biofilm morphology and constitutive fluorescence together to demonstrate localisation of cells within the biofilm matrix (C). Bar = 50 microns

Quantification of percentage abiotic surface coverage by *Salmonella* biofilms was carried out using ImageJ software (MacBiotronics). EF micrographs were converted to 16 bit monochrome images and and percentage surface coverage calculated using pixel data as a percentage of the whole field of view. At least 10 randomly selected fields of view per sample, from triplicate samples, were used to estimate percentage biofilm surface coverage.

2.9.3 EDIC microscopy for the direct visualisation of bacteria at the phylloplane

Individual spinach leaves were rinsed by dipping in sdH_2O and dried under sterile air. Leaf discs (1 cm diameter) were then cut using a cork borer which was sterilised with 70% ethanol in between each use, and mounted onto slides using a 100 µL droplet of water agar (approximately 2% w/v) (Brandl and Mandrell 2002). For visualisation of *S.* Typhimurium 12023 GFP at the spinach phylloplane (Chapter 3) samples were directly observed by EDIC/EF microscopy at x500 and x1000 using long working distance lenses. For visualisation of indigenous bacterial communities at the spinach phylloplane (Chapter 5), mounted leaf discs were stained with DAPI (Sigma-Aldrich, UK). 50 µL DAPI solution (28.5 µM) was pipetted onto each leaf disc and samples stained at room temperature in the dark for 15 minutes. Leaf discs were then rinsed

twice with 100 µL sdH_2O and dried under sterile air. All samples were maintained in cool storage until microscopy within 2 hours of sample preparation.

2.10 Statistical analyses

Experimental procedures were performed independently on a minimum of two occasions where possible. Triplicate replicate samples per occasion were analysed to ensure sufficient data for statistical analysis. Unless otherwise specified, statistical analyses were carried out using analysis of variance (ANOVA) following data transformation where necessary using MiniTab® 16 statistical software.

CHAPTER 3

Investigating the use of nitric oxide for the control of *S.* Typhimurium and *S.* Thompson at the spinach phylloplane

3.1 Introduction

The contamination of leafy salads with zoonotic pathogens has caused a number of serious food poisoning outbreaks (see Table 1). Of particular note is the high incidence of *Salmonella* infection associated with RTE leafy salads (Tirpanalan et al 2011). Contamination of salad leaves can occur pre- or post-harvest from a number of sources, including contact with surface water run-off from contaminated land, irrigation with contaminated ground water and direct defecation by wild animals and birds (Heaton and Jones 2008, Solomon et al 2002a, Solomon et al 2002b). As described in Section 1.2.1.2, whilst it is possible to mitigate the risk of contamination occurring, it is difficult to eliminate it entirely. Once contamination has occurred however, zoonotic pathogens are able to remain strongly associated with leaves and persist throughout the food production process, allowing them to remain viable to the point of product sale where they present food safety hazard (Brandl 2006a). The emergence and popularity of RTE leafy salad products and the discovery of the ability of zoonotic pathogens to survive and persist in the phyllosphere (Brandl 2006b) has highlighted the importance of lettuce as a vector for the transmission of food poisoning bacteria to the food chain (Little and Gillespie 2008). The high and increasing number of food poisoning outbreaks associated with fresh produce (EFSA 2012, Sivapalasingam et al 2004) is evidence that current sanitisation strategies are insufficient and that new methods of decontamination for leafy salads are required to ensure food safety.

3.1.1 Colonisation of spinach leaves by *Salmonella* spp.

Despite it being recognised that zoonotic pathogens such as *Salmonella* spp. cause food poisoning outbreaks when associated with leafy salads, remarkably little is known about the interaction of zoonotic pathogens with leaf surfaces (Brandl 2006b). The colonisation strategies used by bacteria that confer such successful existence at the phylloplane will ultimately determine pathogen survival in the harsh and variable environmental conditions found in the phyllosphere, and the ability of human pathogenic bacteria to persist through the food production process and pose a food safety risk (Brandl 2006a). Further understanding of these strategies is essential for the development of novel decontamination strategies to improve food safety.

This lack of understanding is in part due to limitations of methods available for the study of bacteria at the phylloplane (Morris et al 1998). As described in Section 2.9, EDIC/EF microscopy is an excellent tool for the visualisation of bacteria *in situ* at leaf surfaces and was used here for the visualisation of GFP labelled strains of *S.* Typhimurium and *S.* Thompson in association with the spinach phylloplane (Section 3.3.1).

EDIC/EF microscopy is also an excellent tool for the visualisation of bacterial biofilms (Keevil 2003). As described in Section 1.4 biofilms are ubiquitous in the environment and are notoriously difficult to decontaminate from surfaces (Costerton et al 1995). In addition, environmental biofilms can act as reservoirs for zoonotic pathogens, providing a sheltered environment from external stresses such as shear forces and UV light (Giao et al 2009, Keevil 2002). As described in Section 1.4.3, phylloplane biofilms have been demonstrated at leaf surfaces including spinach, lettuce, basil, parsley and cabbage (Morris et al 1997, Warner et al 2008). For zoonotic pathogens at the phylloplane however, few studies have investigated their existence (Brandl 2006b). For biofilms of *Salmonella* spp. at the phylloplane, even fewer studies exist. Brandl and Mandrell have demonstrated the presence of aggregates of *S.* Thompson at the cilantro phylloplane, whilst Kroupitski *et al*. demonstrated the presence of *S.* Typhimurium biofilms at the Romaine lettuce phylloplane (Brandl and Mandrell 2002, Kroupitski et al 2009b).

3.1.2 Investigating nitric oxide treatment for use as an intervention strategy for the contamination of spinach leaves by *Salmonella* spp.

The dispersal of biofilms from surfaces has recently been demonstrated to occur as a response to the production of reactive oxygen and nitrogen species within biofilm microcolonies (Webb et al 2003b). This process can be triggered in the model biofilm species *P. aeruginosa* by exogenous treatment with the nitric oxide donor SNP, and it is thought that this process also causes the reduction in surface coverage seen for biofilms of *E. coli*, *V. cholerae*, *S. marcescens*, *F. nucleatum, B. licheniformis* and *S. epidermidis* following exogenous SNP application (Barraud et al 2006, Barraud et al 2009b). As described in Section 1.5, it is thought that nitric oxide signalling may be

conserved across many bacterial genera. The use of nitric oxide to induce the dispersal of biofilms from surfaces is being investigated for applications ranging from use in a medical setting for use against *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients (Barraud et al 2012, Howlin et al 2011) to use an industrial setting for the decontamination of drinking water biofilms (Barnes et al 2013, Dye unpublished), and for its antifouling ability for use in marine biosensors (Walker unpublished).

In the case of *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients, biofilm formation provides a mechanism by which bacteria are protected from the cells of the immune system and from treatment with antibiotic concentrations designed for the killing of planktonic cells, to which cells residing within a biofilm may be more resistant (Hassett et al 2002). Bacteria within a biofilm have been shown to be up to 1000 times more resistant to antibiotics than their planktonic counterparts (Ceri et al 1999). In addition to the problems associated with the presence of these thick biofilm structures in the lungs, these biofilms provide a reservoir for the periodic release of highly infectious planktonic *P. aeruginosa* cells from the biofilm. Whilst these planktonic cells are problematic for the patient, they are at least largely susceptible to antimicrobial agents, therefore, if treatment with nitric oxide can cause biofilm dispersal the hypothesis is that the planktonic cells released can then be killed by co-localised administration of antibiotics (Barraud et al 2012). Similarly, in an industrial setting for the decontamination of obstructive biofilms from water filters in drinking water distribution systems, it has been hypothesised that the use of nitric oxide can be used for partial biofilm dispersal and coupled to the use of abrasives for more complete removal of problematic biofilms (Anon, unpublished). The demonstration that this theory of exogenous application of nitric oxide for induction of biofilm dispersal followed by traditional methods for the killing of cells released or the mechanical destruction of the remaining biofilm suggests that nitric oxide treatment can indeed be used for a wide range of bacterial species in a range of environments and may be applicable to use at the phylloplane in combination with soft washing procedures currently in place in the production of leafy salad products, as described in Section 1.2.4.

When considering the development of anti-microbial strategies for fresh food product surfaces such as leafy salads however, some additional considerations are required. The dispersal of bacterial biofilms by nitric oxide investigated thus far (at non-food surfaces) has not demonstrated complete biofilm removal. The most successful reductions have been observed when biofilm dispersal by nitric oxide treatment is coupled to subsequent treatment with antibiotics, chemical sanitisers, or abrasives (Barraud et al 2006). However, for fresh produce, the use of antibiotics and chemical sanitisers is undesirable in terms of environmental impact and product acceptance by the consumer, and the use of abrasive treatments is not possible due to the need to maintain leaf integrity for product quality and shelf life. 'Soft' mechanical washing procedures using potable water are used for the preparation of RTE leafy salads. It is however possible that the treatment of leaves with nitric oxide may disperse pathogenic contaminants and phylloplane biofilms and lead to increased biofilm removal during the soft washing process. As the dispersal action of nitric oxide occurs at low, non-toxic concentrations of the nitric oxide donor SNP (Barraud et al 2009b), this may be suitable for application in a food production environment. Here, treatment with nitric oxide to disperse *Salmonella* spp. from the phylloplane was investigated (Section 3.3.4).

In addition, the specific mechanisms of attachment and early colonisation of the phylloplane by zoonotic pathogens such as *Salmonella* spp. remain poorly understood. Factors determining bacterial surface attachment are known to be under the control of a number of different environmental signals (see Chapter 4) and bacteria have been shown to rapidly respond to changes in environmental conditions at the phylloplane (Lindow and Brandl 2003). Therefore, as the expression of cell surface appendages involved in surface attachment for *Salmonella* spp. is under the control of a c-di-GMP signalling mechanism (Simm et al 2007), by which exogenous treatment with nitric oxide is thought to exhibit its effects (Barraud et al 2009a), the effect of nitric oxide on attachment and colonisation of spinach leaves by *Salmonella* spp. has also been investigated here. Any nitric oxide effect on attachment and colonisation of spinach leaves by zoonotic pathogens would be an important consideration if nitric oxide were to be applied in a food processing environment.

3.2 Experimental procedures

General methodology used for these investigations is described in Chapter 2. Colonisation of the spinach phylloplane by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP was investigated. The effect of nitric oxide treatment on initial attachment (1 hour) and early colonisation (24 hours) of spinach leaves by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311, as well as the effect of nitric oxide treatment on detachment of these strains from the spinach phylloplane, was also investigated.

Spinach leaves were inoculated with approximately 1 x 10^6 CFU mL^{-1} bacteria as described in Section 2.4. Nitric oxide was delivered to leaves where required by the addition of the nitric oxide donor SNP as described in Section 2.6, at final concentrations ranging from 50 nM to 1 µM as detailed in individual results sections.

For experiments carried out at room temperature on the laboratory bench, conditions were selected to include temperature and light fluctuations as would be found in an open spinach field environment. For experiments carried out under controlled light conditions, light was delivered via the use of a full spectrum lamp set on a 12 hour light, 12 hour dark cycle. Controlled light levels were selected to increase the amount of light delivered to the assay system to maximise the light-dependent breakdown of SNP to liberate nitric oxide. For these experiments, an additional lower SNP treatment concentration of 50 nM was included to account for the potential increase in release of nitric oxide from SNP at elevated light levels. For experiments carried out under controlled temperature conditions, experiments were carried out inside a 22°C incubator in order reduce external temperature fluctuations.

Samples for analysis by microscopy were processed and observed by EDIC/EF microscopy as described in Section 2.9.3. For enumeration of *Salmonella*, bacteria were recovered from the phylloplane by Pulsification as described in Section 2.5, serially diluted, and plated onto Rambach® agar as described in Section 2.2.2.2.

3.3 Results

3.3.1 Colonisation of the spinach phylloplane by *S.* Typhimurium and *S.* Thompson shows a progression from attachment of single cells to the development of phylloplane biofilms

The localisation of *S.* Typhimurium 12023 GFP (Figure 19) and *S.* Thompson RM2311 GFP (Figure 20) at the spinach phylloplane after 1, 24 and 48 hours for assessment of initial attachment, early colonisation and establishment of bacteria, respectively was investigated using EDIC/EF microscopy.

Single cells of *S.* Typhimurium and *S.* Thompson can be seen attaching to the spinach phylloplane at both the lower (Figure 19 A, Figure 20 A) and upper leaf surface (Figure 19 D, Figure 20 D) after 1 hour. Initial attachment to the spinach phylloplane occurs at the margins between epidermal cells and in the immediate area surrounding stomata. Single cells appear to attach predominantly to areas of the leaf that have existing 'debris' present on the surface. Attachment progressed to the formation of bacterial aggregates after 24 hours, with stacks or throngs of cells protruding from the phylloplane at both the lower (Figure 19 B, Figure 20 B) and upper leaf surface (Figure 19 E, Figure 20 E). Aggregations were observed at epidermal cell margins and around stomata for both strains.

Colonisation of spinach leaves after 48 hours was observed predominantly around stomata and at the edges of epidermal cells for *S.* Typhimurium (Figure 19 C & F), and at the margins between epidermal cells for *S.* Thompson (Figure 20 C & F). Microcolony-like structures can be seen for both *S.* Typhimurium and *S.* Thompson, and some of these structures appear to have a slimy outer covering indicative of the presence of the EPS matrix characteristic of bacterial biofilms.

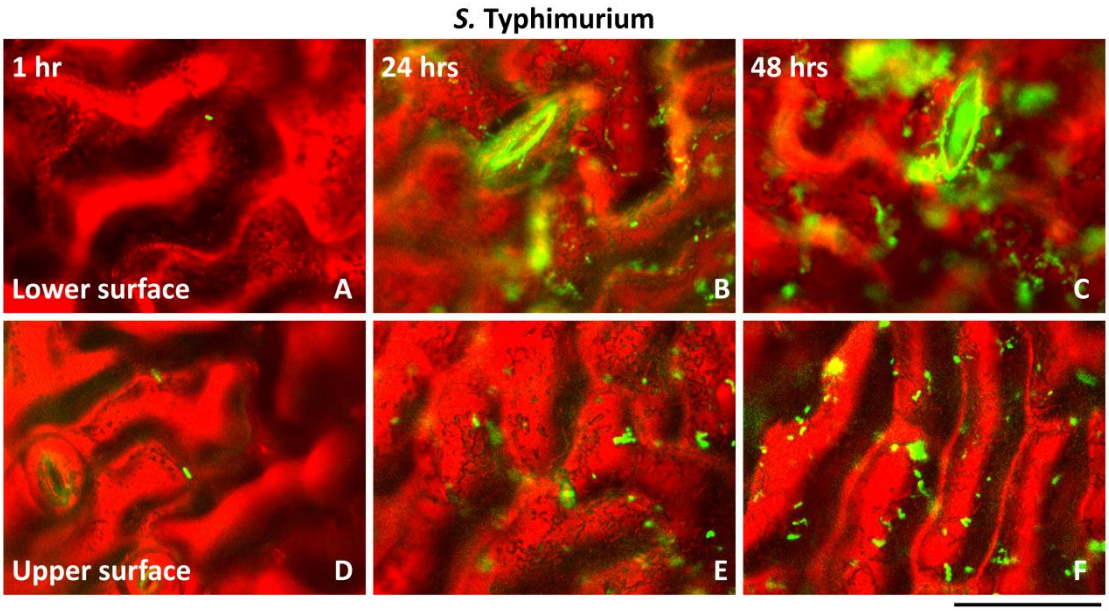


Figure 19 Colonisation of spinach leaves by *S.* Typhimurium 12023 GFP

Representative EDIC/EF micrographs showing localisation of *S.* Typhimurium 12023 GFP at the spinach phylloplane after 1 hour for initial attachment (A & D), 24 hours for early colonisation (B & E) and 48 hours for establishment of cells (C & F). Green = GFP labelled *S.* Typhimurium 12023 GFP. False colour (red) has been applied to the leaf surface for improved visualisation of GFP cells. Bar = 50 microns

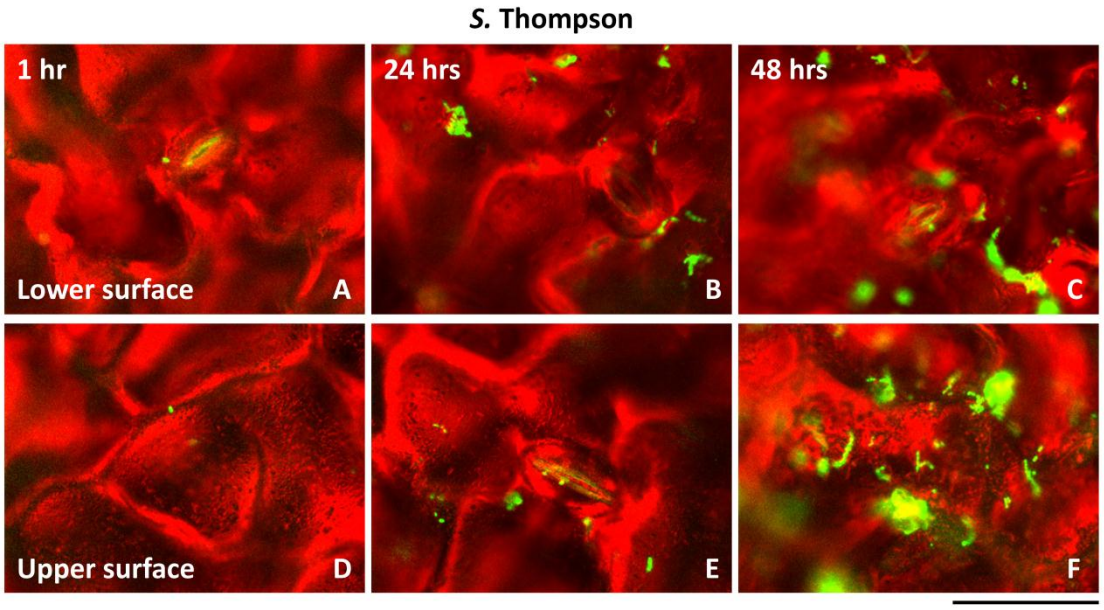


Figure 20 Colonisation of spinach leaves by *S.* Thompson RM2311 GFP

Representative EDIC/EF micrographs showing localisation of *S.* Thompson RM2311 GFP at the spinach phylloplane after 1 hour for initial attachment (A & D), 24 hours for early colonisation (B & E) and 48 hours for establishment of cells (C & F). Green = GFP labelled *S.* Thompson RM2311 GFP. False colour (red) has been applied to the leaf surface for improved visualisation of GFP cells. Bar = 50 microns

3.3.2 Treatment with nitric oxide influences initial attachment of *S.* Typhimurium and *S.* Thompson to the spinach phylloplane

The effect of exogenous application of SNP on the initial attachment of *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP to the spinach phylloplane after 1 hour was investigated.

Initial experiments were carried out at room temperature on the laboratory bench using SNP concentrations of 500 nM and 1 µM in order to investigate whether nitric oxide treatment influences the initial attachment of *S.* Typhimurium to spinach leaves (Figure 21).

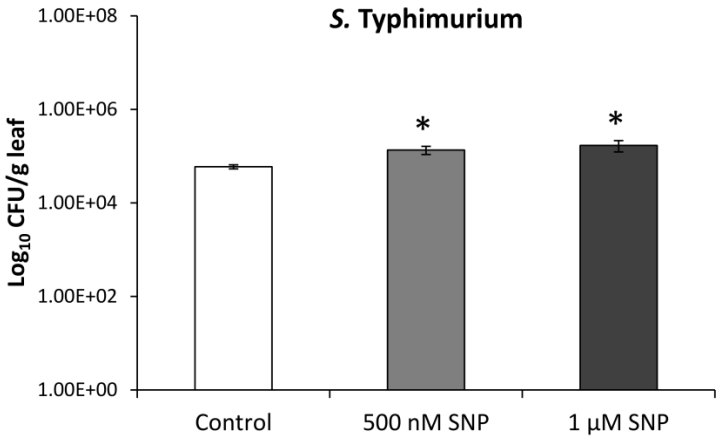


Figure 21 The effect of nitric oxide treatment on initial attachment of *S.* Typhimurium 12023 GFP to spinach leaves

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP and incubated at room temperature on the laboratory bench for 1 hour in the presence (grey bars) or absence (white bar) of the nitric oxide donor SNP. Bacteria were removed from leaves by pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three leaves per treatment.

Treatment of spinach leaves with 500 nM and 1 µM SNP for 1 hour caused a statistically significant (one-way ANOVA; $P < 0.05$), approximate 0.5 log increase in the number of culturable *S.* Typhimurium associated with the spinach phylloplane (Figure 21), which is an undesirable outcome for the application of SNP to spinach leaves in a food production environment.

Further investigations were carried out under controlled temperature and light conditions, as described in Section 3.2, to investigate whether controlling external environmental conditions, and maximising the release of nitric oxide from SNP could lead to reductions in initial attachment of *S.* Typhimurium to the spinach phylloplane. An additional 50 nM SNP treatment group was added, as described in Section 3.2. No increases in the number of culturable *S.* Typhimurium associated with the spinach phylloplane were observed under these conditions, and for 50 nM SNP treated leaves, a statistically significant (one-way ANOVA; $p < 0.05$) 0.5 log reduction in culturable *S.* Typhimurium was observed (Figure 22 A). For *S.* Thompson, a similar statistically significant (one-way ANOVA; $p < 0.05$) 0.5 log reduction in culturable cells was observed for 500 nM SNP treated leaves (Figure 22 B). However, despite these statistically significant reductions, both *Salmonella* strains investigated remained associated with leaves in considerable numbers (1.3×10^4 to 1.2×10^6 CFU/g leaf) following SNP treatment. Interestingly, initial attachment of *S.* Thompson to the spinach phylloplane was 1.5 log greater than initial attachment of *S.* Typhimurium.

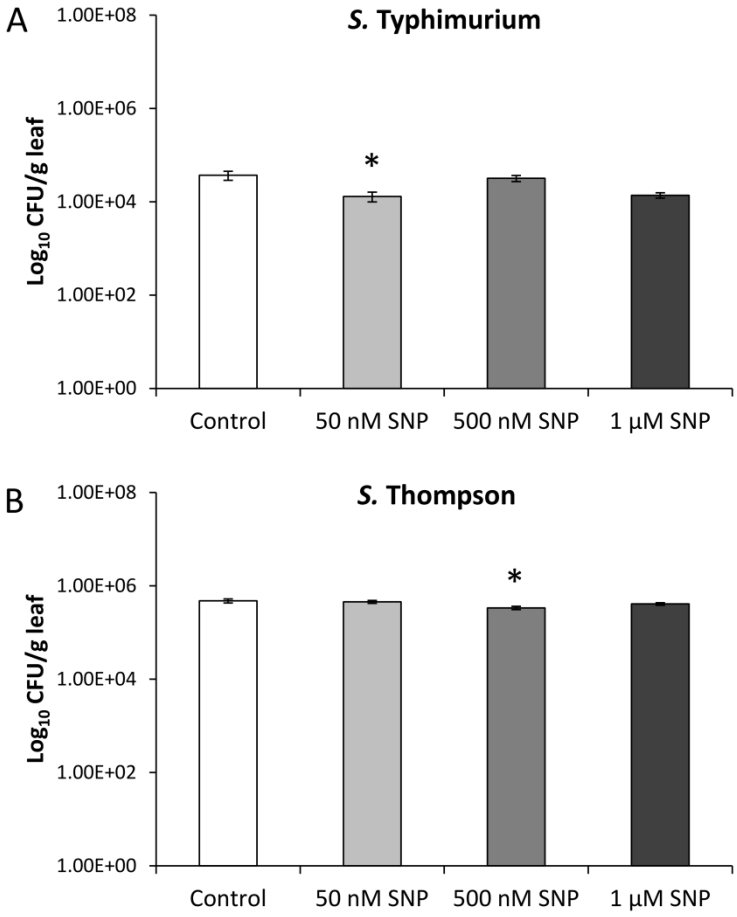


Figure 22 The effect of nitric oxide treatment on initial attachment of *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP to spinach leaves

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP (A) or *S.* Thompson RM2311 GFP (B) and incubated in a 22°C incubator in the light for 1 hour in the presence (grey bars) or absence (white bars) of the nitric oxide donor SNP. Bacteria were removed from leaves by Pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three leaves per treatment.

Microscopy images taken for qualitative assessment of *Salmonella* at the phylloplane for these investigations show the attachment of single cells of *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP after 1 hour in the presence or absence of SNP for all concentrations investigated. No clearly visible differences between Control and SNP treated leaves were seen. Representative microscopy images are provided in Figure 23. A full appendix of microscopy images is provided in Appendix 1.

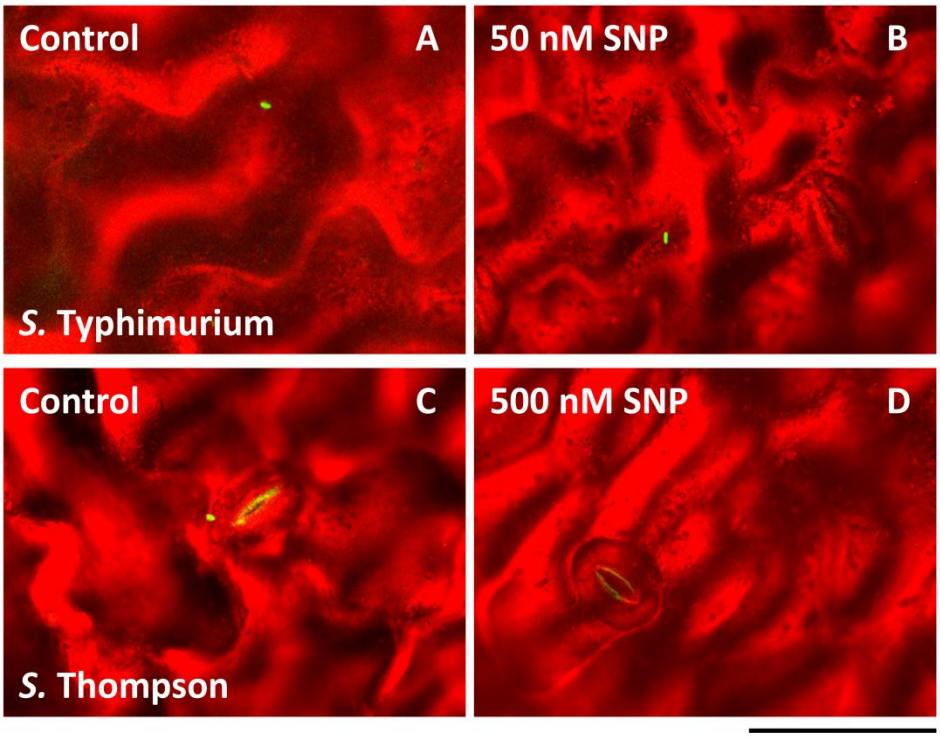


Figure 23 Representative EDIC/EF micrographs showing initial attachment of *S*. Typhimurium 12023 GFP and *S*. Thompson RM2311 GFP to spinach leaves following nitric oxide treatment

Representative EDIC/EF micrographs showing initial attachment of *S.* Typhimurium 12023 GFP (A & B) and *S.* Thompson RM2311 GFP (C & D) to the spinach phylloplane following inoculation with approximately $1x10^6$ CFU mL^{-1} bacteria and incubation at 22°C in the light for 1 hour in the presence (B & D) or absence (A & C) of the nitric oxide donor SNP. Bar = 50 microns.

3.3.3 Treatment with nitric oxide influences early colonisation of the spinach phylloplane by *S.* Typhimurium and *S.* Thompson

The effect of exogenous application of SNP on colonisation of the spinach phylloplane by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP after 24 hours (denoted 'early' colonisation) was investigated.

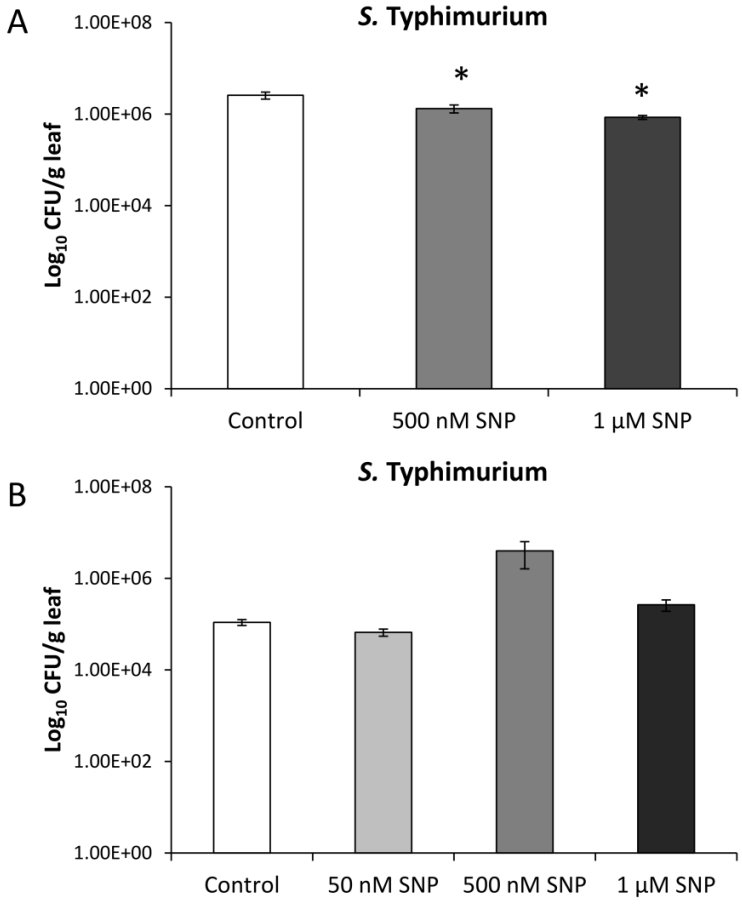


Figure 24 The effect of nitric oxide treatment on early colonisation of spinach leaves by *S.* Typhimurium 12023 GFP

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP and incubated at room temperature on the laboratory bench (A) or in a 22 C incubator in the light (B) for 24 hours in the presence (grey bars) or absence (white bar) of the nitric oxide donor SNP. Bacteria were removed from leaves by Pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three (A) and two (B) leaves per treatment.

Treatment of spinach leaves with 500 nM and 1 µM SNP for 24 hours caused a statistically significant (one-way ANOVA; $p < 0.05$) approximate 0.5 log reduction in culturable *S.* Typhimurium 12023 GFP associated with the spinach phylloplane, when the experiment was carried out at room temperature on the laboratory bench (Figure 24 A). When carried out under controlled temperature and light conditions for the minimisation of environmental variables, a 1.5 log increase in culturable *S.* Typhimurium associated with the phylloplane was suggested for leaves treated with 500 nM SNP (Figure 24 B). Whilst this result did not reach statistical significance (Kruskall-Wallace; $p > 0.05$), the potential for SNP treatment to increase colonisation of spinach leaves by *S.* Typhimurium is an undesirable outcome for the application of SNP to spinach leaves in a food production environment.

For *S.* Thompson, treatment of spinach leaves with 500 nM SNP for 24 hours caused a statistically significant (Mann-Whitney U; $p < 0.01$) approximate 0.5 log reduction in culturable *S.* Thompson associated with the spinach phylloplane, when carried out at room temperature on the laboratory bench (Figure 25 A). In order to investigate whether increasing the exposure of the assay system to light could increase this reduction, the experiment was repeated on the laboratory bench under controlled light conditions, as described in Section 3.2. A statistically significant (one-way ANOVA $p < 0.05$) 0.2 log increase in culturable *S.* Thompson associated with the spinach phylloplane was observed under these conditions (Figure 25 B), which, again is an undesirable outcome for the use application of SNP in a food production environment. In order to further control environmental variables for the delivery of nitric oxide to the assay system, the experiment was carried out under controlled temperature and light conditions. A 50 nM SNP treatment group was also added. No effect of SNP on culturable *S.* Thompson associated with the spinach phylloplane was observed under these conditions (Figure 25 C).

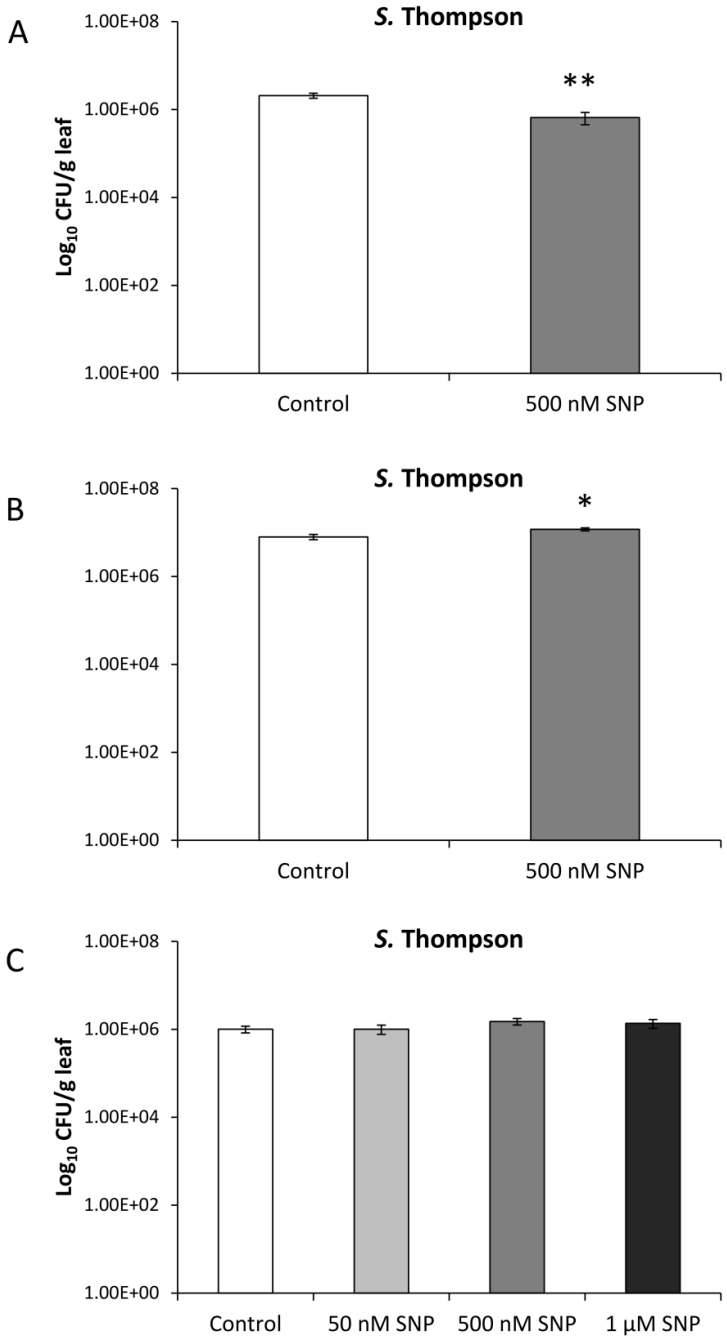


Figure 25 The effect of nitric oxide treatment on early colonisation of spinach leaves by *S.* Thompson RM2311 GFP

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Thompson RM2311 GFP and incubated at 22°C in the dark (A) at room temperature on the laboratory bench (B), or in a 22°C incubator in the light (C) for 24 hours in the presence (grey bars) or absence (white bars) of the nitric oxide donor sodium nitroprusside. Bacteria were removed from leaves by Pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of ten (A), three (B), and five (C) leaves per treatment.

Microscopy images taken for qualitative assessment of *Salmonella* at the phylloplane for these investigations, did not show any clear differences between control and SNP treated leaves for *S.* Typhimurium or *S.* Thompson. Representative microscopy images are provided in Figure 26. Again, levels of *S.* Thompson (Figure 26 C & D) associated with the spinach phylloplane appeared to be higher than levels of *S.* Typhimurium (Figure 26 A & B). A full appendix of microscopy images is provided in Appendix 1.

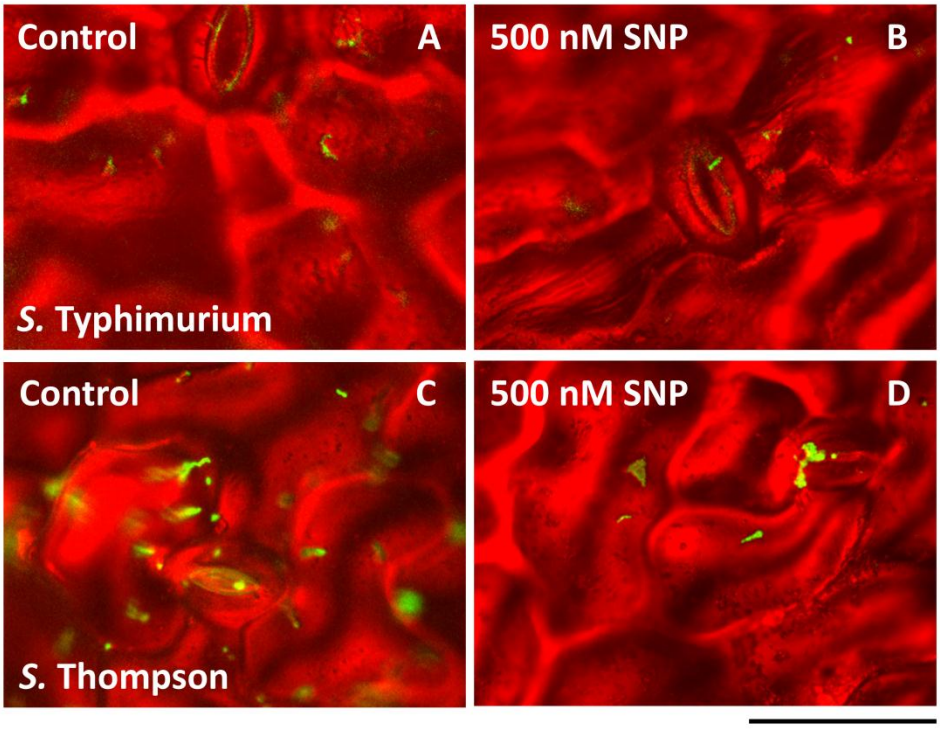


Figure 26 Representative EDIC/EF micrographs showing early colonisation of spinach leaves by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP after 24 hours following nitric oxide treatment

Representative EDIC/EF micrographs showing early colonisation of spinach leaves by *S.* Typhimurium 12023 GFP (A & B) and *S.* Thompson RM2311 GFP (C & D) following inoculation with approximately $1x10^{6}$ CFU mL^{-1} bacteria and incubation at 22°C in the light for 24 hours in the presence (B & D) or absence (A & C) of the nitric oxide donor SNP. Bar = 50 microns.

3.3.4 Treatment of spinach leaves with nitric oxide does not lead to dispersal of *S.* Typhimurium or *S.* Thompson from spinach leaves

The effect of exogenous application of SNP on the detachment of *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP from the spinach phylloplane after 24 hours was investigated.

For *S.* Typhimurium 12023 GFP, no statistically significant effects of SNP treatment on detachment of *S.* Typhimurium from spinach leaves were observed under temperature and light controlled conditions (Kruskall-Wallace; $p > 0.05$), although an approximate 0.7 log increase in numbers of culturable *S.* Typhimurium recovered from the phylloplane following treatment with 50 nM SNP was suggested (Figure 27).

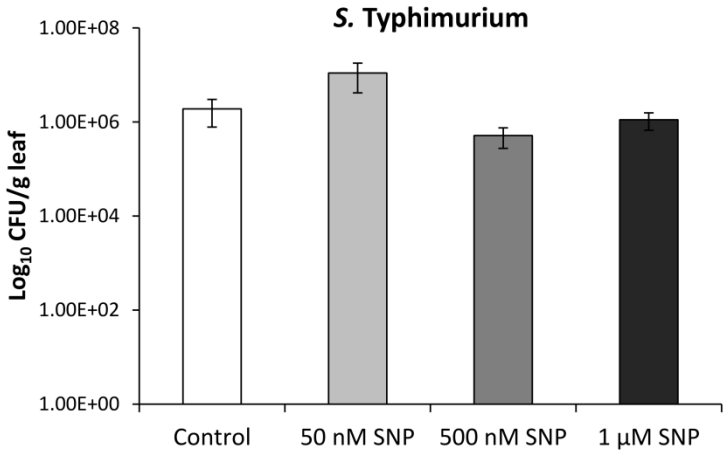


Figure 27 The effect of nitric oxide on dispersal of *S.* Typhimurium 12023 GFP from spinach leaves

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP and incubated in a 22°C incubator in the light for 24 hours to allow for colonisation of leaves by bacteria. Spinach leaves were transferred to new media and incubated in the presence (grey bars) or absence (white bars) of the nitric oxide donor SNP for a further 24 hours. Bacteria were removed from leaves by Pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of five leaves per treatment.

For *S.* Thompson RM2311 GFP (Figure 28) no statistically significant effects of SNP treatment on detachment of *S.* Thompson from spinach leaves were observed when the experiment was carried out either on the laboratory bench (Figure 28 A, Mann-Whitney U; $p > 0.05$), or under temperature and light controlled conditions (Figure 28 B, one-way ANOVA; $p > 0.05$).

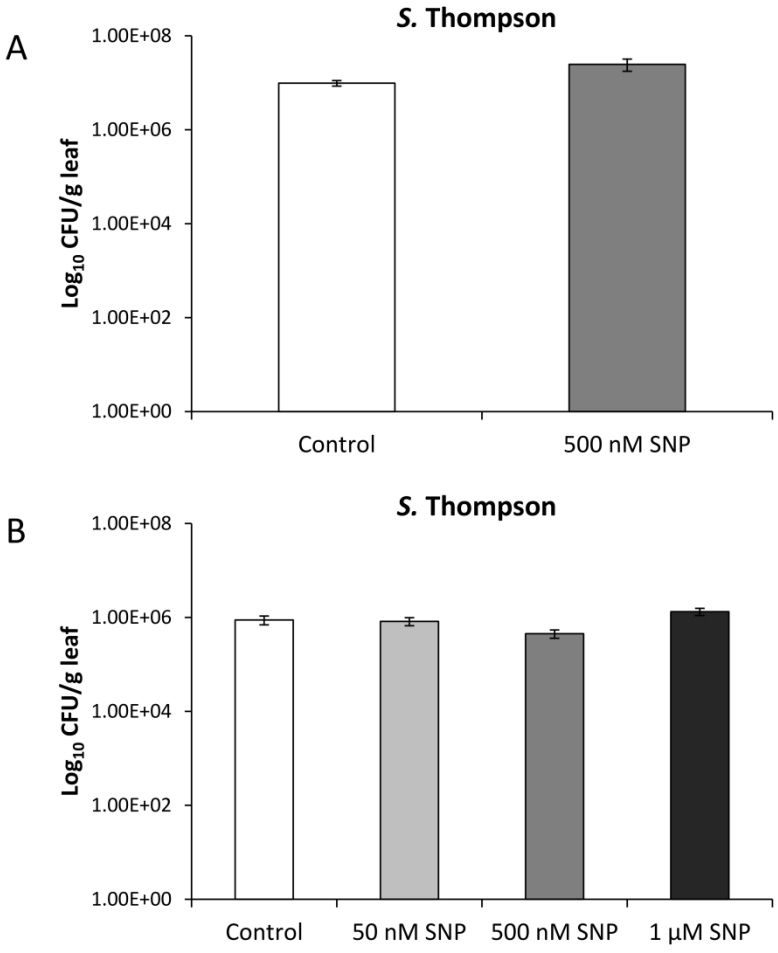


Figure 28 The effect of nitric oxide on detachment of *S*. Thompson RM2311 GFP from spinach leaves

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Thompson RM2311 and incubated at room temperature on the laboratory bench (A) or in a 22°C incubator in the light (B) for 24 hours to allow for colonisation of leaves by bacteria. Spinach leaves were transferred to new media and incubated in the presence (grey bars) or absence (white bars) of the nitric oxide donor SNP for a further 24 hours. Bacteria were removed from leaves by Pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three (A) and five (B) leaves per treatment.

Microscopy images taken for qualitative assessment of *Salmonella* at the phylloplane for these investigations, did not show any clear differences between control and SNP treated leaves for *S.* Typhimurium or *S.* Thompson. Representative microscopy images are provided in

Figure 29. A full appendix of microscopy images is provided in Appendix 1.

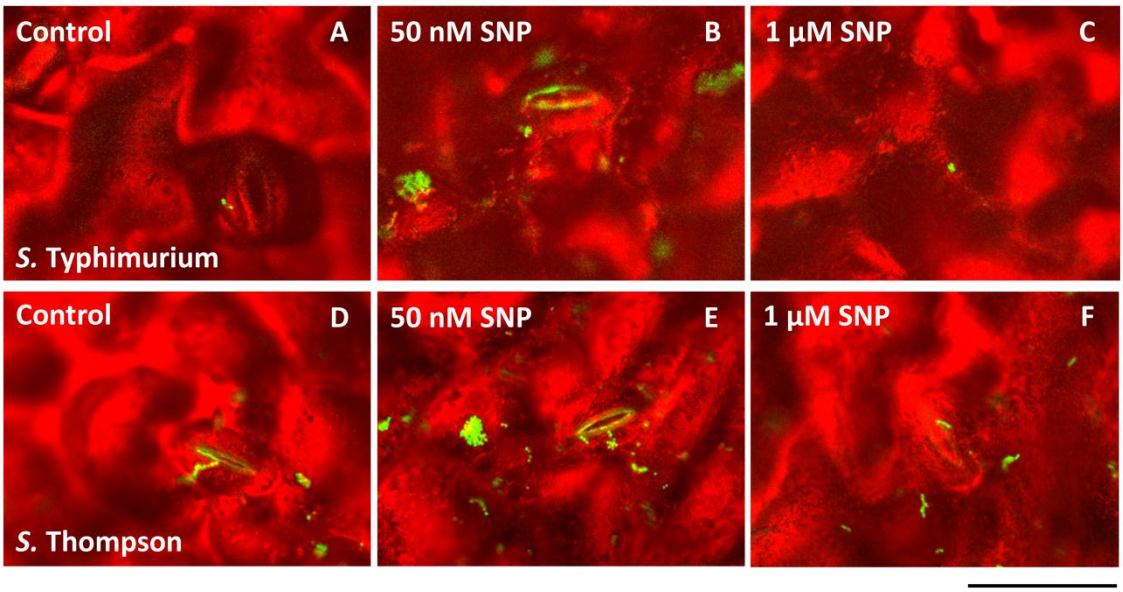


Figure 29 Representative EDIC/EF micrographs showing *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP remaining at the spinach phylloplane following 24 hours treatment with nitric oxide

Representative EDIC/EF micrographs showing *S.* Typhimurium 12023 GFP (A & B) and *S.* Thompson RM2311 GFP (C & D) remaining at the spinach phylloplane following incubation of leaves in a 22°C incubator for 24 hours in the presence (B & D) or absence (A & C) of the nitric oxide donor SNP. Prior to treatment with SNP, leaves had been artificially inoculated with approximately $1x10^6$ CFU mL^{-1} *Salmonella* and incubated in a 22 C incubator for 24 hours in the absence of SNP to allow for the establishment of *Salmonella* at the phylloplane. Bar = 50 microns.

3.4 Discussion

3.4.1 The use of EDIC/EF microscopy for the assessment of GFP labelled *Salmonella* spp. at the phylloplane and the effect of nitric oxide

In previous work by Warner *et al*, the use of EDIC/EF microscopy for the observation of GFP expressing bacteria at the phylloplane was described. A snapshot of colonisation of spinach leaves by *S.* Thompson at 24 hours, following inoculation with a high concentration of inoculate ($1x10^8$ CFU mL^{-1}) was reported, demonstrating the potential for the use of the technique to track pathogens on leaves (Warner et al 2008). Here, EDIC/EF microscopy was used for the first time to visualise *S.* Typhimurium at the leaf surface, and to visualise the attachment and colonisation of spinach leaves by *S.* Typhimurium and *S.* Thompson over a 48 hour time period. Furthermore, EDIC/EF microscopy was used to qualitatively assess the effect of nitric oxide on attachment to, colonisation of, and detachment from the spinach phylloplane by *S.* Typhimurium and *S.* Thompson for the first time.

3.4.1.2 Localisation of fluorescently labelled *S.* Typhimurium and *S.* Thompson at the spinach phylloplane

The use of microscopy methods for the assessment of bacteria at the phylloplane is essential for understanding the localisation of pathogenic contaminants at leaf surfaces, and for providing insights into their interactions with both the leaf surface and with indigenous bacterial populations found there. The demonstration that bacterial biofilms exist at leaf surfaces (Morris et al 1997) necessitates the use of microscopy methods that preserve both the integrity of the leaf and the structure of the biofilm in order to successfully visualise phylloplanes and their associated biofilms as they exist in their natural environments.

As described in Section 2.9, EDIC/EF microscopy is an excellent technique for the visualisation of leaf surfaces and the bacteria that reside there (Warner et al 2008, Warner 2009). The observation of fully hydrated leaf samples and their associated bacterial communities is possible due to the minimal sample processing required for

EDIC/EF microscopy (Warner et al 2008). As both bacterial biofilms and leaf tissues are composed of a large proportion of water, minimal sample processing provides an advantage over other advanced microscopy techniques such as SEM for example, which requires extensive sample preparation that can result in sample dehydration resulting in the distortion of leaf surface and biofilm morphology. This can result in images which are poorly suited to the assessment of localisation of bacteria in relation to leaf surface structures (Keevil and Walker 1992). The episcopic illumination of samples and the use of long working distance lenses in EDIC/EF microscopy mitigates these issues, allows the direct visualisation of opaque samples and eliminates the need for the use of coverslips or oil, therefore making EDIC/EF microscopy an excellent technique for the visualisation of biofilms on leaf surfaces in their natural, unaltered state (Keevil 2003, Warner et al 2008). EDIC/EF microscopy was successfully used here to visualise the colonisation of spinach leaves by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP over a 48 hour period.

Single cells of both *S.* Typhimurium and *S.* Thompson were observed attaching to spinach leaves after 1 hour, with attachment progressing to the formation of aggregates by 24 hours and the establishment of structures resembling early biofilms by 48 hours, suggesting that existence in biofilm communities is an important colonisation strategy for *Salmonella* spp. at leaf surfaces. The precise mechanisms by which leaf surface colonisation occurs remain unknown, but will define not only colonisation efficiency for zoonotic pathogens such as *Salmonella* spp. but also the method required for bacterial removal from the phylloplane. Single cells may attach directly to the phylloplane via specific interactions, adhere to the leaf and form single species biofilms, or incorporate into existing phylloplane communities that are already established there.

Here GFP labelled *Salmonella* were seen attaching to existing bacterial aggregates at the phylloplane, staining of such leaf surface 'debris' with the nucleic acid stain DAPI has previously shown it to be microbial in origin (Warner et al 2008). These findings together suggest that *S.* Thompson and *S.* Typhimurium may preferentially attach to areas of leaf surfaces already colonised by indigenous phylloplane bacteria. Similarly, the attachment of single cells of the plant colonists *Pantoea agglomerans* and

Pseudomonas fluorescens was shown by other researchers to occur preferentially to pre-existing bacterial aggregates at the phylloplane of bean plants, which confers increased survival to these bacteria than when diffusely attached to the leaf (Monier and Lindow 2005b). However, interestingly, in the same study it was also found that attachment of single cells of *Pseudomonas syringae* to pre-existing bacterial aggregates did not increase their survival on bean leaves, suggesting different bacteria exploit different mechanisms of colonisation for survival at the phylloplane and highlighting the diversity of bacterial behaviours found in the phylloplane environment.

For *S.* Thompson, localisation of bacteria observed here is consistent with findings by Warner et al (Warner et al 2008). In addition, the formation of aggregates and microcolonies by *S.* Thompson at the phylloplane, indicative of the formation of bacterial biofilms, is consistent with findings by Brandl and Mandrell, who demonstrated these structures at the cilantro phylloplane (Brandl and Mandrell 2002). For *S.* Typhimurium, this is the first description of visualisation at the phylloplane using EDIC/EF microscopy. In a study by Kroupitski *et al*., localisation of *S.* Typhimurium at the phylloplane of Romaine lettuce was observed in a similar pattern around stomata as observed in this work (Kroupitski et al 2011). In a study by Berger *et al.* a diffuse pattern of attachment was seen for *S.* Typhimurium at the basil phylloplane, suggesting that leaf species may have an influence on pathogen colonisation (Berger et al 2009). In this work Berger *et al*. suggested a direct attachment of *Salmonella* to the phylloplane, rather than to pre-existing communities on leaves. However, their study was carried out using basil plants gown in sterile soil in a controlled laboratory environment, therefore minimising background microflora of the leaves, which may have unintentionally pre-selected leaves to which *Salmonella* would directly attach, rather than interact with existing phylloplane communities that are known to be ubiquitous for environmentally grown leaves (Morris and Monier 2003).

The selection of field grown leaves for the studies presented in this thesis, with pre-existing microbial communities as they would exist in a real-life food production environment, is specifically designed to incorporate the interaction of zoonotic pathogens with natural phylloplane communities into the experimental model. Interactions of pathogens with leaf surfaces, including their indigenous microbial

communities, are an important consideration for decontamination strategies, as it is not yet known whether zoonotic pathogens attach directly to leaf surface, to existing phylloplane biofilms, or set up their own single species biofilms, and this may have implications for their removal. Indigenous phylloplane communities will be considered further in Chapter 6.

A dual fluorescence approach, combining DAPI staining for visualisation of existing phylloplane biofilms with attachment of GFP expressing *Salmonella* was investigated, unsuccessfully, for localisation of *Salmonella* in relation to existing phylloplane aggregates and biofilms, in order to confirm that *Salmonella* preferentially attach to existing phylloplane aggregates and biofilms. Unfortunately the visualisation of GFP labelled *Salmonella* and DAPI stained phylloplane biofilms were unable to be observed together due to the non-target staining of GFP labelled *Salmonella* with DAPI, as shown in Figure 37. However, as *Salmonella* were seen attaching to aggregates on the leaf surface in these investigations (Section 3.3.1), and that these aggregates resemble microbial aggregates identified by DAPI staining (Warner 2008, Chapter 6), it is concluded here that these aggregates are indeed likely to be indigenous phylloplane biofilms.

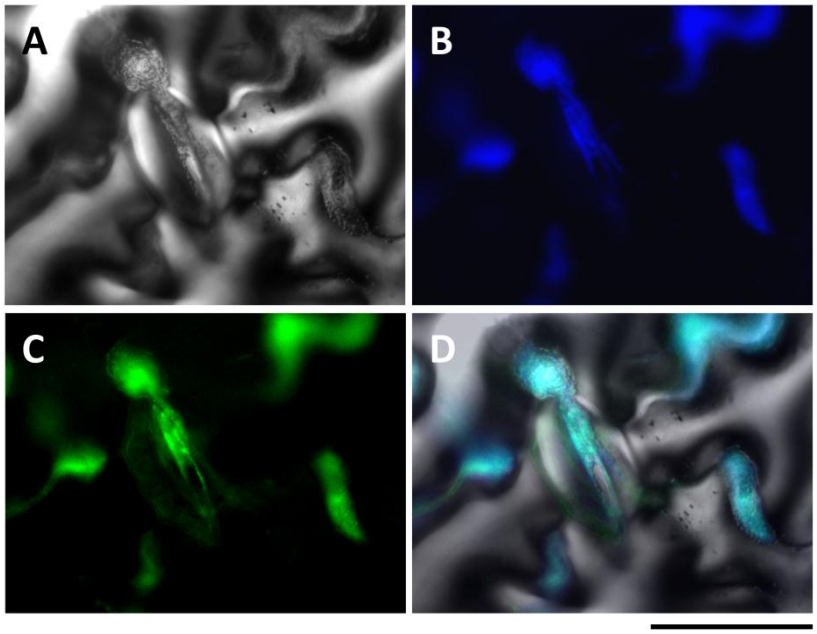


Figure 30 Investigation of a dual stain approach for the visualisation of GFP labelled *Salmonella* in relation to DAPI stained indigenous bacteria at the spinach phylloplane. Representative EDIC/EF micrographs showing the spinach phylloplane (A), DAPI stained bacteria (B) and GFP labelled *S.* Thompson RM2311 GFP (C) and an image overlay demonstrating the unsuitability of this technique for visualisation of the interaction of GFP labelled zoonotic pathogens with DAPI stained indigenous phylloplane bacteria (D)

The mechanism for aggregate and biofilm formation by *Salmonella* spp. at the phylloplane remains unknown, and may be an important target for the development of novel decontamination strategies for leafy salads. In laboratory studies based on the developmental model of biofilm formation (see Figure 4) bacterial aggregation of cells following initial surface attachment has been shown to involve twitching and swarming motility (O'Toole and Kolter 1998a). At the phylloplane, the aggregate formation seen here may be due to a) the additional attachment of single planktonic *Salmonella* to those already attached to the leaf surface, b) the movement of cells together on the surface by twitching and swarming motility, or by the clonal growth of attached cells. Clonal growth is known to occur in the development of microcolonies during the formation of bacterial biofilms (Conibear et al 2009). These researchers constructed a GFP reporter strain of *P. aeruginosa* which lost fluorescence upon cell division demonstrating clonal growth of microcolonies from single attached cells. The investigation of clonal growth at the phylloplane using EDIC/EF using a similar GFP

reporter strain would not presently be possible. A reporter strain with dual fluorescence would be required, one constitutively incorporated to allow bacteria artificially inoculated onto the phylloplane to be distinguished from indigenous bacteria, and a second fluorescent reporter gene that loses its fluorescence upon clonal growth. The construction of such a reporter strain could not be carried out within the scope of this project, however, the construction of such a strain would be a valuable future addition to the study of *Salmonella* biofilms at the phylloplane.

Whilst the methods used here have been carefully selected to best model the interaction of *Salmonella* spp. with the phylloplane in an environmental context and with relevance to further studies requiring the exogenous delivery of SNP to leaves, it is recognised that they are not without their limitations. The inoculation by immersion method used here to introduce *Salmonella* spp. to the phylloplane was designed to allow bacteria equal access to all areas of the leaf to investigate where they preferentially attach, which may not best reflect field growing conditions in the environment. Therefore, inoculation by immersion was considered the best available method, particularly for the introduction of SNP to the phylloplane assay. However immersion times of greater than 48 hours were not possible (which would be desirable to better investigate mature *Salmonella* biofilms at the leaf surface, for example) as leaf tissue degrades too much for EDIC/EF microscopy at immersion times greater than this. In addition, the concentration of inoculum required for detection of GFP labelled bacteria for phylloplane samples is much higher than would be present in the field under normal circumstances. It is however possible that this level of inoculum may represent a worst case scenario if an area of a spinach field had been directly contaminated by direct defecation from wild animals for example. Further work on the development of biofilms by zoonotic pathogens at the phylloplane could be modified to use a lower concentration of inoculum (less of a metabolic burden on the leaf) and a non-immersion based method of inoculation, to allow the formation of bacterial biofilms to be more fully investigated. It is hoped that in combination with traditional microbiological methods, EDIC/EF microscopy can be used to better understand interactions of zoonotic pathogens with the phylloplane and the effect of nitric oxide as novel decontamination strategy for phylloplane biofilms.

3.4.2 Investigating the effect of exogenous application of sodium nitroprusside on the colonisation of spinach leaves by *Salmonella* species

No effects large enough for practical intervention in the attachment to, colonisation of, or detachment of *S.* Typhimurium or *S.* Thompson from spinach leaves in a food production environment were seen here. Findings suggest that exogenous application of SNP to spinach leaves does not significantly affect the ability of *S.* Typhimurium or *S.* Thompson to attach and colonise spinach leaves, or induce dispersal of *Salmonella* spp. from the phylloplane. However, small effects (in the region of 0.5 log changes in numbers of culturable *Salmonella*) that appeared to be influenced by temperature and light were observed, suggesting that nitric oxide treatment does have some activity at the spinach phylloplane. These findings, and the possible reasons for them, are discussed below.

Initial attachment of *S.* Thompson and *S.* Typhimurium to the phylloplane occurs rapidly, within 1 hour of inoculation onto leaves. As nitric oxide induces a signaling response via modulation of c-di-GMP in the model species *P. aeruginosa*, and proteins controlling this process are ubiquitous among bacteria, it is hypothesised that the signaling mechanism may be conserved across bacterial genera (see Section 1.5). Whether this signaling response is in fact conserved and applicable to *Salmonella* spp. remains unknown, although *Salmonellae* do possess the cellular apparatus necessary for a similar nitric oxide response to be possible (Simm 2007). This will be explored further in Chapter 4. It is possible then, that even if this nitric oxide/c-di-GMP signaling mechanism is active in *Salmonella* spp. and that delivery of the correct concentration of nitric oxide can be controlled (see below), a 1 hour treatment time may not be sufficient to allow for the induction of a signaling response that can influence attachment of *Salmonella* spp. to spinach leaves when SNP and *Salmonella* are co-inoculated. In an environmental situation it would not be possible to pre-treat field growing spinach crops with nitric oxide to stop pathogens attaching to leaves, however, the effect of nitric oxide on initial attachment of *Salmonella* to the phylloplane becomes important when considering the re-attachment of pathogens to leafy salads during a wash process.

Increases in initial attachment of *Salmonella* spp. to spinach leaves have also been observed here. Whilst these increases are relatively small at only approximately a 0.5 log change, the demonstration of the potential for exogenous treatment with nitric oxide to increase pathogen attachment to leaves is a highly undesirable outcome for consideration of an intervention strategy for use for leafy salads in a food production environment. This increase in initial attachment may be due to the known window effect of nitric oxide action, where nitric oxide treatment reduces biofilm coverage at a certain concentration, but increases it at others (Barraud et al 2006). Reasons for this window effect remain unclear, but as nitric oxide signalling has been shown to act via the modulation of intracellular levels of c-di-GMP by the induction of PDE activity (see Section 1.5) it is possible that there is an optimum level of PDE activity, above or below which additional signalling occurs to restore c-di-GMP levels. It is emerging that the control of intracellular c-di-GMP levels in bacteria is a complex and highly regulated process (Plate and Marletta 2012, Romling et al 2013) and it is therefore possible that modulation of PDE activity is not the only effect of exogenous application of nitric oxide in the c-di-GMP signalling mechanism. This is discussed further in Chapter 4.

In addition, the release of nitric oxide from SNP occurs in a light-dependent manner (Yamamoto and Bing 2000). Therefore, as light levels in the laboratory fluctuate during a day, and between days, the actual concentration of nitric oxide released from SNP may vary in the laboratory. Furthermore, as temperatures in the laboratory may also vary, and as temperature has also been shown to influence surface attachment by *Salmonella* spp. (Walker et al 1999) further experiments were carried out under controlled temperature and light conditions. This was achieved by using a full spectrum lamp in the 22°C incubator to mitigate any effects of variable temperature and light on the attachment of *Salmonella* to the phylloplane. Under controlled temperature and light conditions, 0.5 log reductions in initial attachment were observed with 50 nM and 500 nM SNP treatment for *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP respectively, suggesting indeed that controlling conditions for the release of nitric oxide from SNP can lead to reductions in the attachment of *Salmonella* to spinach leaves. However, even with a 0.5 log reduction for SNP treated leaves by comparison to untreated Control leaves, numbers of culturable *Salmonella* attaching to leaves were high, at approximately $1x10^6$ CFU/g leaf. In addition,

S. Thompson appears to be a more efficient coloniser of the spinach phylloplane than *S.* Typhimurium under the conditions investigated here, highlighting strain specific differences in attachment ability which may have important implications for food safety. The *S.* Thompson RM2311 GFP strain used for these investigations is constructed from a clinical outbreak strain of *Salmonella* (Brandl and Mandrell 2002), whilst *S.* Typhimurium 12023 is a laboratory strain, demonstrating that clinical isolates may be better adapted than laboratory strains for colonising environmental surfaces such as the phylloplane.

When the effect of nitric oxide treatment on early colonisation of spinach leaves by *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP after 24 hours was investigated, again the effect of nitric oxide appeared to be influenced by external environmental conditions. When experiments were carried out on the laboratory bench approximate 0.5 log decreases in numbers of *S.* Typhimurium and *S.* Thompson associated with spinach leaves were observed, suggesting that nitric oxide treatment may interfere with phylloplane colonisation strategies such as the formation of aggregates and biofilms. However, even with a 0.5 log reduction for SNP treated leaves by comparison to untreated Control leaves, numbers of culturable *Salmonella* attaching to leaves were high, at approximately $1x10^6$ CFU/g leaf for both strains. When experiments were carried out under temperature and light controlled conditions, in contrast to results of initial attachment studies, the nitric oxide effect on early colonisation of leaves was either mitigated or the number of culturable *Salmonella* associated with leaves marginally increased (0.2 log for *S.* Thompson when incubated at elevated light levels on the laboratory bench). Therefore, a nitric oxide effect on early colonisation of spinach leaves appeared to be occurring, but the nature of this effect could not be determined from these investigations.

When exogenous application of nitric oxide was investigated for its ability to cause dispersal of *Salmonella* spp. from the spinach phylloplane, no effects were observed. This may be because optimisation of nitric oxide delivery in this system did not achieve the optimum level to induce biofilm dispersal, or that environmentally grown *Salmonella* microcolonies do not undergo biofilm dispersal events in response to ROS signalling in the same way as in the model species *P. aeruginosa* (see Section 1.4.4.3),

and therefore are not dispersed by exogenous application of nitric oxide. The study of the dispersal effect of nitric oxide on bacteria at the phylloplane is complex, as there are a number of variables that may influence the response of *Salmonella* to nitric oxide treatment that cannot be controlled, such as the microenvironment at the phylloplane, leaf surface topography, nutrient availability and underlying microbial communities already present at the leaf surface. Therefore, in Chapter 4, *Salmonella* biofilms have been investigated using an abiotic model system, in order to allow experimental conditions to be more easily controlled than at the phylloplane, in order to further characterise the influence of nitric oxide treatment on surface colonisation and biofilm formation by *S.* Typhimurium.

3.5 Conclusions

EDIC/EF microscopy was used to demonstrate that *S.* Typhimurium 12023 GFP and *S.* Thompson RM2311 GFP attach efficiently to the spinach phylloplane and form aggregates and microcolonies that are biofilm-like in appearance. However, upon treatment of spinach leaves with nitric oxide, via the exogenous application of the nitric oxide donor SNP, no consistent repeatable effects on attachment and colonisation of spinach leaves by *Salmonella* were seen, nor on the dispersal of *Salmonella* from the phylloplane. This was despite optimisation of experimental conditions for the controlled delivery of nitric oxide to spinach leaves and investigations into optimal timing and concentration of nitric oxide treatment. Therefore it is not possible to conclude from these investigations that nitric oxide treatment can be used for the reduction of colonisation of spinach leaves by *Salmonella* spp. for use in a food production environment.

It remains unknown whether zoonotic pathogens such as the *Salmonella* spp. investigated here attach directly to the leaf surface via specific interactions, establish single species biofilms on the leaf surface, or incorporate into indigenous phylloplane communities and biofilms already present at the phylloplane. EDIC/EF microscopy has been successfully used here to suggest that *Salmonella* spp. may preferentially attach to existing bacterial aggregates at the spinach phylloplane. Therefore in an experimental system using field grown leaves, where local environmental conditions at

the leaf surface may vary with growing conditions as well as leaf species, identifying the specific effect of nitric oxide on *Salmonella* at the spinach phylloplane is difficult. A nitric oxide effect seems to be present but not a large or a consistent one. Therefore a better understanding of the specific interactions governing surface attachment by *Salmonella* and the involvement of nitric oxide, if any, in the development and dispersal of *Salmonella* biofilms, is required to further investigate whether nitric oxide treatment of leaves can be optimised for use against the contamination of leafy salads with zoonotic pathogens such as *Salmonella* spp.

CHAPTER 4

Investigating surface colonisation strategies for *S.* Typhimurium, and the influence of nitric oxide

4.1 Introduction

In the previous Chapter it was found that nitric oxide treatment of had variable effects on the colonisation of spinach leaves by *S.* Typhimurium and *S.* Thompson. Despite laboratory investigations for optimisation of conditions for nitric oxide delivery to leaves, exogenous application of SNP (which has been demonstrated to be efficacious for the reduction of biofilm surface coverage for single species *S. marcescens*, *V. cholerae*, *E*. coli, F. nucleatum, *B. licheniformis* and *S.* epidermidis as well as for multi-species biofilms from drinking water distribution systems (Barraud et al 2009b)) did not influence the attachment, colonisation or detachment of *S.* Typhimurium or *S.* Thompson from spinach leaves in a consistent manner.

Exogenous application of SNP was demonstrated to have small (approximately 0.5 log) influences on the attachment and colonisation of spinach leaves by *Salmonella* spp. to either increase, or decrease attachment and colonisation of spinach leaves for *S.* Typhimurium and *S.* Thompson (see Chapter 3). However, optimisation of conditions for the delivery of nitric oxide with relevance to field growing conditions could not conclusively show a reproducible effect. Possible reasons for this include the following.

Firstly, the effect of nitric oxide treatment directly on biofilm formation and dispersal for *Salmonella* spp. has not yet been reported. Studies carried out using *P. aeruginosa* (a model organism for the study of bacterial biofilms) have described a mechanism of action for nitric oxide signalling via the induction of phosphodiesterase (PDE) activity (Barraud et al 2009a). PDE's hydrolyse c-di-GMP leading to a reduction in overall intracellular c-di-GMP level, which corresponds to downstream signalling events promoting planktonic existence, resulting in biofilm dispersal events and a reduction in biofilm surface coverage, as described in Section 1.5. As outlined above, a reduction in biofilm surface coverage in response to nitric oxide treatment has been described for a number of bacterial species, and for both single and multi-species biofilms (Barraud et al 2009b). It has also been described that c-di-GMP signalling and the proteins that control c-di-GMP synthesis (GGDEF domain containing proteins with DGC activity) and degradation (EAL and HD-GYP domain containing proteins with PDE activity) are

ubiquitous among the bacteria (Romling et al 2013). It is therefore hypothesised that nitric oxide signalling leading to biofilm dispersal, perhaps via the mechanism described in *P. aeruginosa*, may also be conserved across bacterial genera (see Section 1.5). These investigations have not yet been reported for *Salmonella* spp.

S. Typhimurium is known to contain a total of 20 proteins with GGDEF and EAL domains (see Figure 31). Therefore, *S.* Typhimurium appears to contain the necessary cellular apparatus for modulation of c-di-GMP levels by nitric oxide via the induction of PDE activity in a similar way to that seen for *P. aeruginosa*, but whether or not this actually corresponds to downstream signalling events leading to biofilm dispersal and the induction of a planktonic phenotype in *S.* Typhimurium remains unknown. Here, the effect of nitric oxide treatment on biofilms of *S.* Typhimurium has been investigated.

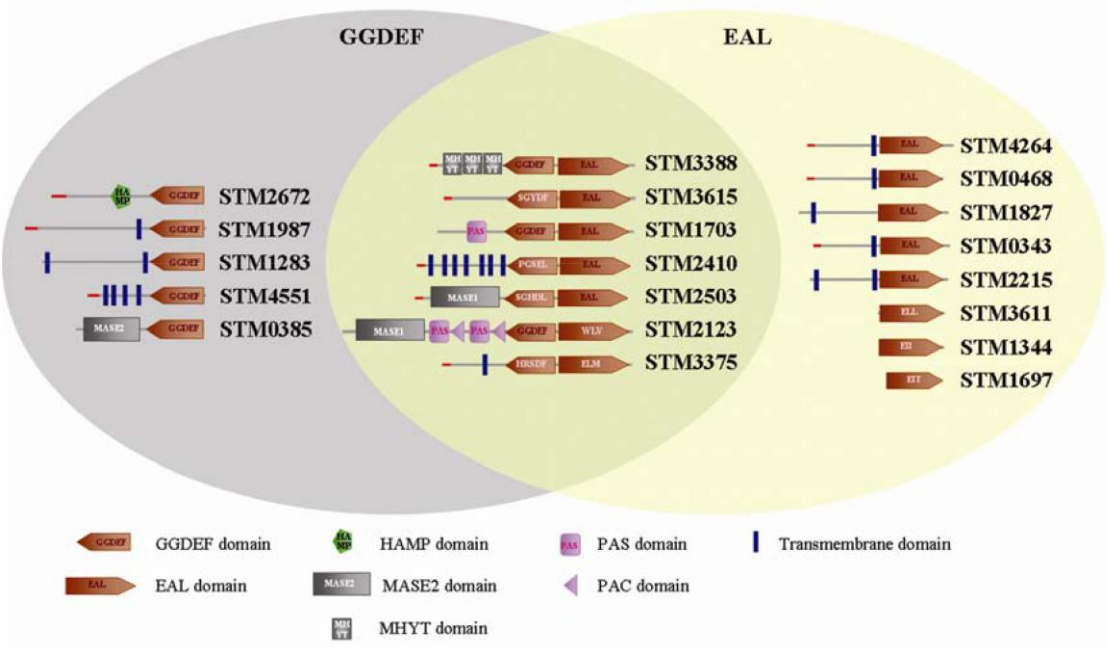


Figure 31 GGDEF/EAL domain proteins in *S.* Typhimurium
from (Simm 2007)

Secondly, the complex nature of interactions between bacteria and leaf surfaces, and their variability in an environmental situation, are not yet well understood for zoonotic pathogens such as *Salmonella* spp. A better understanding of the mechanisms underlying surface colonisation and biofilm formation by *Salmonella* spp. under

environmental conditions, may lead to better targeting of intervention strategies for zoonotic pathogen colonisation, such as nitric oxide treatment, in a highly complex and variable environmental situation such as the phyllosphere.

It is well documented that the expression of cell surface appendages governing bacterial interactions with surfaces vary in response to environmental stimuli (Gomelsky and Hoff 2011, Walker et al 1999) and that biofilm dispersal can occur in response to changes in external environments (see Section 1.5). As the biofilm dispersal action of nitric oxide is thought to occur via modulation of c-di-GMP levels (Barraud et al 2009a), and the expression of cell surface appendages governing surface colonisation are under the control of c-di-GMP (Hengge 2009), it is not unreasonable to hypothesise that exogenous nitric oxide treatment may have a more complex interplay with the transduction of environmental signals than currently appreciated. Indeed, regulation of c-di-GMP levels by proteins with DGC and PDE activity is already known to be under the control of a complex network of feedback signals (Romling et al 2005). Therefore, whilst under carefully controlled laboratory conditions it may be possible to specifically induce PDE activity leading to biofilm dispersal, as in the case of *P. aeruginosa*, it remains to be seen whether this is applicable in an environment such as the phylloplane, where external stimuli leading to the transduction of signals via c-di-GMP cannot be easily controlled.

Here, the effect of the environmental input variable, temperature, on colonisation of spinach leaves by *S.* Typhimurium has been investigated. In addition, the role of flagellar expression (a downstream consequence of c-di-GMP signalling (Wolfe and Visick 2008)) in the colonisation of spinach leaves by *S.* Typhimurium has been investigated. Flagella have recently been reported to directly mediate attachment of *Salmonella* spp. to the phylloplane (Berger et al 2009) but have not yet been considered in the context of phylloplane biofilms. *Salmonella* biofilms have been demonstrated to exist at leaf surfaces (Kroupitski et al 2009b), and flagellar expression is important for the development of bacterial biofilms (O'Toole and Kolter 1998a). However, as the study of biofilm formation at the phylloplane encounters surface variables that cannot be controlled experimentally, an abiotic surface biofilm assay

system has been used here to allow the role of flagellar expression in biofilm formation to be investigated under controlled conditions.

It is hypothesised that a better understanding of the colonisation strategies for *Salmonella* spp. at the phylloplane, and the role of nitric oxide in bacterial biofilm formation and dispersal for *Salmonella* spp., may be able to better inform experimental design and optimise exogenous application of nitric oxide for decontamination of *Salmonella* from the phylloplane.

4.2 Experimental procedures

General methodology used for these investigations is described in Chapter 2. The effect of temperature on colonisation of spinach leaves by *S.* Typhimurium, and the role of flagellar in attachment and biofilm formation have been investigated at both the biotic leaf surface, as described in Section 2.4, and using an abiotic polystyrene 6-well plate experimental model, as described in Section 2.7. The effect of nitric oxide treatment on the dispersal of *S*. Typhimurium biofilms has also been investigated at the abiotic surface using the 6-well plate experimental model (Section 2.7).

For leaf colonisation assays, spinach leaves were inoculated with approximately 1×10^6 CFU mL^{-1} bacteria, as described in Section 2.4, and incubated at 22, 30 or 37°C for 24 hours for the investigation of the effect of temperature on colonisation of the phylloplane by *S.* Typhimurium 14028 WT. For investigations into the role of flagellar expression in the attachment of *S.* Typhimurium to the spinach phylloplane, *S.* Typhimurium 14028 *flh*C, deficient in the production of flagellar, was used (Simon et al 2007).

For abiotic surface biofilm assays investigating the role of flagellar expression in surface attachment by *S.* Typhimurium 14028 WT and *S.* Typhimurium 14028 *flh*C, biofilms were grown statically at 22, 30, and 37 C in $LB_{1/5}$ for 24 hours. For investigations into the effect of SNP on colonisation of the abiotic surface by *S.* Typhimurium 12023 GFP, biofilms were grown in the presence of SNP for 24 hours. Control biofilms were grown in the absence of SNP for comparison. For investigations

into the effect of SNP on detachment of *S.* Typhimurium 12023 biofilms from the abiotic surface biofilms were grown at 22°C in $LB_{1/5}$ for 24 hours in the absence of SNP to allow for biofilm formation before the addition of 50 nM, 500 nM or 1 µM SNP and incubation at 22°C for a further 24 hours. Control biofilms were incubated in the absence of SNP for comparison. For the investigation of biofilm growth in sdH_2O, *S.* Typhimurium 12023 GFP biofilms were grown for between 2 hours and 7 days in the absence of SNP, 500 nM SNP was then added to 7 day-old biofilms for the assessment of detachment.

For leaf assays, bacteria were recovered from the phylloplane by Pulsification as described in Section 2.5, and plated onto Rambach® agar as described in Section 2.2.2.2 for enumeration of culturable *Salmonella*. Abiotic surface biofilm samples were processed and observed by EDIC/EF microscopy as described in Section 2.9.2. For *S.* Typhimurium 14028 WT and *S.* Typhimurium 14028 *flh*C biofilms were stained with Syto®-9 prior to microscopy as described in Section 2.9.2.

4.3 Results

4.3.1 Colonisation of spinach leaves by *S.* Typhimurium is influenced by temperature

The effect of temperature on colonisation of spinach leaves by *S.* Typhimurium 14028 WT was investigated at 22, 30 and 37°C, in order to determine whether temperature fluctuations in an open spinach field environment may influence colonisation of the phylloplane by *Salmonella* spp.

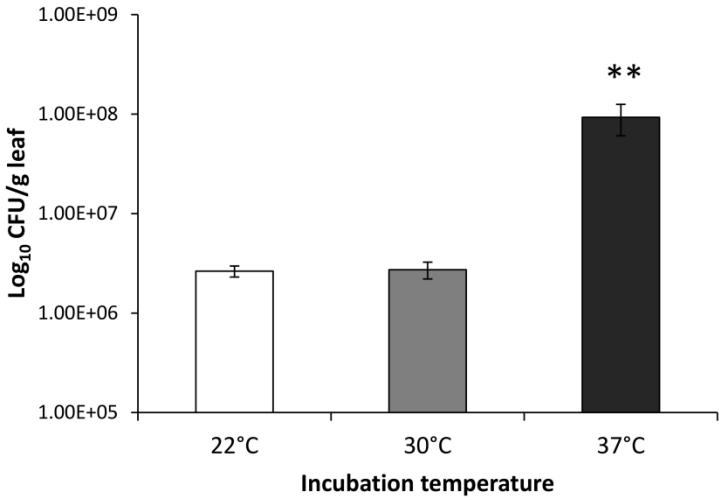


Figure 32 The effect of temperature on colonisation of spinach leaves by *S.* Typhimurium 14028 WT

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 14028 WT and incubated at 22, 30 or 37°C for 24 hours. Bacteria were removed from leaves by pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three leaves per treatment.

A statistically significant (one-way ANOVA; $P < 0.01$) approximate 2.0 log increase in culturable *S.* Typhimurium 14028 WT associated with the spinach phylloplane was observed for leaves incubated at 37°C by comparison to leaves incubated at 30°C and 22°C (Figure 32). No significant difference in culturable *S.* Typhimurium were seen for leaves incubated at 30°C by comparison to leaves incubated at 22°C. Colonisation of spinach leaves by *S.* Typhimurium is therefore influenced by temperature at 37°C (human physiological temperature) by comparison to the environmental temperatures

investigated, but is not influenced by fluctuations between the environmental temperatures of 22°C and 30°C investigated here.

4.3.2 Flagellar expression is involved in, but not essential for, colonisation of spinach leaves by *S.* Typhimurium

The effect of flagellar expression on colonisation of spinach leaves by *S.* Typhimurium 14028 WT and *S.* Typhimurium 14028 *flh*C was investigated at 22, 30 and 37°C to determine whether flagellar expression influences the ability of these bacteria to colonise the spinach phylloplane.

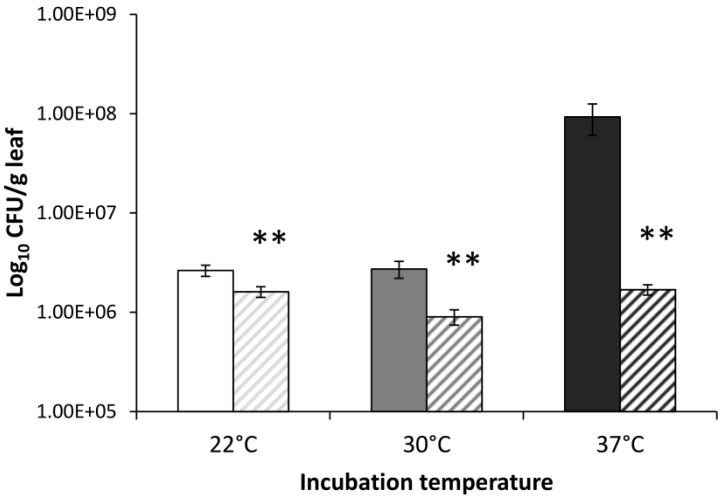


Figure 33 Colonisation of spinach leaves by *S*. Typhimurium 14028 WT and flagellar deficient *S.* Typhimurium 14028 *flh*C at 22, 30 and 37°C

Spinach leaves were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 14028 WT (solid bars) or flagellar deficient *S.* Typhimurium 14028 *flh*C (striped bars) and incubated at 22, 30 or 37°C for 24 hours. Bacteria were removed from leaves by pulsification and plated onto Rambach® agar for enumeration of culturable *Salmonella*. Error bars represent ± standard error of the mean of three leaves per treatment.

Colonisation of the spinach phylloplane by flagellar deficient *S.* Typhimurium is less efficient than colonisation by the wild-type strain at all temperatures investigated, with statistically significant (one-way ANOVA: $P < 0.01$) 0.2, 0.5 and 1.7 log reductions in numbers of culturable *Salmonella* associated with the spinach phylloplane after incubation of leaves at 22, 30 and 37°C, respectively (Figure 33). In addition, the 2.0

log increase in colonisation observed for wild type *S.* Typhimurium at 37°C is not observed for the flagellar deficient mutant, suggesting indeed that flagellar expression facilitates efficient colonisation of spinach leaves by *S*. Typhimurium at 37°C. Whether this is a direct attachment mechanism, an involvement in biofilm formation, or a direct consequence of increased motility is not clear. However, abolishing flagellar expression does not abolish the ability of *S.* Typhimurium to attach to spinach leaves, and therefore flagellar expression is not essential for bacterial attachment to the spinach phylloplane.

4.3.3 Flagellar expression is required for efficient surface colonisation by *S.* Typhimurium at the abiotic surface and is important for biofilm formation

It is hypothesised that biofilm formation is an important strategy for colonisation of the phylloplane by *Salmonella* spp. It has been shown that colonisation of the phylloplane by *S.* Typhimurium is temperature dependent (Section 4.3.1), and that flagellar mediated motility is important for successful colonisation of the phylloplane (Section 4.3.2). Abiotic surface colonisation and biofilm formation by wild-type and flagellar deficient *S.* Typhimurium was investigated under low nutrient conditions to reflect conditions encountered by biofilms in an environmental situation.

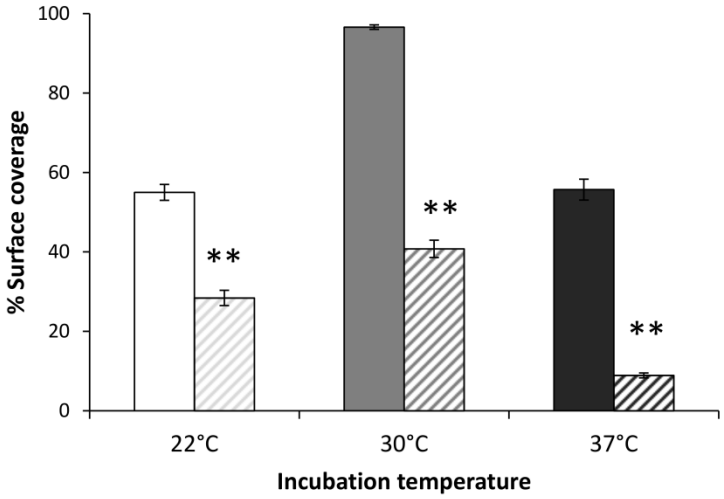


Figure 34 Colonisation of the abiotic surface by *S.* Typhimurium 14028 WT and flagellar deficient *S.* Typhimurium 14028 *flh*C at 22, 30 and 37°C

Abiotic 6 well plates were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 14028 WT (Solid bars) or flagellar deficient *S.* Typhimurium 14028 *flh*C (striped bars) per well and incubated statically in 1/5 strength LB broth at 22, 30 or 37°C for 24 hours. Biofilms were stained with Syto®9 and observed by EDIC/EF microscopy. Percentage biofilm surface coverage was calculated from EDIC/EF micrographs using ImageJ® software. Error bars represent ± standard error of the mean of percentage surface coverage measurements taken from a minimum of ten frames per well from three wells per treatment.

Colonisation of the abiotic surface by flagellar deficient *S.* Typhimurium was less efficient than colonisation by the wild-type strain at all temperatures investigated. Statistically significant (one-way ANOVA; $P < 0.01$) reductions in percentage surface coverage of 48%, 58% and 84% for biofilms grown at 22, 30 and 37°C, respectively, were observed for *S.* Typhimurium 14028 *flh*C biofilms by comparison to *S.* Typhimurium 14028 WT (Figure 34). As seen at the phylloplane (see Section 4.3.2) abolishing flagellar expression did not abolish the ability of *S.* Typhimurium to colonise the abiotic surface, therefore flagellar expression is not essential for surface colonisation by *S.* Typhimurium. Interestingly surface colonisation for the wild type strain at the abiotic surface was optimal at 30°C (>95% biofilm surface coverage) rather than at 37°C as seen at the phylloplane.

EDIC microscopy was used to investigate biofilm morphology at the abiotic surface at 22, 30 and 37°C for wild type and flagellar deficient *S.* Typhimurium (Figure 35). Whilst the flagellar deficient mutant adhered to the abiotic surface at all temperatures,

biofilm morphology was distinctly different from that of the wild type. Wild type *S.* Typhimurium biofilms at all temperatures investigated formed a monolayer of cells interspersed with small microcolonies after 24 hours (Figure 35 A, C & E) whilst virtually no microcolonies were apparent for the flagellar deficient mutant strain (Figure 35 D & F). In addition, biofilm morphology for the flagellar deficient mutant strain was distinctly different at human physiological temperature of 37°C (Figure 35 F) than at lower temperatures of 30°C and 22°C (Figure 35 B & D) more akin to temperatures found in the environment. EDIC microscopy of biofilms grown at 37°C for the flagellar deficient mutant strain appeared to only loosely adhere to the abiotic surface, as demonstrated by the morphology of surface coverage observed following sample processing shown in Figure 35 F. This finding suggests not only that flagellar expression is important for surface colonisation by *S.* Typhimurium at 37°C, but also that an alternative, non-flagellar mediated mechanism for surface colonisation may be in effect at 22 and 30°C that is not expressed at 37°C.

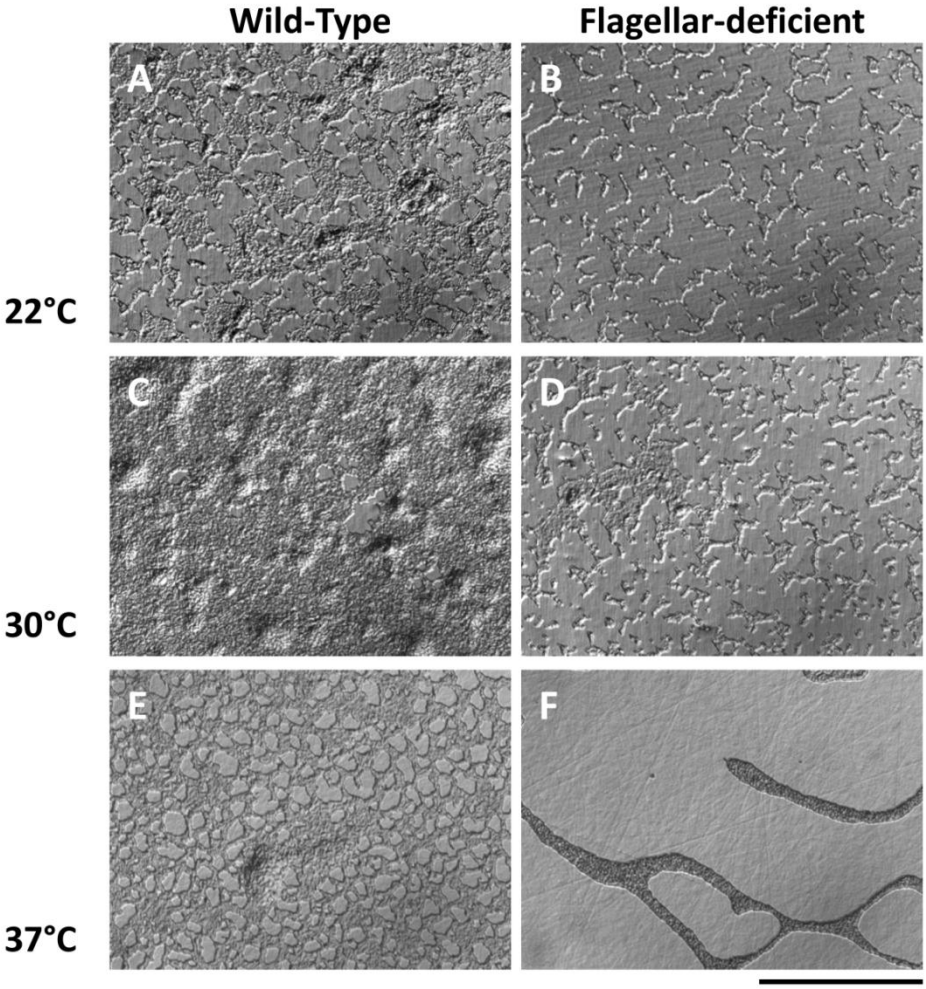


Figure 35 Structure of *S.* Typhimurium 14028 WT and flagellar deficient *S.* Typhimurium 14028 *flh*C biofilms grown under low-nutrient conditions at the abiotic surface at 22, 30 and 37°C

Representative EDIC micrographs showing morphology of *S.* Typhimurium 14028 WT (A, C & E) and *S.* Typhimurium 14028 *flh*C (B, D & F) biofilms grown in 1/5 strength LB broth for 24 hours at 22°C (A & B), 30°C (C & D) and 37°C (E & F). A monolayer-like biofilm with small interspersed microcolonies can be seen for *S.* Typhimurium 14028 WT biofilms, whilst less confluent biofilm coverage can be seen for biofilms of *S.* Typhimurium 14028 *flh*C, particularly at 37°C. Differences in biofilm coverage and morphology can also be seen between WT biofilms grown at 22°C (A), 30°C (C) and 37°C (E). Scale bar = 50 microns

4.3.4 Nitric oxide treatment of *S.* Typhimurium 12023 GFP biofilms does not reduce abiotic surface biofilm coverage

The effect of nitric oxide treatment on colonisation of the abiotic surface by *S.* Typhimurium 12023 GFP and on the detachment of *S.* Typhimurium 12023 GFP biofilms from the abiotic surface was investigated, in order to determine whether exogenous application of SNP influences *S.* Typhimurium biofilms.

Exogenous application of SNP to *S.* Typhimurium 12023 GFP biofilms grown for 24 hours in the presence of 50 nM, 500 nM or 1 µM SNP did not lead to a reduction in abiotic surface coverage (Figure 36 A). No reductions in biofilm surface coverage were observed when SNP was added to *S.* Typhimurium 12023 GFP biofilms after 24 hours for the induction of biofilm dispersal (Figure 36 B). A statistically significant increase in surface coverage by *S.* Typhimurium was observed with 1 µM SNP treatment when added during biofilm formation (Figure 36 A, one-way ANOVA $P < 0.01$), and with 50 nM and 1 µM SNP treatment when added for the induction of biofilm dispersal (Figure 36 B, one-way ANOVA $P < 0.01$). Microscopy images did not show any visible differences in biofilm morphology between Control and SNP treated *S.* Typhimurium biofilms.

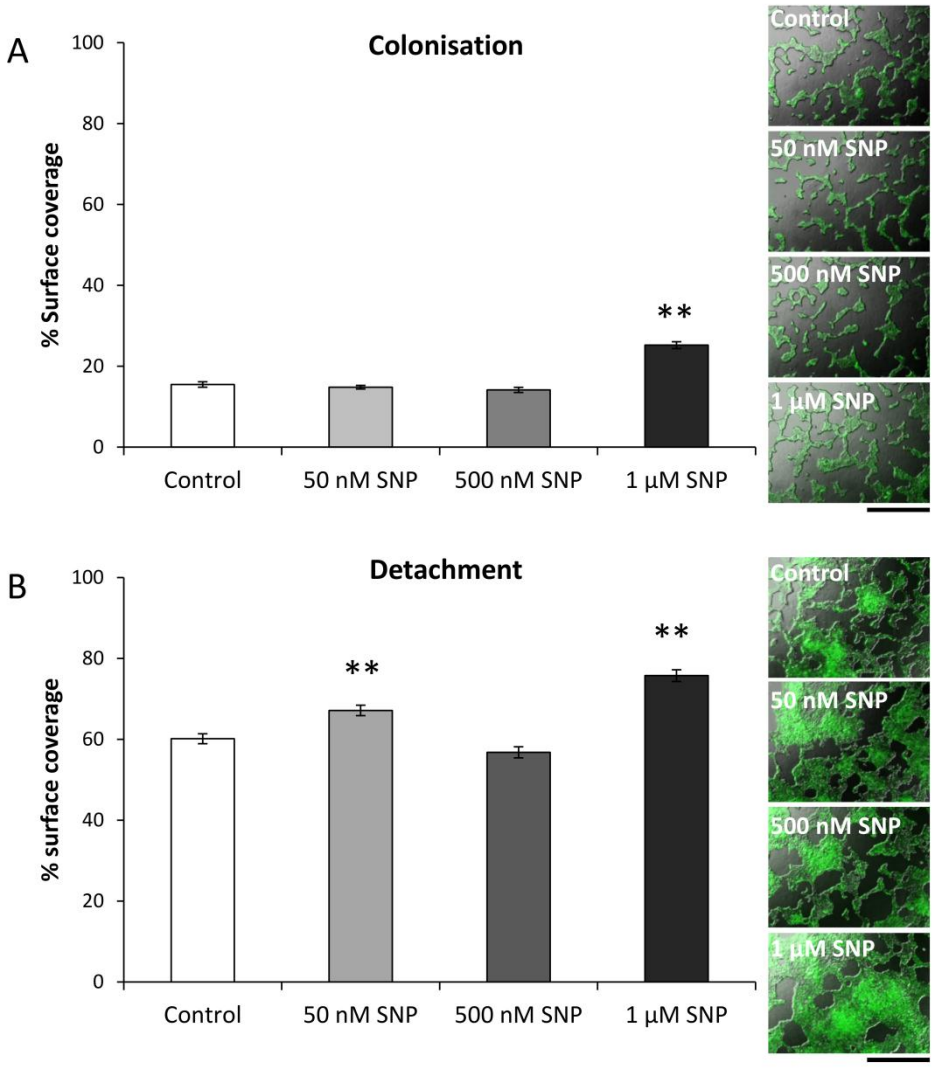


Figure 36 The effect of nitric oxide on biofilm formation by *S.* Typhimurium 12023 GFP at the abiotic surface

Abiotic 6 well plates were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP per well in 1/5 strength LB broth and incubated statically in the presence (grey bars) or absence (white bars) of the nitric oxide donor SNP. For assessment of colonisation, plates were incubated at 22°C for 24 hours. For assessment of detachment, plates were incubated at 22°C in the absence of SNP to allow for surface colonisation by bacteria, before the addition of SNP for a further 24 hours. Biofilms were observed by EDIC/EF microscopy and percentage biofilm surface coverage calculated from EDIC/EF micrographs using ImageJ® software. Error bars represent ± standard error of the mean of percentage surface coverage measurements taken from a minimum of ten frames per well from three wells per treatment. Scale bar = 50 microns

4.3.5 *S.* Typhimurium 12023 GFP forms biofilms when grown under low nutrient conditions but nitric oxide treatment does not have an effect on their dispersal

EDIC/EF microscopy was used to visualise the development of a *S.* Typhimurium 12023 GFP biofilm at the abiotic surface when grown in sdH_2O only at 22°C to model low nutrient environmental growth conditions, and to reflect experimental conditions used in leaf assay systems in Chapter 3. Morphology of *S.* Typhimurium 12023 GFP biofilms was visually assessed and percentage surface covered quantified over a 7 day period. The exogenous application of SNP to 7 day old *S.* Typhimurium 12023 GFP biofilms for the induction of biofilm dispersal was also investigated.

Biofilm development over a 7 day period was observed by EDIC/EF microscopy (Figure 38 A), and percentage biofilm surface coverage was quantified (Figure 37 B). Attachment of single cells to the abiotic surface occurs from 2 to 4 hours post-inoculation and progresses to the formation of aggregates from 4 to 12 hours, with the appearance of a few small microcolony-like structures. Progression of biofilm formation from 24 to 48 hours shows the appearance of small microcolonies with larger microcolonies present after 7 days, as indicated by white arrows. However, by comparison to mature biofilm morphology characteristic of other model species such as *P. aeruginosa*, which are often grown experimentally in high nutrient conditions at human physiological temperatures, (see Section 1.4), biofilm morphology observed here for *S.* Typhimurium is distinctly different, with a dispersed monolayer of cells and flat microcolony architecture as shown in Figure 37 A.

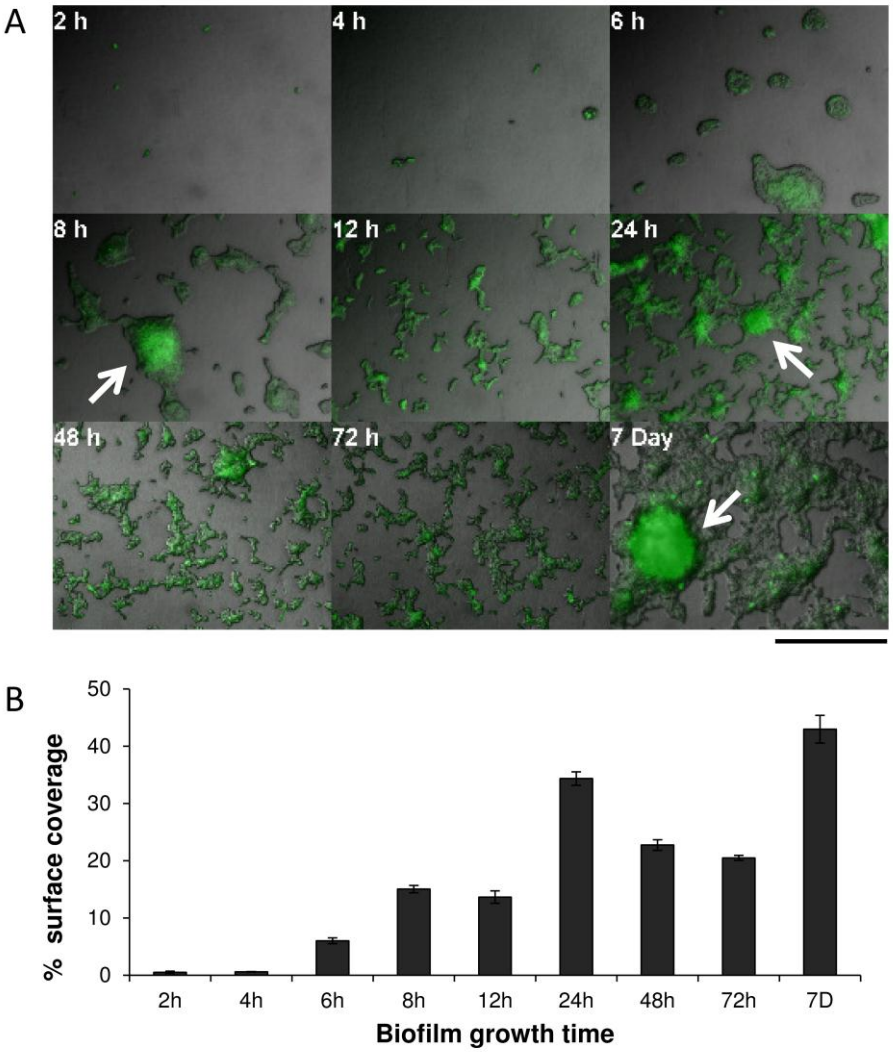


Figure 37 Biofilm morphology (A) and percentage surface coverage (B) of *S.* Typhimurium 12023 GFP biofilms grown in sdH_2O at 22°C

Abiotic 6 well plates were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP per well in sdH_2O and incubated statically at 22°C for 2 hours to 7 days. Biofilms were observed by EDIC/EF microscopy (A) and percentage biofilm surface coverage calculated from EDIC/EF micrographs using ImageJ® software (B). Error bars represent ± standard error of the mean of percentage surface coverage measurements taken from a minimum of ten frames per well from three wells per treatment. Scale bar = 50 microns

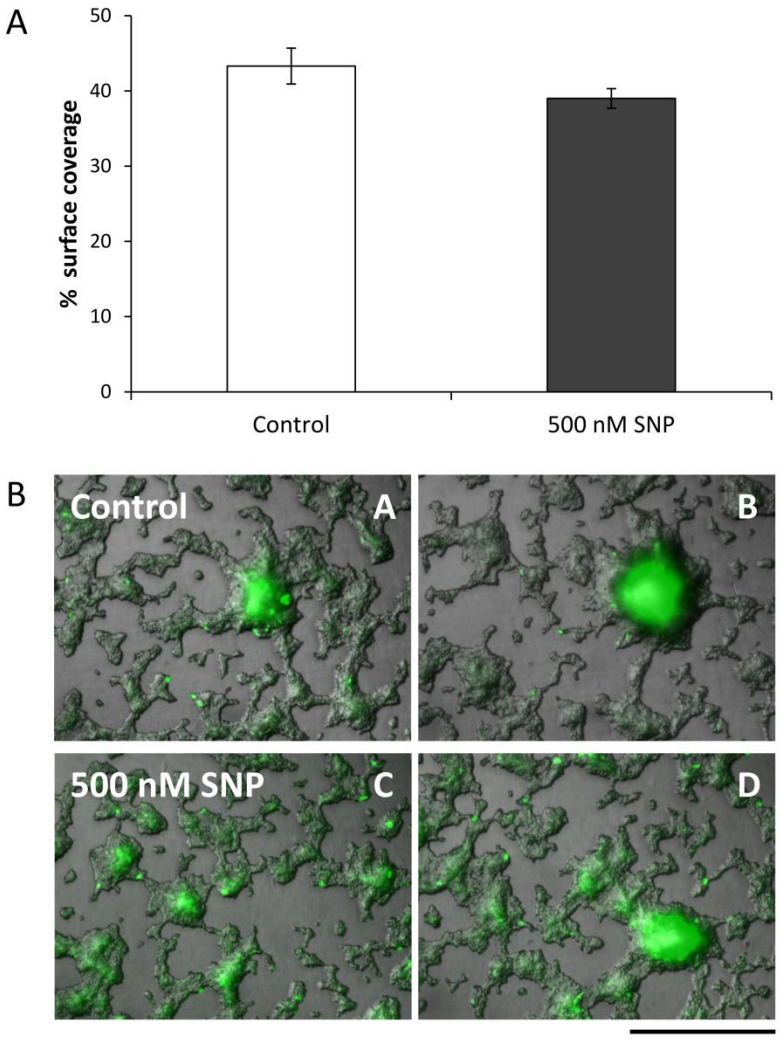


Figure 38 The effect of nitric oxide on dispersal of 7 day old *S.* Typhimurium 12023 GFP biofilms grown in sdH_2O

Abiotic 6 well plates were inoculated with approximately $1x10^6$ CFU mL^{-1} *S.* Typhimurium 12023 GFP per well in sdH_2O and incubated statically at 22°C for 7 days to allow for biofilms to establish. Biofilms were then incubated at 22°C for a further 24 hours in the presence (grey bar) or absence (white bar) of 500 nM of the nitric oxide donor SNP. Biofilms were observed by EDIC/EF microscopy and percentage biofilm surface coverage calculated from EDIC/EF micrographs using ImageJ® software. Error bars represent ± standard error of the mean of percentage surface coverage measurements taken from a minimum of ten frames per well from three wells per treatment. Scale bar = 50 microns

Exogenous application of 500 nM SNP to 7 day old *S.* Typhimurium 12023 GFP biofilms resulted in a suggested approximate 10% reduction in biofilm surface coverage, although statistical significance was not reached (Figure 38 A, one-way ANOVA $P>0.05$). Microscopy images of biofilm morphology did not show any differences following treatment with 500 nM SNP by comparison to untreated Control biofilms (Figure 38 B). Therefore, developed *S.* Typhimurium 12023 GFP biofilms grown under low nutrient conditions do not appear to disperse in response to 500 nM SNP treatment.

4.4 Discussion

4.4.1 The effect of variable external environmental conditions on colonisation of spinach leaves by *S.* Typhimurium

The expression of cell surface appendages governing the attachment of bacteria to surfaces is influenced in response to environmental stimuli, with c-di-GMP playing a central role in signal transduction leading to phenotypic changes that define surface interactions and biofilm development (Hengge 2009, Romling et al 2005, Ryan et al 2006). Phylloplane biofilms are an important colonisation and survival strategy for bacteria at the phylloplane, with zoonotic pathogens such as *Salmonella* spp. having been shown to form biofilms at leaf surfaces (Brandl and Mandrell 2002, Kroupitski et al 2009b), and where external environmental conditions to which leaves are exposed throughout the production process for leafy salads are highly variable. Given that exogenous application of nitric oxide has been shown to influence c-di-GMP signalling in bacteria (Barraud et al 2009a) it is possible that the response of biofilms to nitric oxide treatment could be influenced by c-di-GMP signalling induced by external environmental stimuli in a way that is not yet fully appreciated, as signal transduction pathways for nitric oxide signalling in bacteria have not yet been fully elucidated. It has been shown that large differences in number of GGDEF and EAL/HD-GYP domain containing proteins exist between species (Simm et al 2004). Many signals and effectors feed in to the global regulatory signalling molecule c-di-GMP (Romling and Amikam 2006, Solano et al 2009) which may affect the response of biofilms in the environment nitric oxide treatment.

In Chapter 3 it was observed that external environmental conditions affected the response of *Salmonella* at the phylloplane to exogenous treatment with nitric oxide. Therefore, here the effect of temperature on the colonisation of spinach leaves by *S.* Typhimurium was investigated and found indeed to be temperature dependent. Temperature differences were observed between human physiological temperature and lower temperatures designed to model environmental conditions, but no differences were observed between environmental temperatures investigated (see Figure 32). Results here suggest that temperature fluctuations may not influence

colonisation of leaves by *S.* Typhimurium in the field, or that *S.* Typhimurium has efficient alternative mechanisms in place to deal with these temperature fluctuations at non-physiological temperatures to facilitate colonisation under environmental conditions. Using an abiotic surface model to investigate the effect of temperature on biofilm formation by *S.* Typhimurium, biofilm formation was found to be temperature dependent at the abiotic surface, but that optimal temperature for abiotic surface colonisation is 30°C (by comparison to 37°C for spinach leaves), suggesting that different mechanisms of colonisation are in place at spinach phylloplane by comparison to the abiotic surface.

In *S.* Typhimurium, expression of the rdar (red dry and rough) morphotype for example, characterised by the expression of curli fimbriae and cellulose and important for abiotic surface colonisation, is also under the control of c-di-GMP (Kader et al 2006) in a temperature dependent manner. This could account for the differences in surface colonisation observed between human physiological temperature and environmental temperatures observed at the phylloplane, as described above. However, in work carried out by Warner, whilst deletion of genes involved in the production of curli fimbriae influenced colonisation of abiotic polystyrene by *S.* Typhimurium, no effect on colonisation of spinach leaves was observed (Warner 2009), showing that curli fimbriae are not essential for colonisation of spinach leaves by *S.* Typhimurium. This supports the hypothesis that *S.* Typhimurium may have alternative mechanisms of colonisation in place at the spinach phylloplane by comparison to the abiotic surface, and/or that it can effectively adapt its phenotype to facilitate colonisation of leaf surfaces. Here, the role flagellar expression, which has recently been implicated in the attachment of *Salmonella* spp. to leaf surfaces (Berger et al 2009), was investigated for its role in attachment of *S.* Typhimurium to spinach leaves.

Flagellar expression confers motility to planktonic cells and is also known to be involved in the formation of bacterial biofilms. Recently, the involvement of flagella in attachment of *Salmonella* spp. to basil leaves has been reported (Berger et al 2009). It is well known that temperature affects the expression of cell surface appendages involved in surface colonisation, such as flagella (Walker et al 1999), and that flagellar expression is regulated by c-di-GMP (Wolfe and Visick 2008). Here, the role of flagellar

expression in the colonisation of spinach leaves was demonstrated to be important, but not essential, for the colonisation of spinach leaves by *S.* Typhimurium (see Figure 33), but that like the curli fimbriae (Warner 2009), flagellar expression plays an important role in the development of *S.* Typhimurium biofilms at the abiotic surface. Therefore, as flagellar expression is involved in, but not essential for, colonisation of spinach leaves by *S.* Typhimurium, what role do flagella play? It is possible that flagella are involved in the formation of bacterial biofilms at the phylloplane. An increase in flagellar expression upon surface attachment is known to facilitate the swarming ability of bacteria, required for efficient biofilm formation and be important for the microcolony development characteristic of bacterial biofilms (Verstraeten et al 2008). However, as the direct study of *Salmonella* biofilm formation *in situ* at the phylloplane, by time-lapse microscopy for example, is not possible due to experimental constraints such as the breakdown of leaf tissue upon prolonged exposure to high-intensity light, the involvement of flagellar expression in surface colonisation was investigated here at the abiotic surface, under low nutrient conditions to model an environmental biofilm.

As cell surface appendage expression varies in response to environmental stimuli, it is likely that cell surface appendages play different roles in colonisation under different environmental conditions, thus supporting the targeting of a signalling mechanism such as c-di-GMP for decontamination strategies, as c-di-GMP level controls an array of downstream signalling events that determine the switch between sessility and motility in bacteria (Romling et al 2013). However, mechanisms of synthesis and degradation that control intracellular levels of c-di-GMP are only just beginning to be elucidated, and whilst deemed outside of the scope of these investigations, further understanding of signalling mechanisms controlling intracellular c-di-GMP in the future may contribute to the development of novel decontamination strategies for bacterial biofilms. Whilst the influence of nitric oxide signalling for c-di-GMP has been described in *P. aeruginosa*, the specific role of nitric oxide in the control of c-di-GMP levels on a more global scale for bacteria remains unknown. Here, the effect of nitric oxide on *Salmonella* biofilms at the abiotic surface has been investigated. The phylloplane represents a particularly complex surface for the study of bacterial interactions and biofilm formation given its varied topography, composition and environmental conditions to which it is subject, therefore, the use of abiotic surface

models for understanding the response of zoonotic pathogens to treatment with nitric oxide outside of the phylloplane environment is an important tool for the study of novel decontamination strategies.

4.4.2 The effect of nitric oxide treatment on *S.* Typhimurium biofilms

Nitric oxide treatment has been demonstrated to reduce biofilm surface coverage for a range of bacterial species(Barraud et al 2009b), as described in Section 1.5.3, but it was unable to induce a consistent reduction in the contamination of spinach leaves by *Salmonella* spp. (see Chapter 3).

This is the first time that the effect of nitric oxide on the dispersal of *Salmonella* biofilms has been investigated. Here, as for Barraud *et al*, a reduction in biofilm surface coverage was taken as a measure of biofilm dispersal using an abiotic polystyrene 6-well plate model. However, it has only been shown that the reduction in percentage surface coverage seen for bacteria other than *P. aeruginosa* is a result of programmed biofilm dispersal events for the model organism *P. aeruginosa* (Barraud et al 2009a). In the life cycle of a *P. aeruginosa* biofilm, dispersal events occur naturally when microcolonies get so large that nutrients and oxygen are limited within the centre of these structures, leading to the production of ROS and RNS leading to cell death, the breakdown of the microcolony and the release of a sub-population of surviving cells into the surrounding environment in planktonic form, as described in Section 1.4.4.3. Exogenous treatment of biofilms with SNP to induce biofilm dispersal is designed to induce this signalling response at low, non-cytotoxic levels of nitric oxide (Barraud et al 2006). However, the pre-requisite for these dispersal events, and presumably the reason for the evolution of such complex signalling mechanisms in bacteria such as *Pseudomonads*, is the formation of large biofilm microcolonies (or 'macrocolonies') leading to the production of reactive oxygen and nitrogen species. However, not all bacteria form biofilms with a similar structure to *P. aeruginosa*, particularly not under environmental conditions (see Section 1.4.4.2). Biofilm morphology for *S.* Typhimurium under the low nutrient conditions investigated here is distinctly different to that seen for *P. aeruginosa* biofilms. The formation of biofilms by zoonotic pathogens such as *Salmonella* spp. under environmental conditions at the

phylloplane are not known to produce such 'macrocolonies' and have not been demonstrated to produce the same ROS or RNS. Therefore whether these bacteria modulate responses to ROS and RNS, and therefore respond to exogenous treatment with nitric oxide in the same way as *P. aeruginosa* remains unknown.

Treatment of *S.* Typhimurium biofilms with nitric oxide did not cause a reduction in biofilm surface coverage under any of the conditions investigated here, demonstrating that exogenous application of SNP does not induce a signalling response in *S.* Typhimurium corresponding to biofilm dispersal events under low nutrient conditions. Increases in biofilm surface coverage were however observed suggesting some influence for nitric oxide signalling in *S.* Typhimurium. An increase in colonisation by S. Typhimurium at the abiotic surface as seen here, similar to increases in attachment and colonisation observed at the phylloplane, is an undesirable outcome when considering the use of exogenous application of SNP for the reduction of biofilms in a food production environment, and may indeed mean that nitric oxide is not a suitable decontamination strategy for this application. However, it is highly unlikely that the formation of single species biofilms in complex environmental communities such as those found at the phylloplane is a survival strategy employed by zoonotic pathogens, therefore, as phylloplanes are routinely home to large and complex microbial communities, the interaction of zoonotic pathogens with these communities is an important consideration for furthering the understanding of pathogen contamination of leafy salads.

Designing successful intervention strategies for zoonotic pathogen contamination at the phylloplane will be strongly influenced by how zoonotic pathogens exist at leaf surfaces. If a bacterium were to attach to leaves via a single cell surface appendage, then targeting this for a specific decontamination strategy would theoretically be possible, however, it is evident that bacteria have evolved a range of mechanisms for the establishment of stable communities at phylloplanes. Biofilm formation is one such mechanism that allows bacteria to thrive in otherwise hostile environments. Whether single species biofilms exist for zoonotic pathogens at the phylloplane is unknown, but the existence of large and complex microbial communities at leaf surfaces is well documented, and indigenous bacterial biofilms at phylloplanes have

been described (Morris et al 1997, Warner et al 2008). In Chapter 3 it was suggested that *Salmonella* spp. may preferentially attach to existing bacterial aggregates on the leaf surface (see Section 3.3.1), in agreement with other work carried out at these laboratories (Warner et al 2008). Therefore the interaction of *Salmonella* spp. with existing phylloplane communities is an important consideration for targeting decontamination strategies for zoonotic pathogens at the phylloplane. This is investigated further in Chapter 6.

4.5 Conclusions

Temperature has been shown here to be important for colonisation of the spinach phylloplane by *S.* Typhimurium at human physiological temperature by comparison to environmental temperatures, but does not affect surface colonisation between environmental temperatures investigated. In addition, flagellar expression has been shown to be involved in, but not essential for, the attachment of *S.* Typhimurium to spinach leaves. Upon further investigation at the abiotic surface, it is clear that both temperature and flagellar expression are important for efficient surface colonisation and in the development of bacterial biofilms. As these factors are involved in, but not essential for, attachment to both the abiotic surface and the phylloplane, but are essential for biofilm development, it is likely that biofilm formation plays an important role in surface colonisation by *Salmonella* spp. at the phylloplane.

Exogenous application of SNP at the abiotic surface does not induce biofilm dispersal events leading to a reduction in abiotic surface coverage for *S.* Typhimurium under the conditions investigated here, demonstrating that it may not be a suitable decontamination strategy for *Salmonella* biofilms. *Increases* in surface colonisation were observed upon nitric oxide treatment, which may make treatment with nitric oxide an unsuitable method for use in a food production environment. However, whilst these increases are undesirable in terms of a pathogen control strategy, they do indicate that nitric oxide signalling mechanisms are active in *S.* Typhimurium. The reasons for the lack of biofilm dispersal observed here for *S.* Typhimurium remain unclear but it is possible that nitric oxide signalling in *S.* Typhimurium is distinct from the model species *P. aeruginosa* and that the modulation of intracellular c-di-GMP

levels between species is more complex than currently appreciated. Comparisons between responses to nitric oxide treatment between bacterial species could provide valuable insights into the mechanism of signal transduction by nitric oxide in bacteria. In Chapter 5, the effect of nitric oxide treatment on cells of *S.* Typhimurium has been investigated using a proteomic approach in order to gain insights into signalling mechanisms for nitric oxide in *Salmonella* spp.

In Chapter 6, the reasons for the small effects on attachment and colonisation of the spinach phylloplane by *Salmonella* spp. observed in Chapter 3 have been further investigated in the context of interactions with indigenous phylloplane populations. It has been demonstrated in this Chapter that these findings cannot be solely explained by temperature fluctuations, flagellar expression, or due to the dispersal of single species *Salmonella* biofilms. It has already been shown (Chapter 3), in agreement with other work (Warner 2009), that *Salmonella* spp. may preferentially attach to existing bacterial aggregates at leaf surfaces, and it is known that complex microbial communities exist at the phylloplane, therefore the effect of nitric oxide treatment on indigenous bacteria associated with field grown salad leaves has been investigated in Chapter 6.

Chapter 5

The effect of nitric oxide on *S.* Typhimurium 12023: A proteomic investigation

5.1 Introduction

In Chapter 3 it was found that treatment of spinach leaves with nitric oxide, via the exogenous application of the nitric oxide donor SNP, influenced the attachment and colonisation of spinach leaves by *Salmonella* spp. but that despite optimisation of conditions for the delivery of nitric oxide, these influences a) did not occur in a consistent manner, and b) did not result in a practically significant decrease in numbers of culturable *Salmonella* that could be used for the control of zoonotic pathogens in a food production environment (see Sections 3.3.2 and 3.3.3). It was also found that nitric oxide treatment did not induce dispersal of *Salmonella* species from the spinach phylloplane (see Section 3.3.4). However, as the phylloplane is a complex environment for the study of bacterial biofilms, the effect of exogenous application of SNP on the formation and dispersal of *Salmonella* biofilms was investigated using an abiotic surface model system in Chapter 4 (see Sections 4.3.4 and 4.3.5).

Biofilm formation is an important strategy for bacterial survival and persistence in the environment and, as described in Section 1.4.3, *Salmonella* biofilms have been demonstrated in association with the phylloplane, which may confer resistance to decontamination and pose a food safety hazard. Using this abiotic surface model system, it was found that exogenous application of nitric oxide *increased* biofilm formation by *S.* Typhimurium 12023 GFP (Chapter 4). Clearly, the potential for exogenous application of nitric oxide to increase surface colonisation by *Salmonella* spp. is an undesirable outcome for the use of nitric oxide against zoonotic pathogen contamination in a food production environment. However, the demonstration that nitric oxide treatment can increase biofilm formation by *S.* Typhimurium demonstrates that nitric oxide signalling is active in *Salmonella*.

In order to better understand the role of nitric oxide treatment in governing surface colonisation and biofilm formation by *Salmonella* spp., and to better understand how nitric oxide treatment may influence colonisation of spinach leaves by *Salmonella* spp., a proteomic investigation was carried out to directlyinvestigate the response of *S.* Typhimurium to nitric oxide treatment. Changes in protein expression in planktonic *S.* Typhimurium 12023 in response to treatment with 500 nM SNP was investigated by

label-free mass spectrometry using a shotgun proteomic approach. As described in Section 1.3.3.3, Proteomics is a powerful tool for the functional investigation of molecular composition and signalling pathways in bacteria, and can provide details on signalling pathways and interactions between molecules at the cellular level, and allow gene expression to be characterised at the protein level and show how this links to phenotype. In addition to understanding the influence of nitric oxide treatment on *S.* Typhimurium at the molecular level, this approach may provide a useful tool for comparison to proteomic investigations in other bacterial species to allow mechanisms of action for nitric oxide signalling to be further characterised.

5.2 Experimental procedures

General methodology used for these investigations is described in Chapter 2. The effect of nitric oxide treatment on protein expression by *S.* Typhimurium NCTC 12023 was investigated following exogenous application of the nitric oxide donor SNP during the planktonic growth phase. 500 nM SNP was introduced to overnight culture media for *S.* Typhimurium 12023, as described in Section 2.6, and samples processed for proteomic investigations as described in Section 2.8. Data processing was carried out using the gene ontology tool Blast 2GO (BioBam® Bioinformatics), as described in Section 2.8.

5.3 Results

5.3.1 Protein sample preparation and 1D gel electrophoresis

The growth of *S.* Typhimurium NCTC 12023 in the presence of up to 500 µM SNP was investigated to ensure that SNP treatment does not affect the growth of bacteria used for proteomic investigations (data not shown). It was confirmed that growth is not affected in the presence of 500 nM SNP. Following protein extraction, as described in Section 2.8, protein extracts were run on NuPage® Novex® 4-12% polyacrylamide mini-gels (Invitrogen life technologies, UK) to ensure that the extraction procedure had yielded proteins for further analysis, and to investigate whether any changes in protein expression upon nitric oxide treatment could be visually identified (Figure 39).

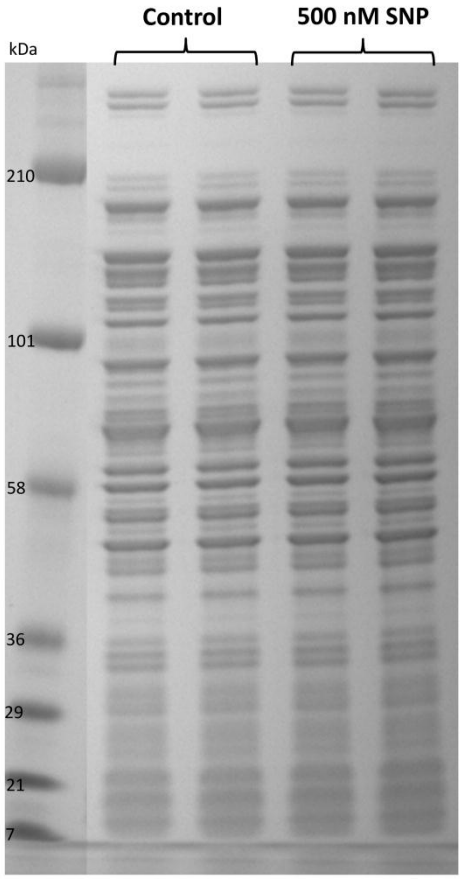


Figure 39 Protein extract profiles of *S.* Typhimurium NCTC 12023 grown in the presence of 0 (Control) and 500 nM SNP

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparing samples for electrophoresis using 4-12% polyacrylamide mini-gels. Gels were stained with the Coomassie blue-based SimplyBlue™ SafeStain before imaging.

It was confirmed that the protein extraction procedure was successful in yielding proteins from *S.* Typhimurium NCTC 12023. No differences between Control and 500 nM SNP treated samples were visible by gel electrophoresis (Figure 39). Protein extracts were sent for further analysis by label-free mass spectrometry at the Centre for Proteomic Research, University of Southampton, UK.

5.3.2 Induction of protein expression in *S.* Typhimurium in response to nitric oxide treatment; detection by label free mass spectrometry

The effect of exogenous application of 500 nM SNP on protein expression by *S.* Typhimurium 12023 was investigated using label-free mass spectrometry. Stringent selection conditions were used for the data presented in this Chapter. All changes in protein expression presented were calculated from the means of all 18 (2x biological and 9x technical) replicate samples. For proteins described as up-regulated, or down-regulated in response to SNP treatment, fold-changes were calculated from means of all 18 replicates from control and SNP treated samples. For proteins described as 'expressed only upon treatment with 500 nM SNP', proteins were present in all 18 replicates for SNP treated samples and below LOD for all 18 controls. For proteins described as 'absent from samples treated with 500 nM SNP', proteins were present in all 18 control replicates and below LOD for all 18 SNP treated replicates.

A full list of proteins whose expression was altered in response to treatment with 500 nM SNP is provided in Appendix 3. The biological processes in which these proteins are involved, are presented in Figures 39 to 42 below.

Key to figures and tables		
Sample description	**Figure number**	**Corresponding Table number (see Appendix 3)**
Proteins expressed only upon treatment with 500 nM SNP	Figure 40	Appendix 3 Table A3.1
Proteins up regulated in response to 500 nM SNP treatment	Figure 41	Appendix 3 Table A3.2
Proteins absent from 500 nM SNP treated samples	Figure 42	Appendix 3 Table A3.3
Proteins down regulated in response to 500 nM SNP treatment	Figure 43	Appendix 3 Table A3.4

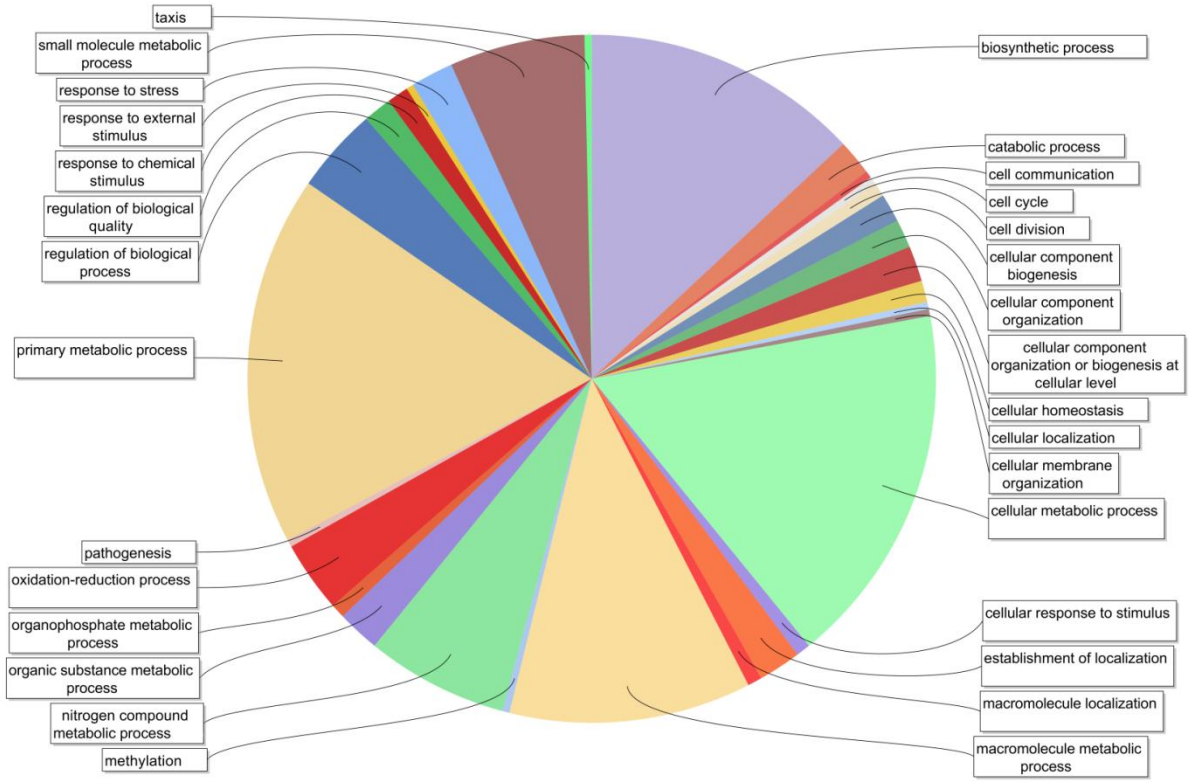


Figure 40 Biological processes associated with *S.* Typhimurium NCTC 12023 proteins expressed only upon treatment with 500 nM SNP

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Data represent a total of 73 proteins which were present in all 18 replicates for SNP treated samples and absent from all 18 Control replicates. Functional analysis of these proteins was carried out using Blast2Go gene ontology software.

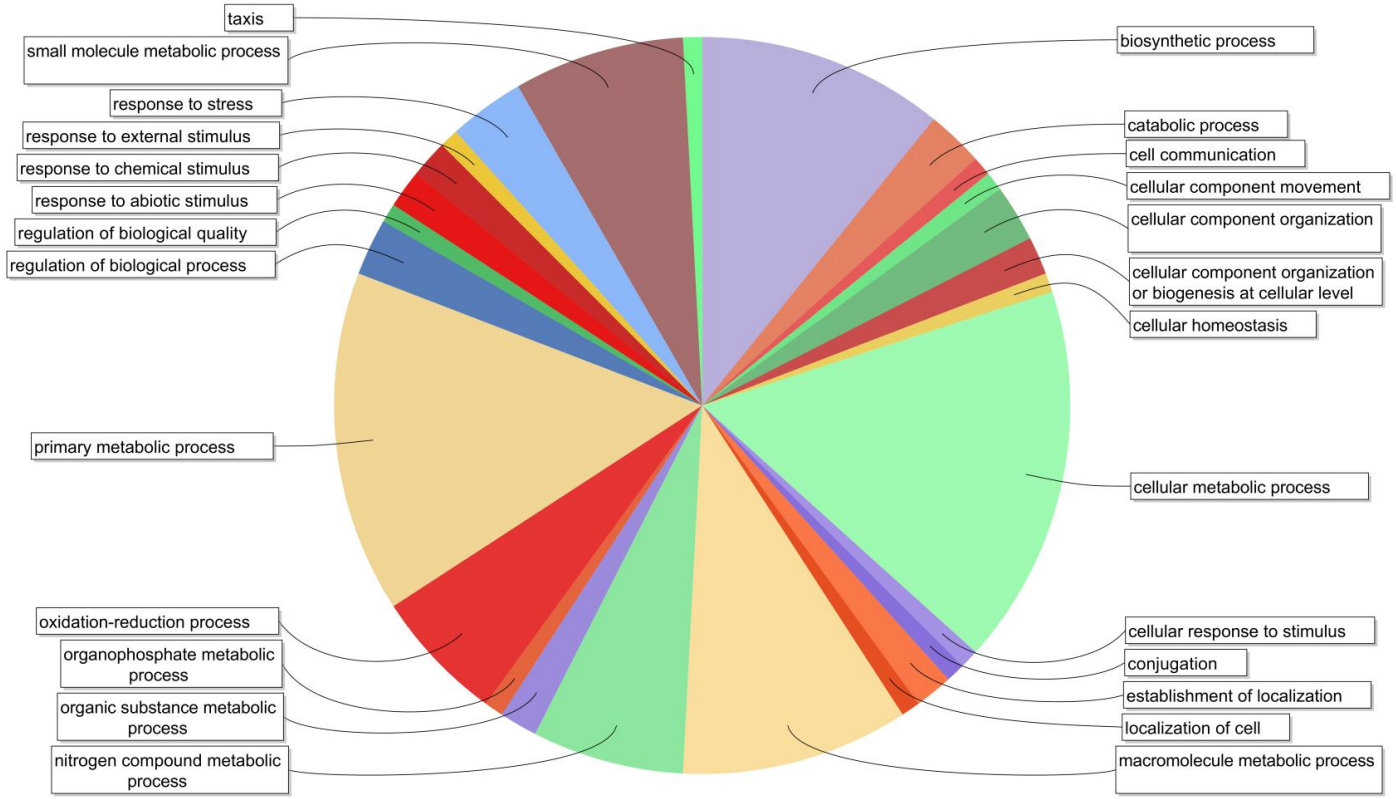


Figure 41 Biological processes associated with *S.* Typhimurium NCTC 12023 proteins up-regulated upon treatment with 500 nM SNP

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Data represent a total of 28 proteins which were up-regulated in all 18 replicates for SNP treated samples by comparison to Controls (n=18). Functional analysis of these proteins was carried out using Blast2Go gene ontology software

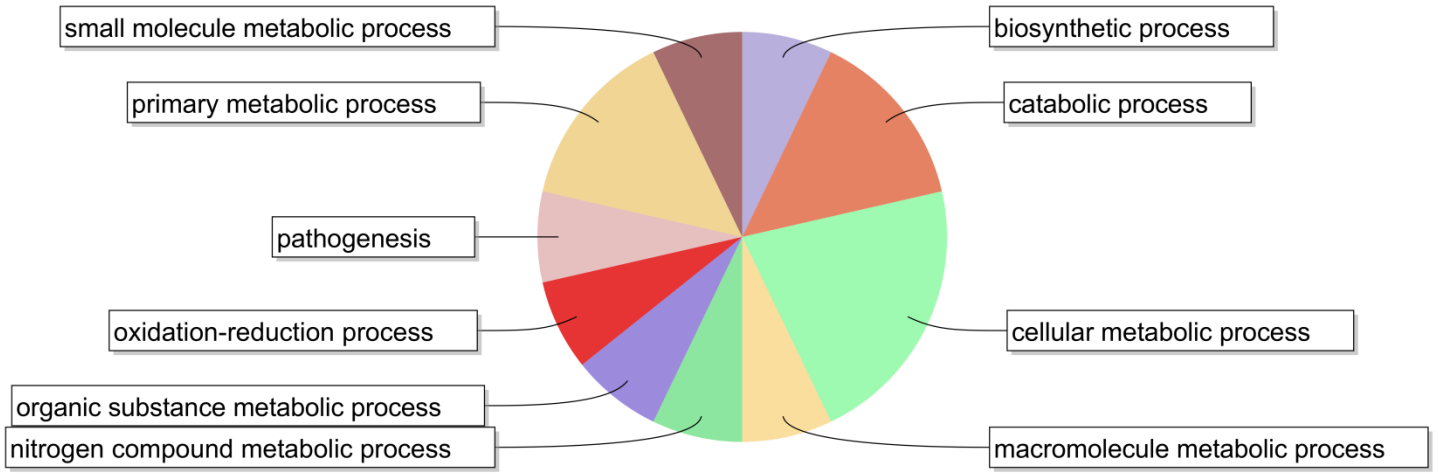


Figure 42 Biological processes associated with *S.* Typhimurium NCTC 12023 proteins absent from samples treatment with 500 nM SNP

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Data represent a total of 7 proteins which were absent from all 18 replicates for SNP treated samples and present in all 18 Control replicates. Functional analysis of these proteins was carried out using Blast2Go gene ontology software.

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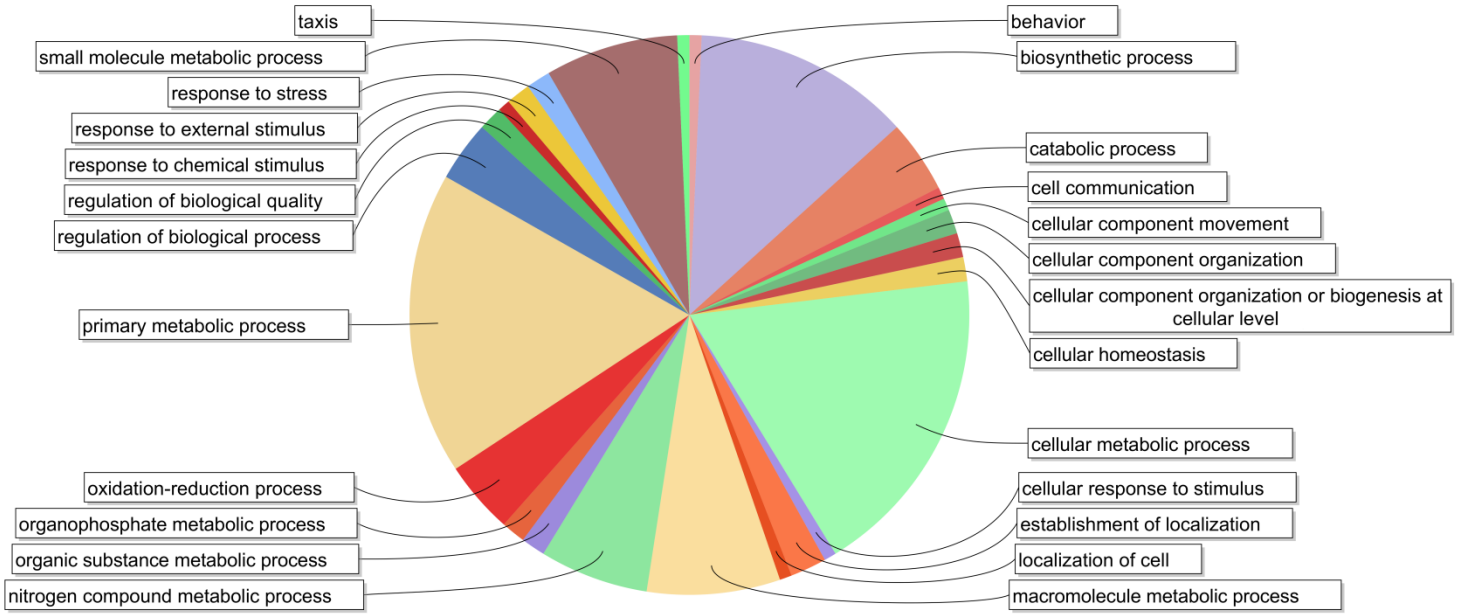

Figure 43 Biological processes associated with *S.* Typhimurium NCTC 12023 proteins down-regulated upon treatment with 500 nM SNP

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Data represent a total of 30 proteins which were down-regulated in all 18 replicates for SNP treated samples by comparison to Controls (n=18). Functional analysis of these proteins was carried out using Blast2Go gene ontology software.

A total of 73 proteins were found to be expressed in response to treatment with 500 nM SNP, and a further 28 proteins were up-regulated in response to treatment with 500 nM SNP by comparison to controls (n=18). Bioinformatic analysis of these proteins using the gene ontology tool Blast 2GO®, demonstrated they are involved in biological processes ranging from the cell cycle and metabolism to the response to external stimuli (Figure 40, Figure 41). A full list of these proteins and their associated GO terms for molecular function and biological process can be found in Appendix 3 (Tables A3.1 and A3.2). As discussed in Section 5.4 below, a general up-regulation of metabolism was observed, and of particular interest are the up-regulation of the chemotaxis proteins, CheA and CheY, the up-regulation of proteins involved in signalling across the bacterial cell membrane, and the up-regulation of the outer membrane protein OMP A which is known to be involved in the formation of bacterial biofilms (Ma and Wood 2009).

A total of 7 proteins were found to be absent from samples treated with 500 nM SNP and a further 30 proteins were down-regulated in response to treatment with 500 nM SNP by comparison to controls (n=18). Again, bioinformatics analysis of these proteins using the gene ontology tool Blast2Go® demonstrated that these proteins are involved in biological processes ranging from the cell cycle and metabolism to the response to external stimuli (Figure 42, Figure 43). A full list of these proteins and their associated GO terms for molecular function and biological process can be found in Appendix 3 (Tables A3.3 and A3.4). As discussed in Section 5.4 below, these findings concur that a general up-regulation of metabolism is observed upon treatment of *S.* Typhimurium with 500 nM SNP. Of particular interest is the loss of methylthioadenoside/ s adenosylhomocysteine nucleosidase which is involved in the production of the quorum sensing signal, autoinducer-2 (AI 2) in *S.* Typhimurium (Jesudhasan et al 2010).

The data reported here shows a general up-regulation of metabolism and the modulation of proteins involved in quorum sensing and chemotaxis, as well as the up-regulation of membrane transporters and the outer membrane protein OmpA, demonstrating that exogenous application of SNP to *S.* Typhimurium induces a signalling response.

5.4 Discussion

Very little work has been carried out to study nitric oxide signalling mechanisms in bacteria using a proteomics approach. In one unpublished study by Treece, the down-regulation of three proteins in response to blocking nitric oxide synthase in *B. subtilis* was found (Treece 2008). This is the first time that a shotgun proteomic approach using label-free mass spectrometry has been applied to study the effects of exogenous application of low, non-toxic concentrations of the nitric oxide donor SNP in *Salmonella.* To put results into context by comparison to the study by Treece, a total of 138 proteins significantly altered in *S.* Typhimurium in response to treatment with nitric oxide were identified here using this shotgun proteomics approach.

A general up-regulation of DNA replication, cellular processes and metabolic proteins involved in glycolysis was demonstrated in response to exogenous application of SNP and a corresponding down-regulation of proteins involved in energy storage were observed. These findings are consistent with the hypothesis that nitric oxide signalling in bacteria is involved in the switch between sessile and planktonic existence in response to environmental stimuli (via c-di-GMP), as in order to colonise new environments an up regulation of metabolism would be required to drive processes such as motility. However, it has not been specifically confirmed here that exogenous application of SNP to *S.* Typhimurium alters the expression of c-di-GMP or the GGDEF or EAL domain containing proteins that control the intracellular level of this central signalling molecule, which in turn controls the switch between sessility and motility in bacteria (Simm et al 2004). Whether a nitric oxide/c-di-GMP signalling mechanism is active in *Salmonella* spp. remains to be specifically determined. However, interestingly, an up-regulation of OmpA was observed in these investigations. OmpA has been shown to promote biofilm formation in *E. coli* (Barrios et al 2006, Ma and Wood 2009) and in *S.* Typhimurium, OmpA is involved in the sensing of environmental signals via transcription of the *csd*G promotor (Gerstel and Romling 2003). The *csd*G promotor controls expression of curli and cellulose and the multicellular 'rdar' biofilm morphotype in *S.* Typhimurium in a process mediated by c-di-GMP (Gerstel and Romling 2003). It is therefore suggested here, that c-di-GMP signalling may be

involved in the response of *S.* Typhimurium to exogenous application of SNP, although this has not been specifically confirmed.

Additionally, an up-regulation of the chemotaxis proteins CheA and CheY was observed in these investigations. CheA and CheY are involved in the sensing of environmental signals by bacteria including *S.* Typhimurium (Krell et al 2010). CheA senses ligand binding to chemoreceptors at the cell membrane and transduces the signal via the phosphorylation of response regulators, including CheY (Scott et al 2012). CheY binds to the bacterial flagellar motor and causes a switch in the direction of flagellar rotation and consequently affects motility, causing cells to tumble, rather than swim (Scott et al 2012). Interestingly, a decrease in the expression of CheZ was also observed, which is a protein involved in termination of the CheY signal, which causes cells to revert back to swimming over tumbling (Scott et al 2012). This finding suggests that the effect of exogenous application of SNP on the flagella switch in *S.* Typhimurium may be prolonged, as the CheY signal may not be terminated by CheZ if CheZ is present at decreased levels. It is interesting that motility is affected during biofilm formation, and it is possible that this change in flagellar rotation may be part of the switch between motility and sessility in bacteria. CheA and CheY have been demonstrated to be up-regulated in *Salmonella* biofilms (Hamilton et al 2009). The effect of exogenous application of SNP on flagella mediated motility has not been specifically investigated here, but may be an interesting investigation in the future.

The expression of a number of additional proteins of interest involved in bacterial signalling were significantly altered in response to treatment with SNP. A down regulation of methylthioadenoside/s adenosylhomocysteine nucleosidase (Pfs), involved in the production of the quorum sensing molecule AI-2, was observed. AI-2 is a key signalling molecule for bacterial biofilm formation (Beeston and Surette 2002). Therefore, as a decrease in Pfs correlates with a decrease in AI-2 (Beeston and Surette 2002, Kim et al 2006, Prouty et al 2002), a decrease in biofilm formation and a preference for planktonic existence might be expected for *S.* Typhimurium cells following SNP treatment here, although a decrease in surface colonisation upon treatment with 500 nM SNP was not observed phenotypically in Chapter 4. This hypothesis for a decrease in biofilm formation is also in contrast to the hypothesis for

an increase in biofilm formation via an up-regulation of OmpA described previously. These findings support the theory that a complex interplay of signals for and against biofilm formation in response to environmental stimuli govern the lifestyle decisions made by bacteria, and that there may not be a single signal molecule responsible for biofilm formation. Indeed, it is likely that bacteria have evolved such complex responses to environmental stimuli in order to survive in harsh and variable environments, and that this is the key to their survival. Therefore, the targeting of one signalling mechanism for the decontamination of bacterial biofilms, may not be a viable approach to the control of problematic biofilms. This is discussed further in Chapter 7.

Whilst the shotgun proteomics approach is a powerful tool for the analysis of protein expression in bacteria, it is not without its limitations. The efficiency of the extraction procedure for capturing the whole proteome of *S.* Typhimurium has not been evaluated here, but it is expected that a significant proportion of proteins, particularly the insoluble membrane-associated proteins, which are of interest to key processes for attachment and biofilm formation, such as the expression of cell surface appendages and EPS, would have been lost using the protein extraction technique used in these investigations. It is possible, for future investigations, that whilst a whole-proteome approach has provided great insights into the induction of signalling in response to nitric oxide treatment here, that protein extraction could be targeted to membrane proteins, or specifically for the extraction of c-di-GMP to investigate the signalling effects of nitric oxide further in *Salmonella*. Additionally, nitric oxide treatment was applied to planktonic cells in these investigations, to characterise its possible effects in relation to *Salmonella* at the phylloplane; however, it would be interesting to compare these findings to a proteomic investigation of *Salmonella* biofilms. In addition, due to limitations in the number of samples that could be analysed, no controls were included to ensure that the response to nitric oxide being observed were in fact due to nitric oxide treatment, and not an artefact of treatment with SNP. Therefore, in order to make these results more robust, additional experiments using either an alternative nitric oxide donor, or a nitric oxide scavenger are required. Nevertheless, some important insights into nitric oxide signalling in *Salmonella* have been gained in these investigations.

5.5 Conclusions

A proteomics approach has proved a powerful approach for the investigation of the bacterial response nitric oxide in *S.* Typhimurium. Currently there is little evidence in the literature to demonstrate the induction of signalling in response to treatment with low, non-toxic concentrations of nitric oxide in *Salmonella* spp. It has been demonstrated here that signalling is indeed induced in *S.* Typhimurium in response to the exogenous application of nitric oxide, including the modulation of proteins involved in quorum sensing and chemotaxis, and the expression of a diverse set of proteins involved in metabolism are influenced by nitric oxide treatment, which is an exciting discovery for future investigations. However, there remains much more to be understood regarding nitric oxide signalling mechanisms in *Salmonella* spp. and the role of nitric oxide signalling in *Salmonella* biofilms. Improved extraction procedures for membrane components, bacterial EPS and targeted extraction procedures for proteins of interest would be useful additions to further characterise the response and to investigate the involvement, if any, in the specific modulation of intracellular levels of c-di-GMP, which has been hypothesised as the 'universal' mechanism of action for induction of biofilm dispersal by nitric oxide.

However, it is interesting that nitric oxide signalling is active in *Salmonella* spp., but that that the changes observed do not correlate with significant changes in attachment and colonisation of salad leaves, or the dispersal from surfaces. This suggests that complex interactions either directly with the phylloplane, or with the indigenous microbial communities that reside there, are fundamental to the interaction of zoonotic pathogens and for their removal using novel decontamination methods. Therefore, in Chapter 6, the ability of exogenous application of nitric oxide to disperse indigenous bacterial communities from the phylloplane has been investigated to further characterise these effects.

CHAPTER 6

Laboratory and field investigations into the effect of nitric oxide on indigenous bacteria at the spinach phylloplane

6.1 Introduction

Indigenous phylloplane populations are known to exist in biofilm communities in the environment (Morris et al 1997, Warner et al 2008). Phylloplane biofilms may not only harbour zoonotic pathogens such as *Salmonella* spp., but indigenous bacterial populations can put a metabolic burden on the leaf causing degradation of leaf tissue and product spoilage (Magnuson et al 1990). Product spoilage for leafy salads, and the corresponding reduction in product shelf life, is not only a major source of financial loss for the food industry, but also, and arguably more importantly, contributes to the large amounts of food discarded as waste from both supermarkets and households (IMechE 2013, Weber et al 2011). Therefore, targeting novel decontamination strategies for the reduction of indigenous phylloplane biofilms is an important consideration for both food safety and product quality.

The exogenous application of nitric oxide to spinach leaves was investigated for its ability to cause dispersal of indigenous bacterial biofilm populations. If dispersal of phylloplane biofilms can be achieved, leading to a reduction in total microbial load at the phylloplane of leafy salads, this has potential benefits for improvement of product quality and extension of product shelf life via a reduction in the metabolic burden placed on leaves by large populations of bacteria. In addition, if phylloplane biofilms harbour zoonotic pathogens on leaf surfaces, these would be dispersed also, leading to an improvement in product safety. In the previous Chapters, small nitric oxide effects were seen for *Salmonella* at the phylloplane (Chapter 3), which could not be accounted for by the induction of dispersal events for single species *Salmonella* biofilms (Chapter 4). It was also shown, in agreement with other work (Warner 2009) that *Salmonella* may preferentially attach to existing bacterial aggregates at the phylloplane (Chapter 3), and these aggregates are phylloplane biofilms. Here, it is hypothesised that the small effects seen in Chapter 3 could be down to the action of nitric oxide on indigenous phylloplane biofilms, in which *Salmonella* may be incorporated.

As described in Section 1.2.2, phylloplane bacterial populations are large and composed of a diverse array of species (Hirano and Upper 2000). Estimates of bacterial numbers on leaves are in the region of 10^7 cells per cm^2 leaf (Lindow and

Leveau 2002) with studies indicating the presence of a diverse range of bacterial species and is variable depending on plant age, species and growing environment (Whipps et al 2008). In addition, studies to characterise the identities of phylloplane inhabitants have been historically hindered by the fact that many phylloplane bacteria are not able to be isolated by culture-based detection (Yang et al 2001). It has also emerged that bacteria exist in biofilm communities at leaf surfaces (Morris et al 1997).

The biofilm dispersal effect of nitric oxide is thought to involve the modulation of intracellular c-di-GMP levels which is controlled by the balance of synthesis by DGCs and degradation by PDEs, as described in Section 1.5. In Chapter 4 it was introduced that different bacteria contain different numbers of GGDEF and EAL/HD-GYP containing proteins, suggesting that differences in control of intracellular levels of c-di-GMP exist between bacterial species. The function of this difference remains unknown but given the relatively large number of GGDEF and EAL domain containing proteins in bacteria that usually reside in the environment, by comparison to the relatively small number present for the human enteric pathogen *S.* Typhimurium (Simm 2007) it may be that bacteria adapted for survival under environmental conditions require more complex regulation of c-di-GMP levels in order to respond effectively to their surrounding environments. Therefore, it is hypothesised here that the exogenous application of nitric oxide to indigenous phylloplane biofilms may have a dispersal effect similar to that seen by Barraud *et al.* for non-*Salmonella* spp. (Barraud 2009).

6.2 Experimental Procedures

General methodology used for these investigations is described in Chapter 2. The effect of nitric oxide treatment on indigenous phylloplane bacteria associated with spinach leaves was investigated in the laboratory and in the field to determine whether exogenous treatment with nitric oxide can reduce total microbial load at the phylloplane, and thus be further developed for use in a food production environment for improvement of both food quality and food safety (Sections 6.3.1 to 6.3.4). Additionally, in response to findings observed in preliminary field studies (Section 6.3.4.1), the effect of nitric oxide treatment on culturability of indigenous bacteria recovered from the spinach phylloplane was investigated (Section 6.3.5).

For laboratory experiments, leaf samples were obtained as described in Section 2.3.1. Spinach leaves were dip inoculated with SNP (unless otherwise specified) at concentrations ranging from 500 nM to 10 µM. Indigenous phylloplane bacteria were recovered from leaves by pulsification (Section 2.5) for enumeration by culture based detection on R2A (Section 2.2.2.2) or by microscopy (Section 2.9.1). Indigenous microbial populations were observed directly at the phylloplane by EDIC/EF microscopy following staining with the nucleic acid stain DAPI (Sigma UK), as described in Section 2.9.3. For field experiments, spinach plots were treated with nitric oxide via the exogenous application of SNP and samples obtained as described in Section 2.3.2. The preparation and delivery of SNP solutions for these investigations in described in Section 2.6.

6.3 Results

6.3.1 Nitric oxide treatment increases the release of indigenous bacteria from spinach leaves under laboratory conditions

The effect of exogenous application of SNP on recovery of indigenous phylloplane bacteria was investigated using field grown spinach leaves in the laboratory, in order to determine whether nitric oxide treatment can increase the release of indigenous bacteria from the spinach phylloplane.

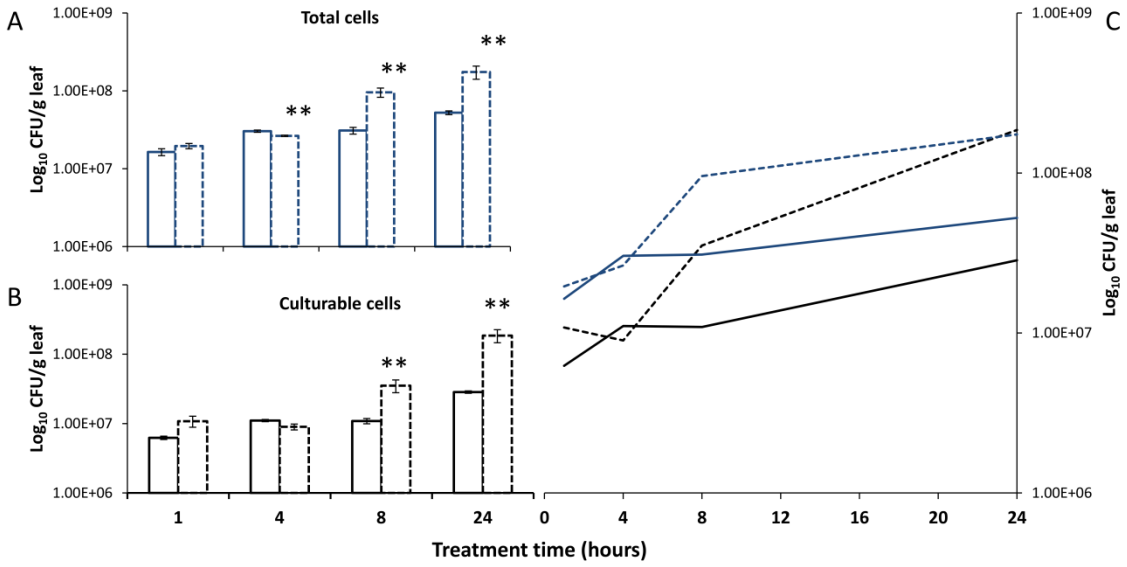

Figure 44 The effect of nitric oxide treatment on recovery of indigenous bacteria from field grown spinach leaves

10 g samples of spinach leaves were incubated at 22°C for 1, 4, 8 & 24 hours in the presence (dotted bars) or absence (solid bars) of 500 nM SNP by immersion. Bacteria were recovered from the phylloplane by Pulsification for enumeration of culturable cells by plating onto R2 agar (B) or enumeration of total recovered cells by microscopy (A). Figure 41 C shows culturable cells (black lines) and total cells (blue lines) over time in the presence (dotted lines) or absence (solid lines) of 500 nM SNP. Error bars represent ± standard error of the mean of three 10 g leaf samples per treatment.

A statistically significant (one-way ANOVA; $P < 0.01$) 0.5 log increase in recovery of indigenous bacteria from the spinach phylloplane was observed following 500 nM SNP treatment for treatment times of ≥ 8 hours by comparison to untreated Control leaves, for both culturable and total cells (Figure 44 A & B). For untreated Control leaves, an

approximate 0.5 log greater recovery of total cells by comparison to culturable cells was observed at all timepoints investigated (Figure 44 C, solid lines), demonstrating that not all bacteria recovered from the phylloplane are culturable. For 500 nM SNP treated leaves, a similar approximate 0.5 log increase was observed after 1, 4 and 8 hours treatment for total cells by comparison to culturable cells (Figure 44 C, dotted lines). Interestingly, after 24 hours treatment with 500 nM SNP, no difference in numbers of total and culturable cells recovered from the phylloplane was observed, suggesting that SNP may have an additional effect on culturability of recovered indigenous phylloplane bacteria (Figure 44 C, dotted lines).

The increased release of indigenous bacteria from spinach leaves following SNP treatment under laboratory conditions demonstrates that exogenous application of nitric oxide to leaves may disperse bacteria from leaves and lead to a reduction in total microbial load at the phylloplane. Further investigations were therefore carried out to determine a) whether the increase in release of bacteria observed here by pulsification corresponds to a decrease in total microbial load at the phylloplane (by microscopy) and b) whether this could be developed for potential application to leafy salads for reduction of total microbial load in a food production environment.

6.3.2 Does the increase in release of bacteria observed by pulsification correspond to a decrease in total microbial load at the phylloplane and can DAPI staining be used to accurately quantify indigenous bacterial communities at the phylloplane?

In order to investigate whether the effect of nitric oxide on recovery of indigenous leaf associated bacteria seen above corresponds to a decrease in total microbial load at the phylloplane, leaves were treated with 500 nM SNP for 24 hours before direct observation of bacteria at the phylloplane by EDIC/EF microscopy as described in Section 2.9.3.

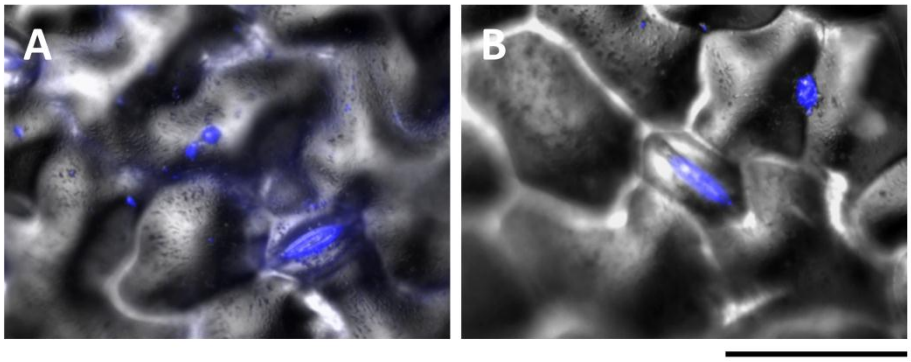

Figure 45 Representative EDIC/EF micrographs of DAPI stained spinach leaves for assessment of nitric oxide treatment on total microbial load at the phylloplane

Individual spinach leaves were dip-treated with 500 nM SNP and incubated at 22°C for 24 hours. Control leaves were dip-treated with sdH_2O only for comparison. Samples were observed by EDIC/EF microscopy following staining with the nucleic acid stain DAPI. DAPI stained indigenous phylloplane bacteria are shown in blue. Scale bar = 50 microns.

Following treatment of leaves with 500 nM SNP for 24 hours, there was an indication that total microbial load at the phylloplane may be lower for 500 nM SNP treated leaves than for Controls (Figure 44). However, it was not possible to accurately quantify this reduction by microscopy, as discussed in Section 6.4.2.

Therefore, for further investigations into the effect of nitric oxide on indigenous phylloplane bacteria, quantification of cell numbers was carried out following recovery of bacteria from the phylloplane by Pulsification, as described in Section 2.5. Recovered cells were quantified by culture-based detection on R2 agar, as described in

Section 2.2.2.2, and by counting of total cells by microscopy, as described in Section 2.9.1. Direct microscopy of leaves using EDIC/EF microscopy following DAPI staining of indigenous phylloplane populations, as described in Section 2.9.3, was used for qualitative assessment of indigenous phylloplane bacteria. This combined approach was carefully selected to gain the best overall picture of the effect of SNP treatment on indigenous phylloplane bacteria associated with spinach leaves.

6.3.3 Nitric oxide treatment increases the release of indigenous bacteria from spinach leaves under laboratory conditions adapted for application to the food production process

Nitric oxide treatment of spinach leaves in the laboratory was found to increase the recovery of indigenous bacteria from the phylloplane (Section 6.3.1), which may correspond to a reduction in total microbial load at the spinach phylloplane and therefore may have implications for product quality and shelf life. However, when considering how this could potentially, in the future, be applied to the food production process, how and where nitric oxide treatment may be achieved to fit in with food production practices must also to be taken into account. Figure 1 describes the production process for an RTE bag of leafy salad and it was decided, in consultation with Vitacress salads Ltd that pre-harvest application was the most appropriate point to treat leaves for trial experiments, in order to minimise disruption to the production process.

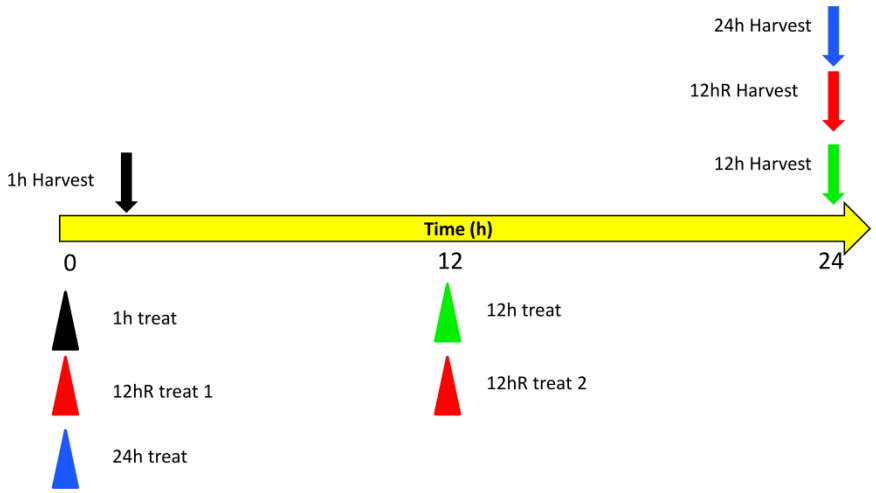

Figure 46 A schematic representation of nitric oxide treatment and sampling (harvest) points, as designed to fit in with farming practices

As spinach leaves are usually harvested in the early morning, before temperatures in the field rise and increase water loss by transpiration leading to wilting of the leaves, nitric oxide treatment timings were selected at a) shortly before harvest (1 hour), b) the night before harvest (12 hours) or c) the day before harvest (24 hours). A repeat application, with SNP treatments at 24 hours and at 12 hours pre-harvest on the same set of leaves, was also investigated. A range of SNP concentrations were selected to determine the optimal concentration for increasing the recovery of indigenous bacterial populations from leaves. A schematic representation of experimental design is provided in Figure 46. Leaves were treated with SNP by dipping in SNP solution to mimic spraying in the field, incubated at room temperature on the laboratory bench to reflect field conditions as far as possible and processed for enumeration of culturable cells at the timepoints denoted “harvest” in Figure 46.

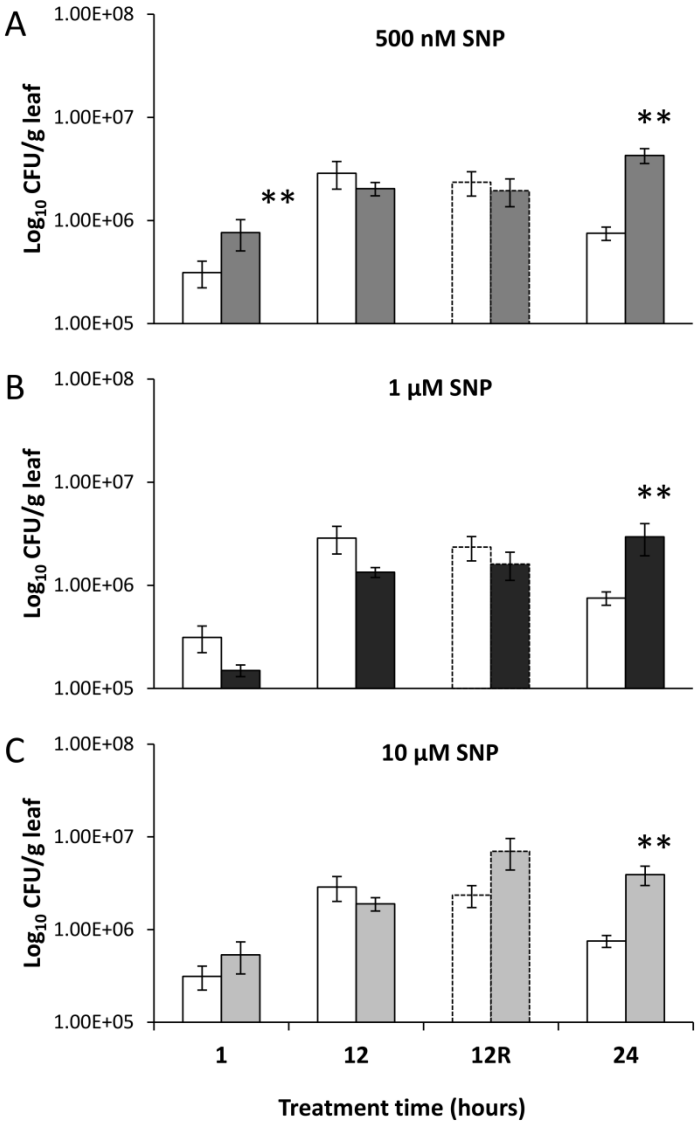

Figure 47 The effect of nitric oxide treatment on recovery of indigenous bacteria from spinach leaves, under laboratory conditions modified for use in the field.

25 g spinach samples were individually dip-treated with 500 nM (A), 1 µM (B), or 10 µM (C) SNP (grey bars), transferred to stomacher bags and incubated at room temperature on the laboratory bench for 1, 12 and 24 hours. 12hR samples were dip-treated at 0h and 12h and processed at 24h. Control leaves were dipped in sdH_2O only for comparison (white bars). Bacteria were removed from leaves by Pulsification and plated onto R2 agar for enumeration of culturable cells. Error bars represent ± standard error of the mean of three 25 g leaf samples per treatment.

Treatment of leaves with 500 nM SNP for 1 hour (Figure 47 A), and treatment of leaves for 24 hours for all concentrations investigated (Figure 47 A, B & C) caused statistically significant (one way ANOVA; $P < 0.01$) increases in the recovery of indigenous bacteria from the spinach phylloplane. Therefore, treatment timings of 1 hour and 24 hours at concentrations of 500 nM, 1 µM, and 10 µM SNP were selected for further investigation in the field.

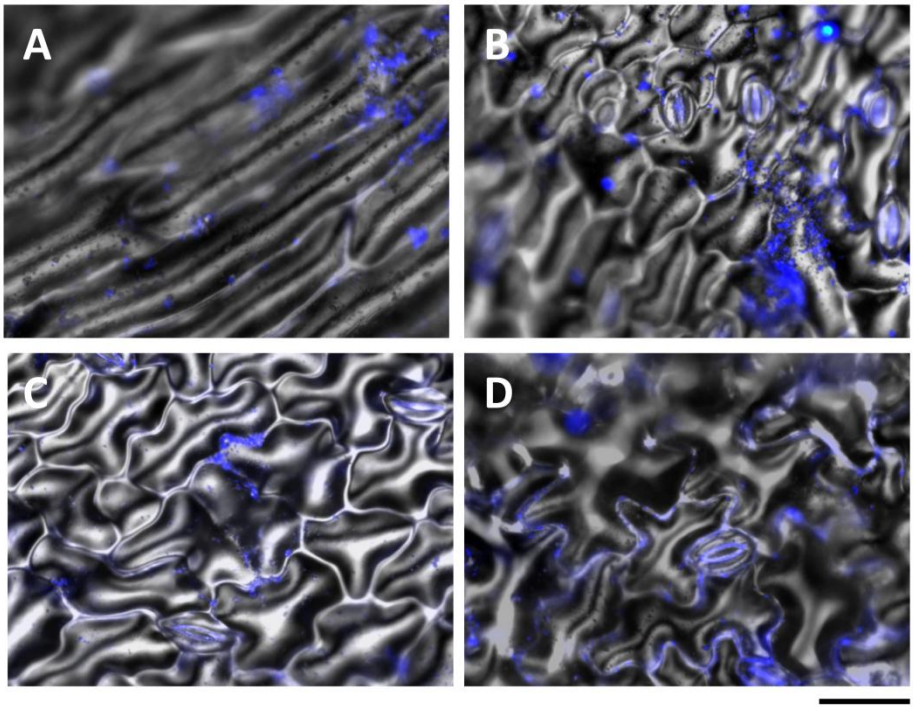

Figure 48 Representative EDIC/EF micrographs of indigenous phylloplane bacteria (blue) associated with spinach leaves following 24 hours treatment with nitric oxide

Spinach leaves were dip-treated with 500 nM (B), 1 µM (C) and 10 µM SNP and transferred to stomacher bags for incubation on the laboratory bench for 24 hours. Control leaves were dip-treated with sdH_2O only (A). Samples were observed by EDIC/EF microscopy following staining with the nucleic acid stain DAPI. Scale bar = 50 microns.

Qualitative assessment of representative microscopy images did not show any clear differences between Control (Figure 48 A) and SNP treated (Figure 48 B, C & D) leaves, although fewer bacterial aggregates may have been present at the phylloplane after 24 hours treatment with 1 µM (Figure 48 C) and 10 µM SNP (Figure 48 D).

6.3.4 Field based investigations into the effect of nitric oxide on indigenous bacteria at the spinach phylloplane

6.3.4.1 Preliminary field studies

Field grown spinach leaves were treated with SNP at concentrations of 500 nM, 1 µM, and 10 µM in order to determine whether pre-harvest treatment of spinach leaves with nitric oxide increased the release of indigenous bacteria from the phylloplane.

No conclusive differences between control and nitric oxide treated samples were observed (data not shown), due to an unexpectedly low number of bacteria recovered from leaves, which resulted in low sample numbers being available for data interpretation. This was despite careful selection of experimental procedures based on a siting sample taken from the same field 1 week prior to the preliminary field study. However, as field samples were retained and maintained in refrigerated storage, samples were re-plated after 7 days at 4°C, when an unexpectedly *high* number of cells were present in recovered samples, particularly for SNP treated samples (data not shown). The reasons for this change in number of bacteria recovered before and after sample storage at 4°C were therefore investigated further (see Section 6.3.5).

Results of this preliminary field trial were inconclusive, but small effects of SNP treatment on recovery of bacteria from leaves were suggested, therefore, the effect of nitric oxide treatment in the field was re-investigated in the following growing season at concentrations of 500 nM and 1 µM SNP (see Section 6.3.4.3).

6.3.4.2 The use of Violet Red Bile Agar for assessment of the effect of nitric oxide treatment on recovery of coliforms from spinach leaves

The use of Violet Red Bile Agar (VRBA) was also investigated to assess the effect of nitric oxide treatment on total coliforms recovered from field grown spinach leaves (data not shown). VRBA is a colorimetric agar used in the food and dairy industry as a presumptive indicator of the presence of coliforms that works on the basis of identification of lactose fermenting organisms including the *Enterobacteriaceae*. It was

found that a resuscitation step was required for bacteria recovered from the phylloplane, which was achieved using a layer of TSA underneath the colorimetric VRBA, as recommended by the US Food and Drug Administration for the detection of coliforms in environmental samples (Feng et al 2002). However, due to the high number of non-coliform bacteria present in samples recovered from the phylloplane, and the relatively low number of presumptive coliforms, it was not possible to enumerate coliforms from phylloplane samples using this method. Therefore, the assessment of the effect of nitric oxide treatment on the recovery of coliforms from field grown leaves was not achieved.

6.3.4.3 Nitric oxide treatment results in negligible changes in the release of indigenous bacteria from spinach leaves in the field

The effect of nitric oxide treatment on the recovery of indigenous bacteria from the spinach phylloplane was re-investigated in the field following pre-harvest treatment of leaves with 500 nM and 1 µM SNP. Leaves were treated and harvested as described in Section 6.2.

A negligible but statistically significant (one-way ANOVA; $P < 0.05$) 0.15 log increase in the release of indigenous bacteria from the spinach phylloplane was observed following pre-harvest treatment of spinach leaves with 1 µM SNP (Figure 49 A). A negligible but statistically significant (one-way ANOVA; $P < 0.05$) 0.2 log decrease in the release of indigenous bacteria from spinach leaves was observed after 24 hours treatment with 500 nM SNP (Figure 49 B).

Microscopy images did not show any clear differences between indigenous phylloplane populations for Control leaves by comparison to SNP treated leaves after 1 hour or 24 hours treatment with SNP, although there was an indication that there may be fewer indigenous phylloplane isolates present on SNP treated leaves by comparison to untreated Controls. Representative microscopy images are provided in Figure 50 and Figure 51 following 1 hour and 24 hour SNP treatment, respectively. A full appendix of microscopy images can be found in Appendix 2.

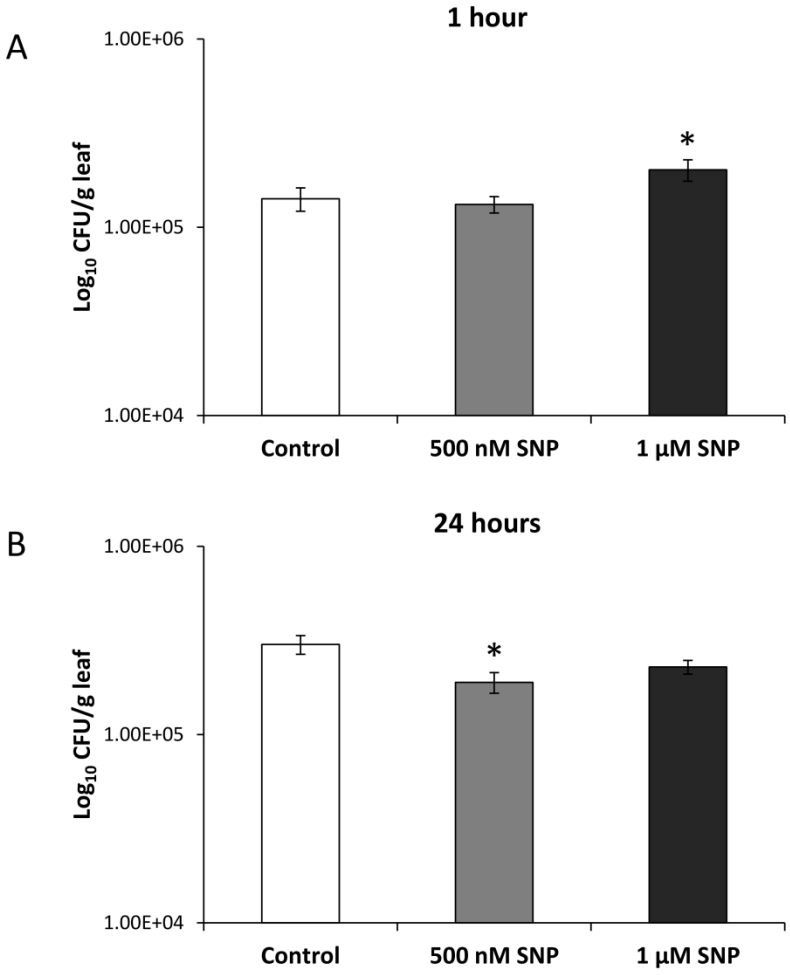

Figure 49 The effect of field based nitric oxide treatment on recovery of indigenous bacteria from spinach leaves

Spinach plots were treated with nitric oxide in the crop field. 50cm^2 plots were sprayed with 1 L sdH_2O (Control), 500 nM SNP or 1 µM SNP to ensure complete coverage of leaves, and sampled after 1 hour or 24 hours. Leaves were harvested by hand and transported back to the laboratory for analysis on the day of harvest. Bacteria were recovered from leaves by Pulsification and resulting suspensions stored for 7 days at 4°C before enumeration of culturable cells by plating on R2A. Error bars represent ± standard error of the mean of nine 25 g leaf samples per treatment.

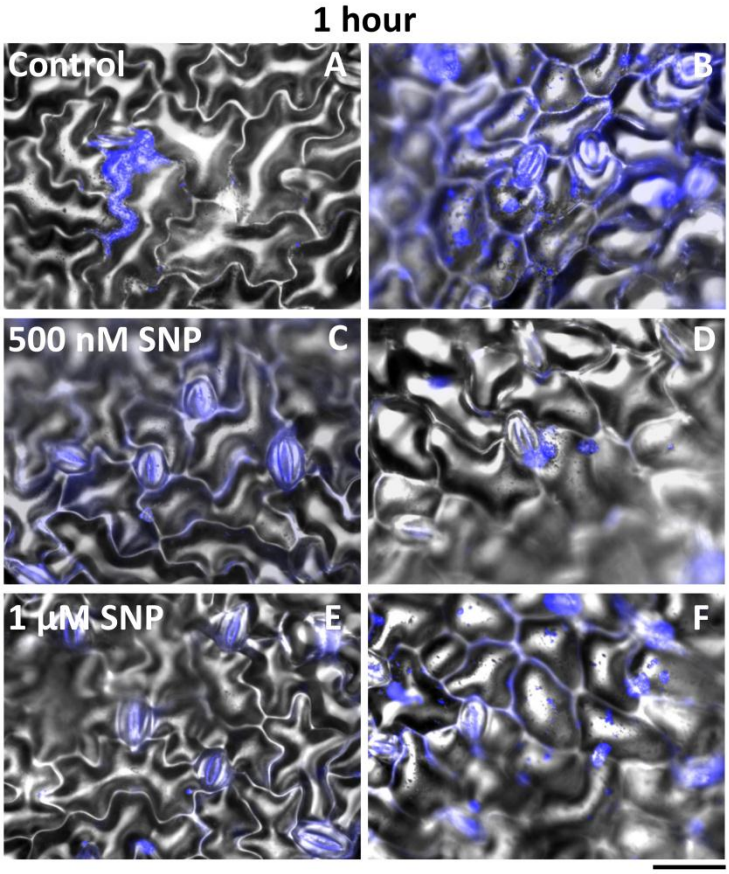

Figure 50 Representative EDIC/EF micrographs showing colonisation of spinach leaves by indigenous phylloplane bacteria following nitric oxide treatment for 1 hour in the field

Spinach plots were treated with nitric oxide in the crop field. 50cm^2 plots were sprayed with 1 L sdH_2O (A & B), 500 nM SNP (C & D) or 1 µM SNP (E & F) to ensure complete coverage of leaves, and sampled after 1 hour. Leaves were harvested by hand and transported back to the laboratory for observation by EDIC/EF microscopy following staining with the nucleic acid stain DAPI on the day of harvest. DAPI stained indigenous phylloplane bacteria are shown in blue. Scale bar = 50 microns.

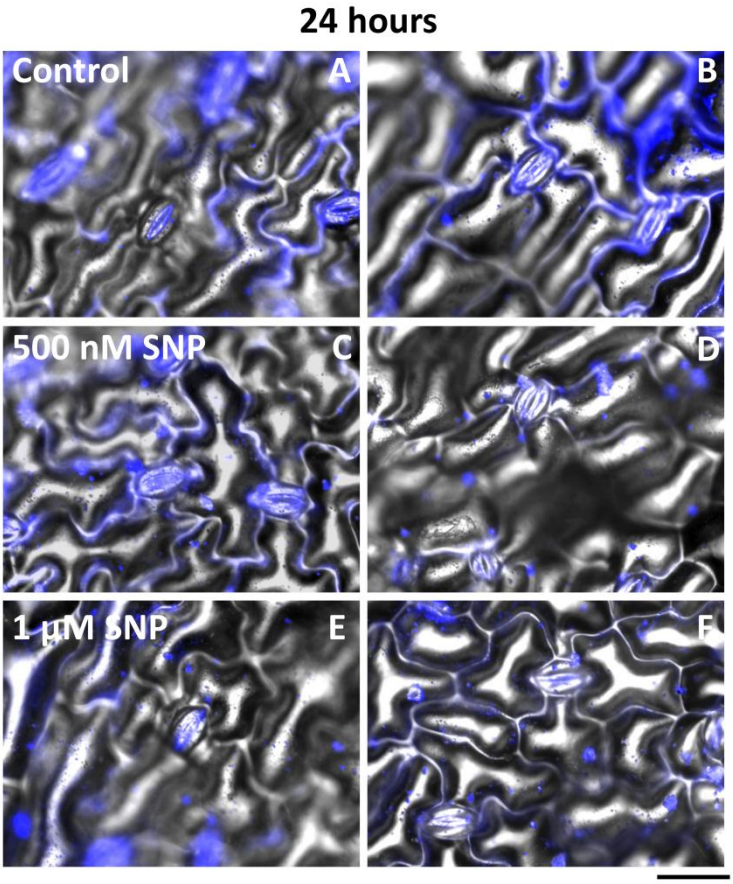

Figure 51 Representative EDIC/EF micrographs showing colonisation of spinach leaves by indigenous phylloplane bacteria following nitric oxide treatment for 1 hour in the field

Spinach plots were treated with nitric oxide in the crop field. 50cm^2 plots were sprayed with 1 L sdH_2O (A & B), 500 nM SNP (C & D) or 1 µM SNP (E & F) to ensure complete coverage of leaves, and sampled after 24 hours. Leaves were harvested by hand and transported back to the laboratory for observation by EDIC/EF microscopy following staining with the nucleic acid stain DAPI on the day of harvest. DAPI stained indigenous phylloplane bacteria are shown in blue. Scale bar = 50 microns.

6.3.5 Laboratory based investigations into the effect of refrigerated storage on indigenous phylloplane bacteria recovered from spinach leaves following nitric oxide treatment

As described in Section 6.3.4.1, preliminary field trial results indicated that, for bacteria recovered from nitric oxide treated spinach leaves, numbers of culturable cells increased over time during refrigerated storage. This was further investigated following SNP treatment of spinach leaves in the laboratory.

6.3.5.1 Culturability of indigenous bacteria recovered from spinach leaves by pulsification, but not by rinsing, increases during refrigerated storage

Spinach leaves were dip-treated with 500 nM SNP and incubated at room temperature on the laboratory bench for 24 hours before recovery of cells by pulsification. Samples of rinse solutions were also retained. Recovered samples were then transferred to refrigerated storage for 1, 4 or 7 days before enumeration of total cells by microscopy (Section 2.9.1) or for enumeration of culturable cells by plating onto R2A (Section 2.2.2.2).

Consistent with previous laboratory investigations (Sections 6.3.1 and 6.3.4.3), exogenous application of 500 nM SNP here causes a negligible but statistically significant (one-way ANOVA, $P<0.01$) 0.1 log increase in the recovery of indigenous phylloplane bacteria from the spinach phylloplane (Figure 52 A & B). Detection of bacteria by culture yielded a lower recovery of cells than total cell counts by microscopy, for both samples rinsed from leaves and samples recovered by pulsification, demonstrating that not all bacteria recovered from spinach leaves were culturable (Figure 52 C & D).

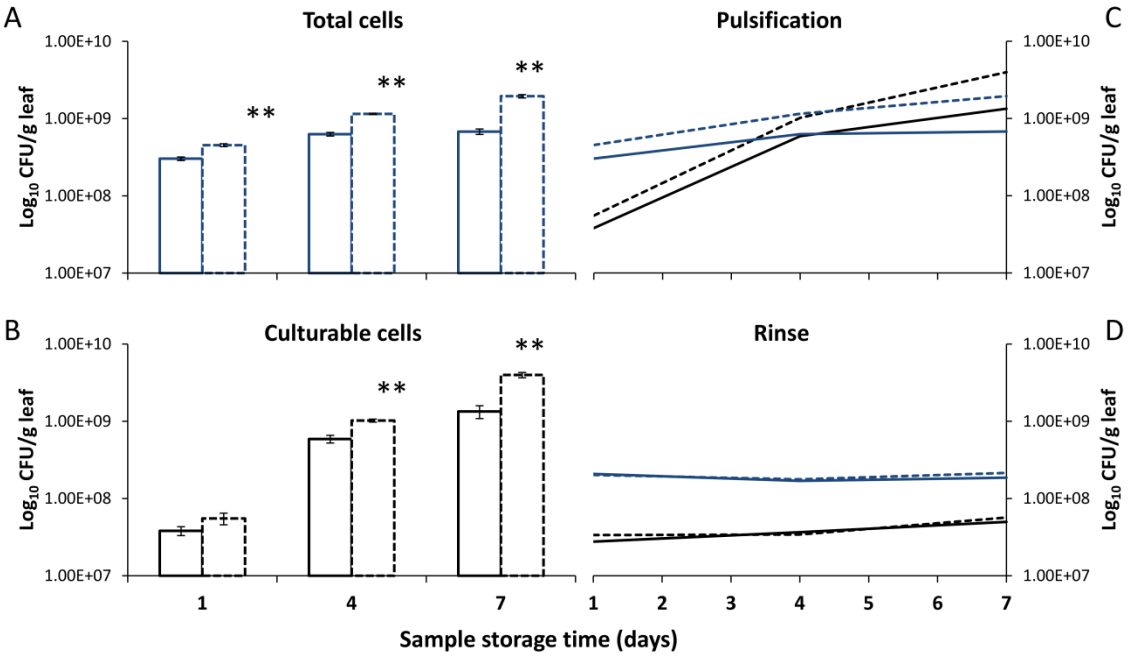

Figure 52 The effect of refrigerated storage on total (A) and culturable (B) numbers of indigenous bacteria recovered from the phylloplane following nitric oxide treatment, after recovery by pulsification (A, B & C) and by rinsing (D).

25 g spinach samples were individually dip-treated with 500 nM SNP (dashed bars), transferred to stomacher bags and incubated at room temperature on the laboratory bench for 24 hours. Control leaves were dipped in sdH_2O only for comparison (solid bars). Bacteria were recovered from the phylloplane by Pulsification for enumeration of culturable cells by plating onto R2 agar (B) or enumeration of total cells by microscopy (A). Figure 52 C and 50 D show culturable cells (black lines) and total cells (blue lines) over time after dip-treatment with 500 nM (dashed lines) or 1 µM (dotted lines) or with dH2O only (solid lines) and following recovery from the phylloplane by Pulsification (C) or by rinsing (D). Error bars represent ± standard error of the mean of three 25 g leaf samples per treatment.

In addition, an increase in culturable cell numbers occurred during refrigerated storage over 7 days for bacteria recovered from the phylloplane by pulsification (Figure 52 C) which was not observed for culturable cells recovered from leaves by rinsing (Figure 52 D). Given that there was little change in total cell numbers during refrigerated storage over 7 days, an effect on culturability of bacteria recovered from leaves by pulsification is suggested, rather than a growth effect. It is also suggested that this increase in culturability of cells is greater for bacteria recovered from SNP treated leaves. This finding is investigated further in Section 6.3.5.2.

6.3.5.2 Exogenous application of SNP to spinach leaves increases the restoration of culturability to recovered phylloplane isolates during refrigerated storage

Previously (Sections 6.3.1 and 6.3.5.1) it was suggested that culturability of bacteria recovered from the spinach phylloplane by pulsification was increased during refrigerated storage following exogenous application of SNP to leaves. Here, elevated light levels (on a 12 hour light, 12 hour dark cycle) were used to increase the light dependent release of nitric oxide from SNP, in order to investigate the effect of exogenous application of SNP on culturability of recovered cells.

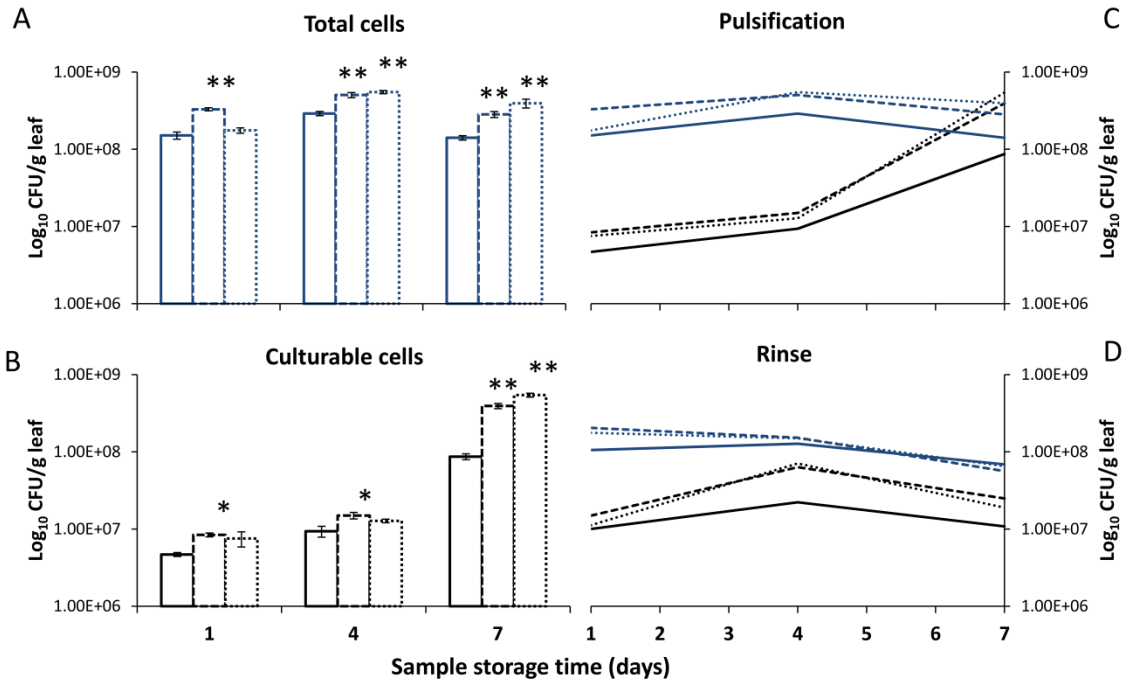

Figure 53 The effect of refrigerated storage on total (A) and culturable (B) numbers of indigenous bacteria recovered from the phylloplane following nitric oxide treatment, after recovery by pulsification (A, B & C) and by rinsing (D)

25 g spinach samples were individually dip-treated with 500 nM SNP (dashed bars) or 1 µM SNP (dotted bars), transferred to stomacher bags and incubated at room temperature on the laboratory bench for 24 hours. Control leaves were dipped in sdH_2O only for comparison (solid bars). Bacteria were recovered from the phylloplane by Pulsification for enumeration of culturable cells by plating onto R2 agar (B) or enumeration of total recovered cells by microscopy (A). Figure 52 C and 50 D show culturable cells (black lines) and total cells (blue lines) over time after dip-treatment with 500 nM (dashed lines) or 1 µM (dotted lines) or with dH_2O only (solid lines) and following recovery from the phylloplane by Pulsification (C) or by rinsing (D). Error bars represent ± standard error of the mean of three 25 g leaf samples per treatment.

Consistent with previous laboratory investigations (see Sections 6.3.1, 6.3.4.3 and 6.3.5.2), exogenous application of SNP increased the recovery of indigenous phylloplane bacteria from spinach leaves (Figure 53 A & B), and a lower recovery of bacteria was seen for culturable cells than for total cells when recovered from leaves by both pulsification and by rinsing (Figure 53 C & D).

In addition, at elevated light levels for increased photolytic release of nitric oxide from SNP, culturable cell numbers recovered from SNP treated leaves was increased further than culturable cell numbers recovered from Control leaves over 7 days refrigerated storage (Figure 53 C), suggesting nitric oxide treatment does indeed have an effect on culturability of cells recovered from leaves by pulsification but not on cells recovered from leaves by rinsing (Figure 53 D).

6.4 Discussion

The effect of exogenous application of SNP to spinach leaves was investigated in the laboratory and in the field, for its ability to cause dispersal of indigenous phylloplane bacteria and a reduction in total microbial load at the phylloplane, which may correspond to an increase in product quality and shelf life for salad leaves. In Chapter 3, it was hypothesised that an explanation for the small changes in culturable numbers of *Salmonella* associated with spinach leaves following nitric oxide treatment could be influenced by the presence of indigenous phylloplane biofilms and the effect of nitric oxide treatment on these biofilm communities (see Chapter 3). The effect of nitric oxide treatment on recovery of indigenous bacteria from the phylloplane is discussed in Section 6.4.1. Additionally, in this Chapter, it was discovered that changes in culturable bacterial numbers occurred in samples recovered from spinach leaves following nitric oxide treatment that had been stored for 7 days in refrigerated storage at 4°C. These findings are discussed in Section 6.4.3.

6.4.1 Nitric oxide treatment is not suitable for practical application in a food production environment for increasing the recovery of indigenous phylloplane bacteria from spinach leaves

A consistent small increase in the recovery of indigenous phylloplane bacteria was observed following exogenous application of nitric oxide to spinach leaves. It was also found that numbers of culturable cells recovered from the spinach phylloplane were consistently lower than numbers of total cells, confirming that not all phylloplane bacteria can be isolated using culture based approaches.

The small increase in recovery of phylloplane isolates was predominantly observed following treatment of leaves with 500 nM SNP for 24 hours in the laboratory, although concentrations ranging from 500 nM to 10 µM at timings ranging from 1 to 24 hours also had an influence on recovery of indigenous bacteria from spinach leaves. In the field, 1 µM SNP treatment for 1 hour increased recovery of indigenous bacteria, whilst 500 nM SNP treatment for 24 hours decreased the recovery of indigenous bacteria from spinach leaves. However, in terms of practical application for reduction

of total microbial load at the phylloplane, it is unlikely (although not investigated here) that the approximate 0.2 log changes observed in the field would be sufficient to improve product quality or safety.

Whilst treatment with nitric oxide influences the recovery of indigenous phylloplane population from leaves, variations were observed in response to different concentrations and timings of SNP treatment between experiments. These variations could be due to a number of factors. Firstly, a diverse array of bacterial species has been demonstrated in association with the phylloplane, which has been shown to vary in response to factors including leaf species, age and growing conditions (see Section 1.2.2). Therefore, differences in growing conditions for the commercial leaf samples used here and differences in their underlying indigenous bacterial populations could explain the variation in response to SNP treatment seen, as different bacterial species have been shown to be dispersed in response to different concentrations of nitric oxide (Barraud et al 2009b). However, for the target application investigated in this Chapter - a reduction in total microbial load at the phylloplane - it was not considered necessary to fully understand the mechanisms behind a reduction effect if an overall reduction in biofilm surface coverage could be induced by treatment of leaves with nitric oxide (assuming the NO/c-di-GMP signalling pathway is conserved across the bacteria, see Section 1.5). However, it was not the case that bacterial populations at the phylloplane were altered in numbers great enough to be beneficial in a food production environment.

Whether the increased recovery of bacteria seen here was in fact due to a reduction in total microbial load via the induction of dispersal events at the phylloplane could not be confirmed by direct quantification at the phylloplane (see Section 6.4.2), and no increases or decreases in phylloplane populations large enough to qualitatively demonstrate a change in total microbial load at the phylloplane were observed. Despite the observed small increase in recovery of bacteria from the phylloplane, and therefore an assumed decrease in bacteria remaining associated with the leaf, DAPI staining of phylloplane communities show that a large number of bacteria remain associated with spinach leaves following nitric oxide treatment.

Variations in the response to SNP seen in these investigations could also be explained by the effect of light on the release of nitric oxide from SNP. The release of nitric oxide from SNP is light-dependent (see Figure 10), and levels of light in the environment are variable (experimental conditions used here were specifically designed to model an environmental situation), therefore the liberation of nitric oxide from SNP may vary depending on light levels, resulting in the delivery of variable nitric oxide concentrations to the phylloplane from a single controlled concentration of SNP. If nitric oxide concentration is an important determinant of biofilm dispersal, this will be important for the dispersal of indigenous phylloplane isolates from leaves and the reduction of total microbial load. It has been shown that there is an optimal concentration for the induction of biofilm dispersal by exogenous application of SNP, above or below which the opposite effect is observed and surface coverage can actually be increased rather than decreased (Barraud et al 2006). Therefore, the delivery of variable concentrations of nitric oxide to leaves will not only define whether a response to nitric oxide treatment occurs but may also define whether that response is a beneficial or a detrimental one in terms of controlling indigenous phylloplane bacterial populations on leaves. When the additional potential for different responses of individual bacterial species within a phylloplane population to respond to nitric oxide treatment at different concentrations is considered, it becomes clear that much more remains to be understood about the identity of indigenous phylloplane bacteria and their responses to nitric oxide treatment before the effects of nitric oxide treatment on total bacteria at the phylloplane can be fully understood. Interestingly, the control of GGDEF and EAL domain containing proteins controlling the level of intracellular c-di-GMP (the conserved nature of which are central to the hypothesis that nitric oxide may be used to decontaminate multi-species biofilms) is achieved by regulatory domains containing redox sensing PAS domains, which are also intricately involved in the bacterial response to light (Taylor and Zhulin 1999). Therefore under uncontrollable experimental conditions such as those found in the field, light levels may have a complex interplay with the transduction of signals via c-di-GMP in bacteria, thus possibly mitigating any effect of nitric oxide on dispersal.

In addition to the effect of SNP on recovery of indigenous phylloplane bacteria, for Control leaves, cell numbers quantified by filtration of recovered samples onto

membranes for counting by EDIC/EF microscopy were higher than culturable cell numbers on all occasions investigated. This finding is consistent with those of other researchers, who commonly report reduced culturability of bacteria adapted for survival in under environmental conditions (see Section 1.3.3.4). Interestingly, recovery of culturable cells was increased to numbers in line with that of total cells for 500 nM SNP treated leaves (Figure 44), suggesting that nitric oxide treatment has an effect on either the culturability of recovered cells, or the metabolic state of the cells recovered from SNP treated leaves, which may be indicative of how they survive at the phylloplane (i.e. in biofilm communities).

It is clear that the exogenous application of nitric oxide to spinach leaves has the potential to influence indigenous phylloplane populations but these investigations did not find changes sufficient to justify practical application in an industrial food setting. Whether the small changes in bacterial numbers associated with the phylloplane observed correlate to improvements in product safety and quality have not been determined here, however, it has emerged that further understanding of the mechanism of action of nitric oxide signalling for bacterial species found at the phylloplane, and therefore a better understanding of the identities of bacteria present at the phylloplanes of leafy salads, are required in order to further characterise this response. This may be more of an academic interest than of commercial benefit at this stage, but further understanding of microbial communities present in association with fresh leafy produce would be beneficial for the food industry for the development of future decontamination strategies.

6.4.2 DAPI staining for accurate quantification of indigenous phylloplane bacteria directly at the leaf surface using EDIC/EF microscopy

It was concluded that whilst EDIC/EF microscopy of leaves following DAPI staining is a useful tool for the qualitative assessment of indigenous phylloplane bacteria, it is not a suitable method for accurate quantification of phylloplane populations for spinach leaves. Figure 54 A & B demonstrates that despite an appearance consistent with the presence of microbial phylloplane aggregates, DAPI staining does not stain these structures 100% of the time. In addition, even when DAPI staining does occur,

microbial contamination is often in areas of the leaf unsuitable for microscopy due to leaf surface topography so that images are not in focus (Figure 54 C). This means that selection of fields of view that are suitable for EDIC microscopy pre-selects for flat areas of the leaf, which are not representative of the whole leaf surface. The capacity of the EDIC/EF microscope to perform z-scans for subsequent generation of multi-image stacks is a powerful tool for studying biofilms on surfaces. However, for leaf tissue, the prolonged exposure to high intensity light required to scan samples in the z-plane, leads to a degradation of the integrity of the leaf structure, causing the sample not only to degrade, but to move in between each image capture as the sample dehydrates. As rough areas of leaves appear to be where large areas of DAPI staining occur (Figure 54 D) a quantitative estimate of bacterial phylloplane populations based only on flat areas of the leaf would lead to an inaccurate estimate of phylloplane population sizes. Therefore, the development of minimal sample preparation techniques that maintain the integrity of the leaf tissue throughout exposure to high intensity light is a possibility for future investigations in order to overcome this.

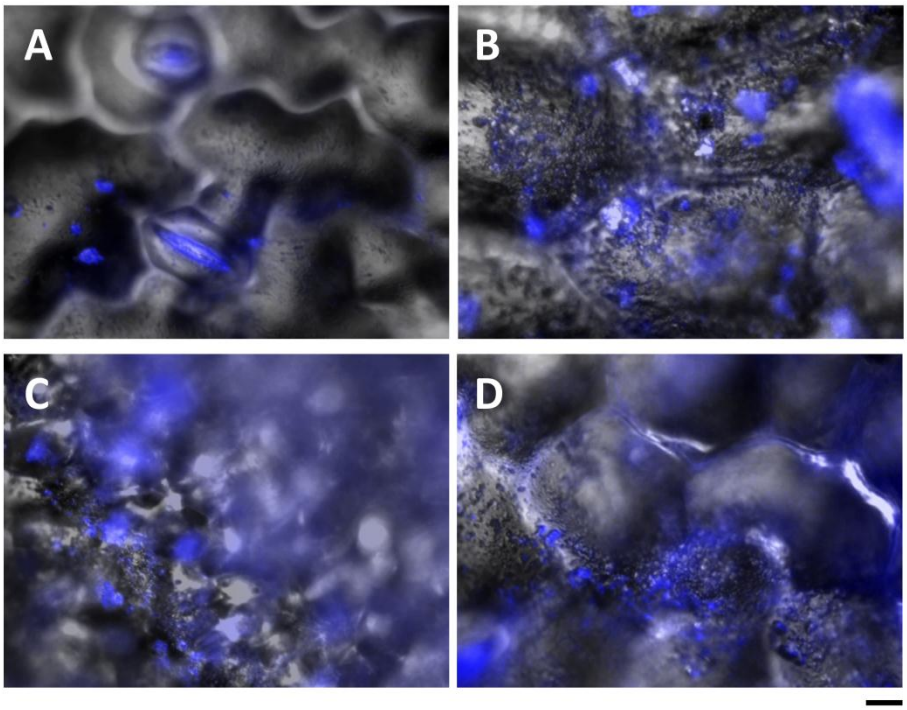

Figure 54 EDIC/EF micrographs of indigenous microbial populations at the spinach phylloplane following staining with DAPI

Spinach leaves were observed by EDIC/EF microscopy following staining with the nucleic acid stain DAPI. A 'flat' area of a spinach leaf showing colonisation by indigenous phylloplane bacteria which could be quantified is shown in Figure 54 A. Figure 54 B, C & D show 'rough' areas of a spinach leaf where indigenous phylloplane populations observed following DAPI staining are predominantly seen to reside and which cannot be accurately quantified without the ability to scan in the z-plane. Scale bar = 50 microns.

The features of EDIC/EF microscopy following DAPI staining of leaves described above demonstrate the disadvantage of the minimal sample preparation used for EDIC microscopy, which is otherwise so well suited for the observation of biotic samples in their natural state. If experimental samples are, by nature, not flat, clear visualisation by EDIC microscopy coupled with EF is not possible without scanning in the z-plane. For samples more robust than leaves, it is possible to take stacks of images in the z-plane, and indeed these can be taken for leaf samples using EDIC microscopy alone. However, even with careful sample preparation to maintain hydration of leaf samples during microscopy, the intensity of light required for EF microscopy coupled with the prolonged exposure required for z-stacking, causes degradation of the leaf tissue leading to structural changes that make this an unsuitable method for quantifying

phylloplane bacteria on rough areas of the leaf. These limitations for the use of EDIC/EF at leaf surfaces are why methods that rely on removal of bacteria from the phylloplane for quantification still remain important tools for the study of phylloplane bacteria.

6.4.3 The effect of nitric oxide on recovered indigenous phylloplane bacteria from spinach leaves during subsequent refrigerated storage

Recovery of bacteria from spinach leaves by pulsification followed by refrigerated storage of samples, resulted in an increase in culturable cell numbers over a 7 day storage period. This effect was not observed for total cells, or for bacteria recovered from the phylloplane by rinsing. In addition, this effect was greater for SNP treated leaves by comparison to control leaves, and was further increased by the elevation of light levels to liberate nitric oxide from SNP.

It is possible that nitric oxide treatment directly increases the culturability of recovered phylloplane isolates, however, given that SNP is not likely to remain associated with leaves during sample processing and remain present in the final refrigerated sample, unless phenotypic changes were induced in phylloplane bacteria that were maintained throughout refrigerated storage once nitric oxide was removed, this seems an unlikely explanation. In addition, only culturable cell numbers, and not total cell counts by microscopy, were increased suggesting an effect on culturability rather than growth. A more plausible explanation, and one that addresses the fact that culturability of bacteria recovered from control leaves is also increased during storage, but to a lesser extent than bacteria recovered from SNP treated leaves, is that pulsification may increase the recovery of indigenous bacteria existing in aggregates and biofilm communities on leaves, which, upon removal from the leaf surface are in a non-culturable state which then become more culturable as they revert to planktonic existence in solution during refrigerated storage, suggesting indeed that nitric oxide has an effect on the recovery of indigenous bacterial biofilms from spinach leaves. As described above, given the large and complex communities present at the phylloplane, it is possible that there are populations of responders and non-responders to nitric oxide treatment. Identification of bacteria isolated from spinach leaves, deemed

beyond the scope of these investigations, would provide insights into the specific effects of nitric oxide on individual species present at the phylloplane and is an important investigation for the future.

6.5 Conclusions

Nitric oxide treatment of spinach leaves leads to small increases the recovery of indigenous phylloplane isolates from spinach leaves, although in the field these increases are negligible, and although not investigated here, are unlikely to lead to improvements in product safety or quality for leafy salads. Further work on improvement of product quality and shelf life is required before it can be decided whether these small effects are worth investigating further for this application, as it is known that only a small reduction in total microbial load is required for product quality to be improved and for shelf life to be increased, which may be of financial benefit to the leafy salads industry. However, it is unlikely that the small changes observed would influence product safety.

In order to fully appreciate the nature of effects of nitric oxide treatment on indigenous phylloplane bacteria, the identification of isolates and their responses to nitric oxide treatment is necessary. It is likely that differences in response to nitric oxide treatment exist between phylloplane bacteria, as they do for other organisms, with some bacteria being responders to the dispersal action of nitric oxide and some not, with the possibility that this response occurs at different concentrations of nitric oxide. Nitric oxide treatment of phylloplane bacteria may also have non-target effects on both bacteria and the leaf itself. Here phylloplane biofilms are considered a burden on the leaf surface and a means by which zoonotic pathogens evade decontamination by washing, but what about beneficial microorganisms? It may be that disrupting established microbial communities at the leaf surface does not have a beneficial effect on leaf quality and that leaf surface biofilms may serve to protect the leaves in some way. Perhaps also, treatment of leaves in the field is not in fact the most appropriate point for intervention, and a post-harvest treatment may be more beneficial to product quality. This would also have the added advantage of allowing conditions for nitric oxide delivery to be more carefully controlled. If it is decided that it is beneficial

to further investigate nitric oxide as a decontamination strategy for leafy salads, a far greater understanding of effects of nitric oxide on individual bacterial species, and on the leaf itself is required before application for use in a food processing environment.

CHAPTER 7

General Discussion

The study of zoonotic pathogens in association with the surfaces of fresh produce is in its infancy (Brandl 2006b). The importance of fresh produce as a vector for zoonotic pathogen entry to the food chain and as a cause of serious food poisoning outbreaks has highlighted the need for further understanding of the interactions of zoonotic pathogens in fresh-produce associated environments such as the salad leaf phylloplane. This understanding is fundamental to the development of novel decontamination methods to aid the production of safe foods.

The use of EDIC/EF microscopy for the investigation of bacteria at the phylloplane has recently been reported (Warner et al 2008, Warner 2009). EDIC/EF microscopy is an advanced light microscopy technique that is advantages over other available microscopy techniques such as SEM and CLSM as it requires minimal sample processing and does not require the use of coverslips of oil; ideal features for the visualisation of biotic leaf samples in their natural, unaltered state. Additionally, EDIC is low cost and high throughput by comparison to other microscopy methods allowing the efficient processing of leaf samples soon after their removal from the field, for example. EDIC/EF microscopy was used here to demonstrate that *Salmonella* spp. are efficient colonisers of the spinach phylloplane, to show that *Salmonella* spp. form aggregates and biofilms at the phylloplane, and to visualise indigenous phylloplane biofilms in association with salad leaves.

The demonstration that bacteria exist in biofilm communities at the phylloplane (Morris et al 1997) and that zoonotic pathogens form biofilms at the phylloplane (Brandl and Mandrell 2002, Kroupitski et al 2009b) is a discovery that has huge implications for food safety. The persistence of bacteria throughout food production leading to food poisoning outbreaks may be due to the protection conferred by surface attachment and biofilm formation. Therefore, it is important to consider the biofilm mode of the growth for the development of novel decontamination strategies. This work has been one of the first investigations of its kind to not only consider the biofilm mode of growth as the predominant strategy for bacterial persistence in the phyllosphere, but to investigate a molecular signalling based approach to control indigenous phylloplane biofilm populations to improve food safety. Furthermore, this

work has been considered in the context of a practical approach to controlling microbial contamination of leafy salads in an industrial food production environment.

Bacteria at the phylloplane and the effect of nitric oxide

In this study, the progression of attachment and colonisation of spinach leaves by *S.* Thompson and *S.* Typhimurium was investigated for the first time using EDIC/EF microscopy. Although EDIC/EF microscopy had previously been used to demonstrate the technique for visualisation of pathogens at the phylloplane, only a snapshot showing *S.* Thompson on leaves after 24 hours had been observed. In this work, the use of EDIC/EF microscopy as a technique to investigate the progression of biofilm formation at the phylloplane by *S.* Thompson and *S.* Typhimurium has demonstrated these pathogens to be efficient colonisers of spinach leaves, to form aggregates with a slimy appearance indicative of bacterial biofilm formation, and agree that they may preferentially attach to areas of the leaf already colonised by indigenous bacterial populations at the phylloplane (Monier and Lindow 2005b, Warner 2009), suggesting that interactions with indigenous phylloplane bacteria may play an important role in the colonisation of spinach leaves by zoonotic pathogens. A dual fluorescence approach using DAPI for staining of indigenous phylloplane biofilms and GFP labelled *Salmonella* strains for simultaneous detection of confirmed indigenous phylloplane biofilms and their interaction with *Salmonella* spp. was unfortunately unsuccessful. However, the demonstration of indigenous phylloplane biofilms at the spinach surface, using the DAPI staining technique developed by Warner (Warner 2009), strongly indicated that *Salmonella* strains were interacting with indigenous phylloplane populations.

It would be interesting to investigate the formation of bacterial biofilms at the phylloplane using an alternative inoculation approach to the immersion in high concentration inocula used here. This approach was selected for subsequent detection of attached cells by EDIC/EF microscopy, however, the investigation of biofilm formation using this method is limited by the fact that colonisation times greater than approximately 48 hours cannot be investigated due to degradation of the integrity of the leaf in solution. An alternative inoculation method, such as dip-

inoculation, followed by an extended period of incubation of leaves at controlled temperature, would allow for biofilm formation over perhaps up to 7 days, which may lead to a better understanding of biofilm formation at the leaf surface under conditions more akin to those encountered by leaves in the environment.

Having demonstrated indigenous phylloplane biofilms, and the progression of colonisation of spinach leaves by *Salmonella* species to aggregated structures resembling early biofilms, the use of the biofilm dispersal agent, nitric oxide, was investigated for its effects on dispersal of both *Salmonella* spp., and the indigenous phylloplane communities with which they are thought to interact at the phylloplane. The theory behind the use of nitric oxide for biofilm dispersal is based on discoveries made by Barraud *et al*., who demonstrated that biofilms of *P.* aeruginosa disperse in response to exogenous application of nitric oxide when introduced to biofilm culture media at low concentration (Barraud et al 2006), which induces a signalling mechanism in *P.* aeruginosa via bacterial c-di-GMP (Barraud et al 2009a). Such signals are used by bacteria within biofilms to induce dispersal when either conditions outside the biofilm become favourable or conditions within it become unfavourable (Webb et al 2003b).

In these investigations, the effect of exogenous application of nitric oxide to spinach leaves for the control of contamination by *Salmonella* spp. was interesting, with nitric oxide effects being observed for attachment and colonisation, but with no clear pattern of a consistent response between experiments, and with no effect on dispersal of *Salmonella* from the phylloplane. The possible reasons for these observations underpinned all subsequent investigations in an attempt to gain a more detailed understanding of the clearly highly complex interaction of both a) *Salmonella* spp. with the phylloplane and the indigenous bacterial populations that reside there, and b) nitric oxide signalling in *Salmonella* spp.

Leaf samples were selected to best reflect the food production environment and the most appropriate point in the food production process at which an anti-microbial treatment could be applied. Therefore, growing region, growing conditions, chemical applications during growth as well as variety of spinach leaf was variable between samples. All of these factors have been demonstrated to affect indigenous bacterial

populations at the phylloplane (Brandl 2006a, Hirano and Upper 1989, Hirano and Upper 2000, Lindow and Leveau 2002, Lindow and Brandl 2003, Yaun et al 2002). Therefore, the complex interplay of *Salmonella* spp., with indigenous bacterial communities associated with spinach leaves is likely to have been variable between samples. Do zoonotic pathogens in the environment attach directly to the leaf surface, to existing bacterial aggregates on leaves, incorporate into existing phylloplane biofilms, or establish their own single species biofilms at leaf surface? These are all possible mechanisms for colonisation of spinach leaves by *Salmonella* species.

These investigations have demonstrated that *Salmonella* appear to attach to both the leaf surface itself, and to existing bacterial aggregates present on leaves. Therefore the response of *Salmonella* spp. to decontamination by nitric oxide treatment will depend not only on its own response to nitric oxide treatment, but on the interactions with indigenous phylloplane communities and how these also respond to decontamination by nitric oxide treatment. It may also be that indigenous phylloplane communities protect plants against contamination by zoonotic pathogens. It has been suggested that specific sites on the leaf surface may provide an anchor for bacteria (Beattie and Lindow 1999), but if these sites are covered in a microbial community and EPS matrix, these sites will be blocked and the biofilm itself becomes the surface with which a contaminating pathogen must interact in order to colonise the leaf. In this way, indigenous communities may harbour zoonotic pathogens, as has been shown for *Legionella* in drinking water biofilms for example (Giao et al 2009). The ability to remove phylloplane biofilms of indigenous species is therefore also an important consideration for food safety.

In these investigations, the effect of nitric oxide treatment on dispersal of indigenous phylloplane bacteria was demonstrated to increase the recovery of bacteria from spinach leaves, but only by a negligible amount. Therefore, it is not possible at this stage to conclude that nitric oxide treatment of leafy salads would be beneficial for product safety and quality in a food production environment. In addition the effect of these small changes in microbial load on product quality and shelf life has not been investigated here, but may be an interesting consideration for future work.

It was also observed, however, that exogenous application of nitric oxide to spinach leaves can increase the attachment and colonisation of spinach leaves by *Salmonella* species on some occasions. Therefore, unless conditions can be optimised further for consistent delivery of nitric oxide to leaves at a concentration that is efficacious for pathogen removal across species, exogenous application of nitric oxide, using SNP for the delivery of nitric oxide, may not be suitable strategy for decontamination of salad leaves.

The use of SNP for the delivery of nitric oxide in these investigations was selected based on its proven ability to induce biofilm dispersal for a range of bacterial species, and its potential applicability in a food production environment. However, it is recognised that the optimal conditions for release of nitric oxide from SNP is somewhat undetermined at low concentration. In studies using a nitric oxide probe for the measurement of nitric oxide in solution as released from 250 µM SNP, nitric oxide release establishes a steady state concentration approximately 1000 fold less than the concentration of SNP (Barraud et al 2009b). The extrapolation of this to low concentrations is assumed to be linear, but due to the inability to measure nitric oxide in solution at such low concentration this has not been established. Additionally the establishment of a steady state concentration relies on the establishment of an equilibrium between nitric oxide in solution and in association with SNP in solution, therefore if external environmental conditions are variable, as in a phylloplane environment, nitric oxide release from SNP may be influenced and concentrations may vary, although this has not been investigated. In addition, the release of nitric oxide from SNP is a light-dependent reaction, therefore, in an environment such as the phyllosphere where light levels are variable, the concentration of nitric oxide released from SNP may also vary.

SNP as a nitric oxide donor for use in the food industry

SNP was selected for use in these investigations for proof of concept because of its proven ability to induce biofilm dispersal, and for its potential application to the food industry in terms of chemical cost, handling and storage. However, when considering the potential for use of SNP in food production, its toxicity must also be taken into

account. The main mechanism for SNP toxicity is the release of cyanide (see Figure 10), which inhibits the enzyme cyclooxygenase in the mitochondrial respiratory chain, thus blocking oxygen useage, causing hypoxia and leading to metabolic acidosis (Way 1984). In Chapter 6, the application of SNP in the field was investigated. It was calculated that even assuming complete dissociation of the SNP molecule, releasing all 5 cyanide moieties per molecule (worst case scenario), at the highest concentration of SNP tested (10 µM SNP) the level of cyanide would be present at levels below the natural levels of cyanide found in soils, and therefore was assessed as not being an environmental risk in these investigations. However, when considering the application of such a treatment on a larger scale, a more extensive risk assessment would be required. It may be that pre-harvest application is not the most appropriate point in the production process for introduction of nitric oxide. It was decided that pre-harvest application for a trial investigation would have minimal impact on commercial production for these investigations. This is not to say that application of nitric oxide for decontamination is limited to pre-harvest application, and it may be that an alternative treatment point is more suitable in a commercial production environment. However, the concept that nitric oxide induces a significant reduction in populations of phylloplane bacteria has not been proven here, and therefore, the practical aspects of nitric oxide treatment in commercial food production have not been fully evaluated, but for commercial context, if a 20 hectare field were to be treated with 500 nM SNP (at 1L per m^2) a total of 30 kg of SNP would be required at a cost of £350 per kg, which is a) unlikely to be commercially viable, and b) would be unacceptable in terms of cyanide toxicity on a large scale.

In addition, whilst it has not been demonstrated here that exogenous application of SNP to biofilms in the phylloplane environment can lead to significant biofilm dispersal events, it is interesting to consider the potential consequences of finding a universal biofilm dispersal agent for application in the environment. Nitric oxide (targeting c-di-GMP signalling), acyl homoserine lactones (targeting quorum sensing), and cis-2-decanoic acid (targeting fatty acid signalling) are examples of molecules that have been investigated for their 'universal' ability to disperse bacterial biofilms (Kaplan 2010, Karatan and Watnick 2009). However, not only is it emerging that in the absence of one signalling mechanism, bacteria respond with another to achieve the same

outcome for survival (biofilm formation for example), but such ability to adapt to changing in environmental conditions has proven fundamental to the successful evolution of bacteria in harsh and inhospitable environments throughout history allowing their survival and persistence (Hall-Stoodley et al 2004). In addition, because of the problems biofilms cause in medicine and industry, mechanisms for their dispersal are widely researched. But what about the protective effects of bacterial biofilms? Biofilms in the mammalian gut for example may exist in symbiosis with their hosts to aid digestion and nutrient uptake (Hooper and Gordon 2001), biofilm formation in the body may help the immune system contain and manage the presence of otherwise infectious bacteria, although they are commonly reported as causing chronic inflammation and being reservoirs for infectious pathogens (Tlaskalova-Hogenova et al 2004). Environmental biofilms, for example of the nitrogen fixing bacteria on the roots of plants, play essential roles in nutrient cycling and maintenance of ecosystems balance (Danhorn and Fuqua 2007). Additionally, biofilm dispersal agents could be considered as just another form of antibiotic, to which bacteria notoriously develop resistance. It is therefore important that biofilm control agents are carefully contained and targeted to problematic biofilms, as although highly unlikely to be found, the release of a universal successful biofilm dispersal agent into the environment could have a detrimental impact on the wider microbial biofilm population.

The effects of nitric oxide treatment on *S.* Typhimurium 14028; surface colonisation strategies and protein expression

In addition to the effect of nitric oxide on bacteria at the phylloplane, the effect of temperature, a variable known to fluctuate in the environment, was investigated for its effect on surface colonisation and found to have an influence on the colonisation of both spinach leaves and the abiotic surface by *S.* Typhimurium. This factor may add to the variability of phylloplane populations and the formation of bacterial biofilms at the phylloplane in the environment. The effect of flagellar-expression was also investigated for its ability to influence surface colonisation by *S.* Typhimurium at both the leaf surface, and at the phylloplane, as it had been suggested that flagellar directly anchor *S.* Typhimurium to leaves (Berger et al 2009). However, it was found here that

whilst flagellar are involved in successful surface colonisation by *S.* Typhimurium they are not essential for attachment. Flagellar expression was however required for biofilm formation at the abiotic surface at 37°C, and it is therefore suggested that rather than a direct attachment mechanism, flagellar expression may influence the formation of phylloplane biofilms by *Salmonella* species.

Whilst the majority of investigations into the effect of nitric oxide on *Salmonella* in association with leaves in this work focused on the biotic and variable nature of the leaf surface which may have mitigated the effects of nitric oxide, it was also recognised that nitric oxide treatment may simply not induce the dispersal of *Salmonella* biofilms. This was investigated at the abiotic surface and indeed, it was found that treatment with nitric oxide actually increased percentage surface coverage when added during both surface colonisation, and for dispersal. However, as biofilm formation had been influenced by treatment with nitric oxide, and as reductions in colonisation of spinach leaves by *S.* Typhimurium had been observed following SNP treatment, a proteomic investigation was carried out to determine the effect on protein expression by *S.* Typhimurium. It was found that an up-regulation of energy production consistent with the planktonic lifestyle in bacteria was induced upon treatment with SNP. The fact that nitric oxide signaling is active in *Salmonella*, yet does not correspond to a decrease in biofilm surface coverage or an induction in biofilm dispersal under the environmental conditions investigated here is a very interesting observation that demonstrates the complexity of nitric oxide signaling in bacteria. These findings also support the emerging consensus that the regulation of intracellular levels of c-di-GMP, which controls the switch between sessility and motility in bacteria, is achieved via the complex interplay of molecular signals that are not all conserved across bacterial species (Romling et al 2013). It will be interesting to compare the results of the proteomic investigations seen here, to emerging studies concerning nitric oxide signaling in bacterial species that are known to undergo biofilm dispersal events in response to nitric oxide treatment, to gain further insights into a mechanism of action for nitric oxide signaling, and how this may or may not be conserved across bacterial species.

Outlook and further work

Reducing microbial food spoilage and increasing product shelf life is key to reducing the amount of food discarded as waste in developed nations (IMechE 2013). It is estimated that 30-50% of food produced globally is discarded as waste. As the increasing global population increases the demand for food, the pressures on food production rise. The space available for food production and the high demand for food lead to intensification of farming practices, increased use of fertilisers and pesticides and a high demand for clean water for irrigation in an attempt to maximise output without compromising product safety. In addition, consumer driven markets in developed nations demand health ready-to-eat convenience foods, such as the leafy salads investigated here, which have short product shelf lives by comparison to other food produce (Clarkson et al 2003). Therefore, minimising loss from food spoilage is essential for improving the sustainability of production of such RTE products. Here, the potential for use of a low concentration of the molecular signalling molecule, nitric oxide, was investigated for its ability to reduce total microbial load at the phylloplane. However, it was not possible to conclude from these investigations that the application of nitric oxide induces a reduction in microbial populations at leaf surfaces large enough to be used for the improvement of product quality or product safety for leafy salads in a commercial food production environment.

Whilst the use of exogenous application of SNP to leafy salads for decontamination of *Salmonella* biofilms and for reduction of total microbial load at the phylloplane has not proven a viable option for commercial use in a food production environment at this stage of our understanding, there are some interesting insights into nitric oxide signalling in bacteria to be learned from these investigations, and into considerations for future decontamination strategies for leafy salads. The underpinning theme throughout these investigations is the fact that variability between environmental leaf samples, in terms of leaf factors such as exposure to variable growth conditions, and in terms of the underlying indigenous microbial communities present, is a major factor in determining pathogen interactions with leaves, and therefore one of the major hurdles to overcome for the design novel approaches to intervention in pathogen contamination of leafy salads. Community analysis of bacterial populations associated

with spinach leaves throughout the food production process would provide a more informed background for the design of novel intervention strategies for use for leafy produce. In particular, the response of individual environmental isolates to exogenous application of nitric oxide in terms of biofilm dispersal could be investigated. In addition, the negligible increases in recovery of indigenous phylloplane bacteria from nitric oxide treated leaves observed for these investigations may or may not lead to an increase in product shelf life, but this requires clarification with product shelf life trials.

This study has provided an extensive investigation into the effects of nitric oxide treatment, via the exogenous application of SNP, on both indigenous phylloplane bacteria, and *Salmonella* in association with spinach leaves. It has also provided a detailed analysis of the molecular response of *Salmonella* to nitric oxide treatment, providing some fascinating insights into the potential molecular signalling responses induced. However, whilst this is interesting in terms of the nitric oxide/c-di-GMP story, and may provide insights into the differences in nitric oxide signalling across bacterial species, perhaps a more high-throughput screening approach to novel decontamination strategies is required for application to a food production environment. This could be carried out using the procedures outlined in this thesis which were based on work by Warner (Warner 2009).

Two new projects related to the work undertaken for this PhD project are due to commence soon. A BBSRC CASE funded PhD studentship in collaboration with Vitacress Salads Ltd. to further investigate decontamination strategies for biofilms and zoonotic pathogens in association with salad leaves, this project will further consider the VBNC state for bacteria at the phylloplane. Secondly, a collaborative project is underway between the University of Southampton and industrial partners, including Vitacress Salads Ltd., funded by the Technology Strategy Board to investigate a novel wash water system for pre-packaging sanitisation of leafy salad crops.

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APPENDIX 1

Appendix 1

Microscopy images for the investigation of nitric oxide treatment on *Salmonella* Typhimurium and *Salmonella* Thompson at the spinach phylloplane.

A Untreated Controls

B 50 nM SNP

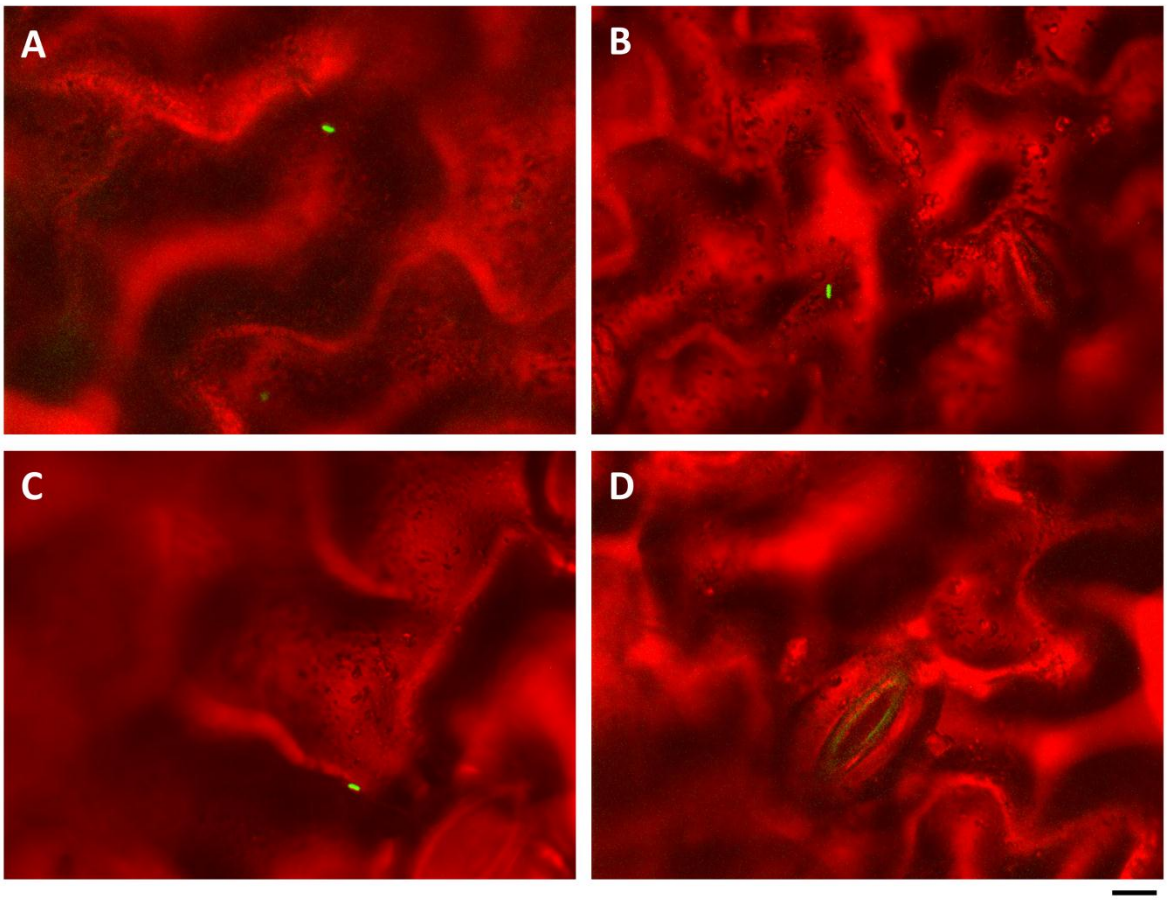
C 500 nM SNP

D 1 µM SNP

Bar = 10 microns

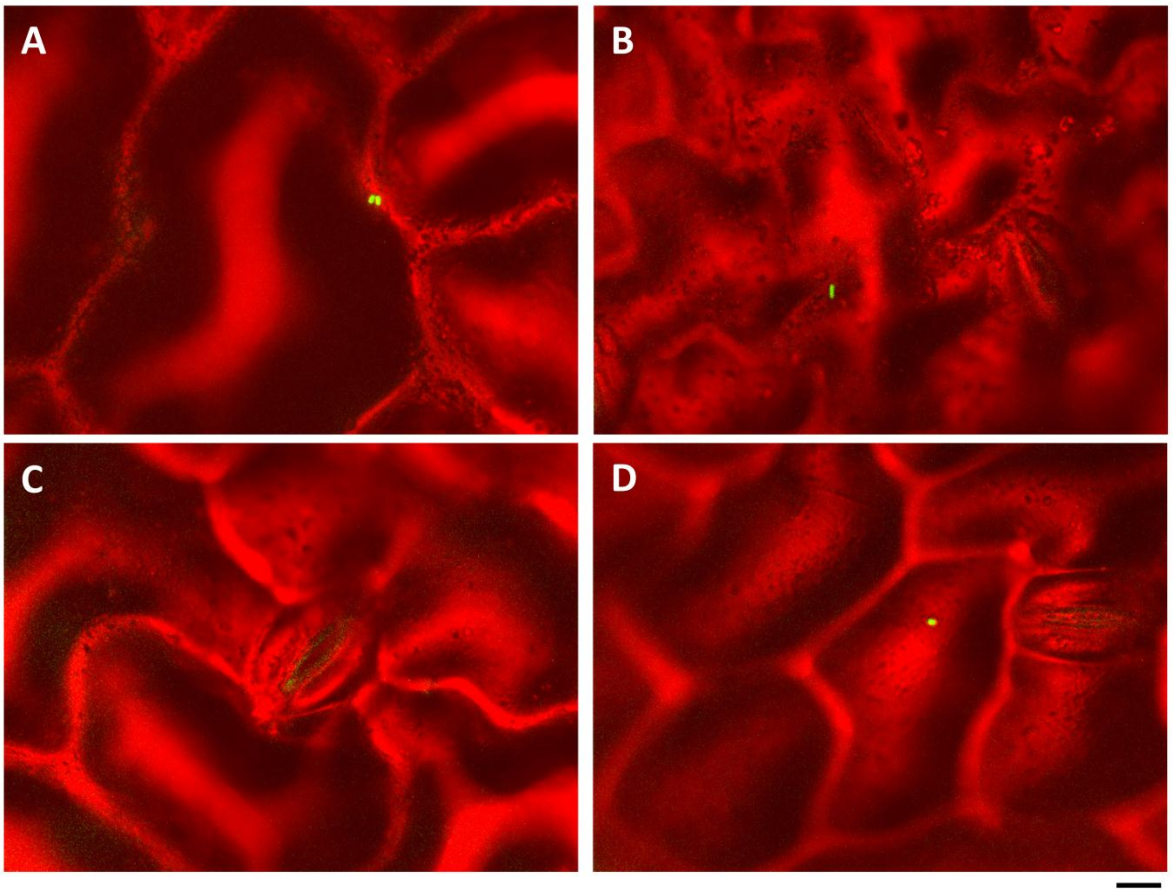
The effect of nitric oxide treatment on initial attachment of *Salmonella* Typhimurium to the spinach phylloplane

Lower surface



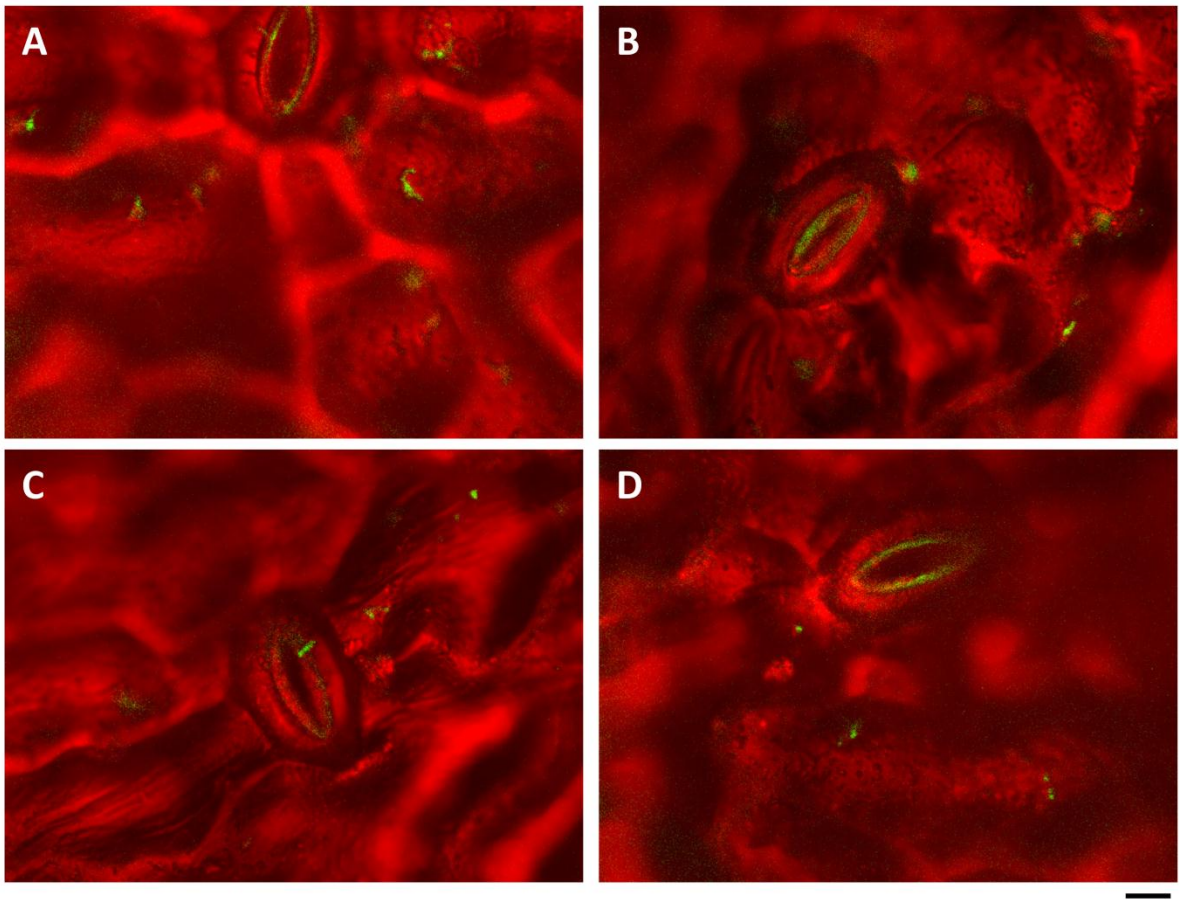
The effect of nitric oxide treatment on initial attachment of *Salmonella* Typhimurium to the spinach phylloplane (continued)

Upper surface



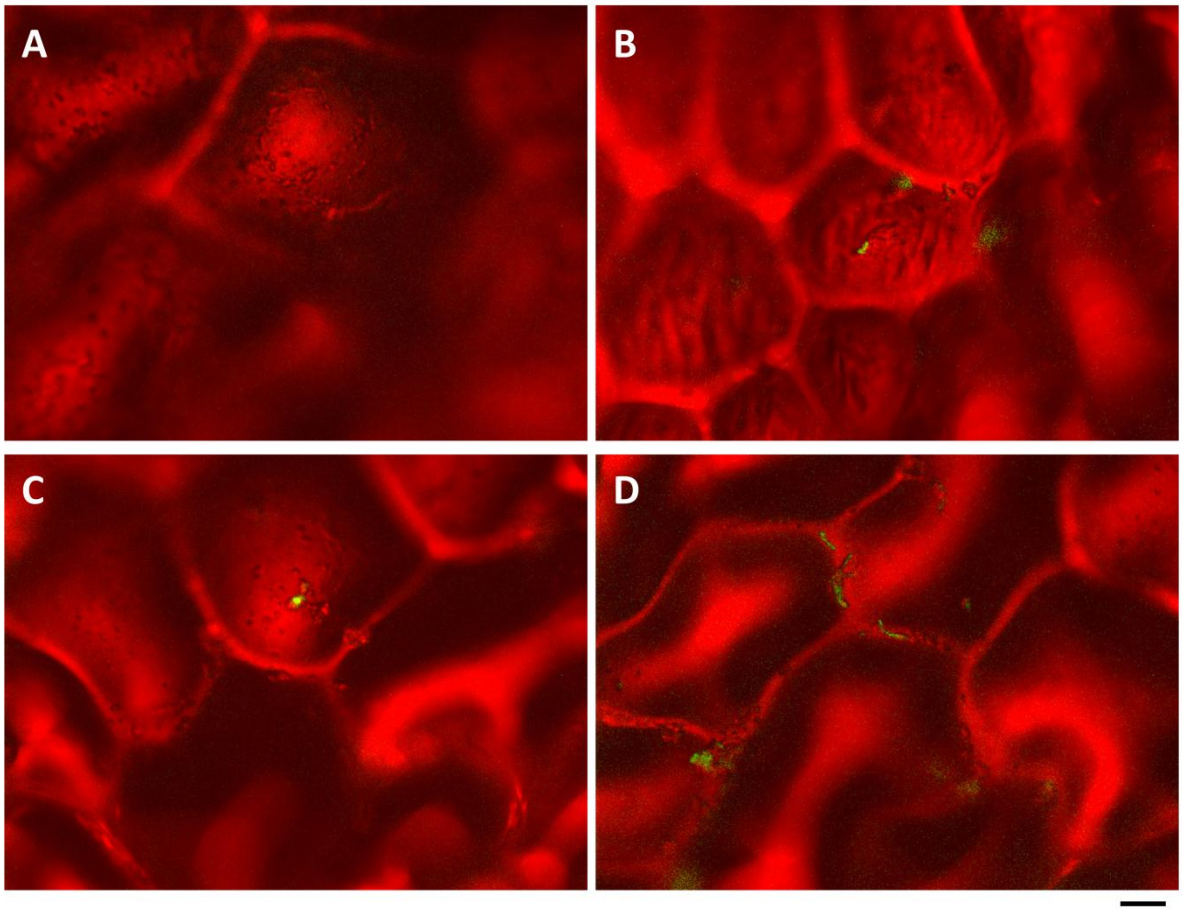
The effect of nitric oxide treatment on early colonisation of the spinach phylloplane by *Salmonella* Typhimurium

Lower surface



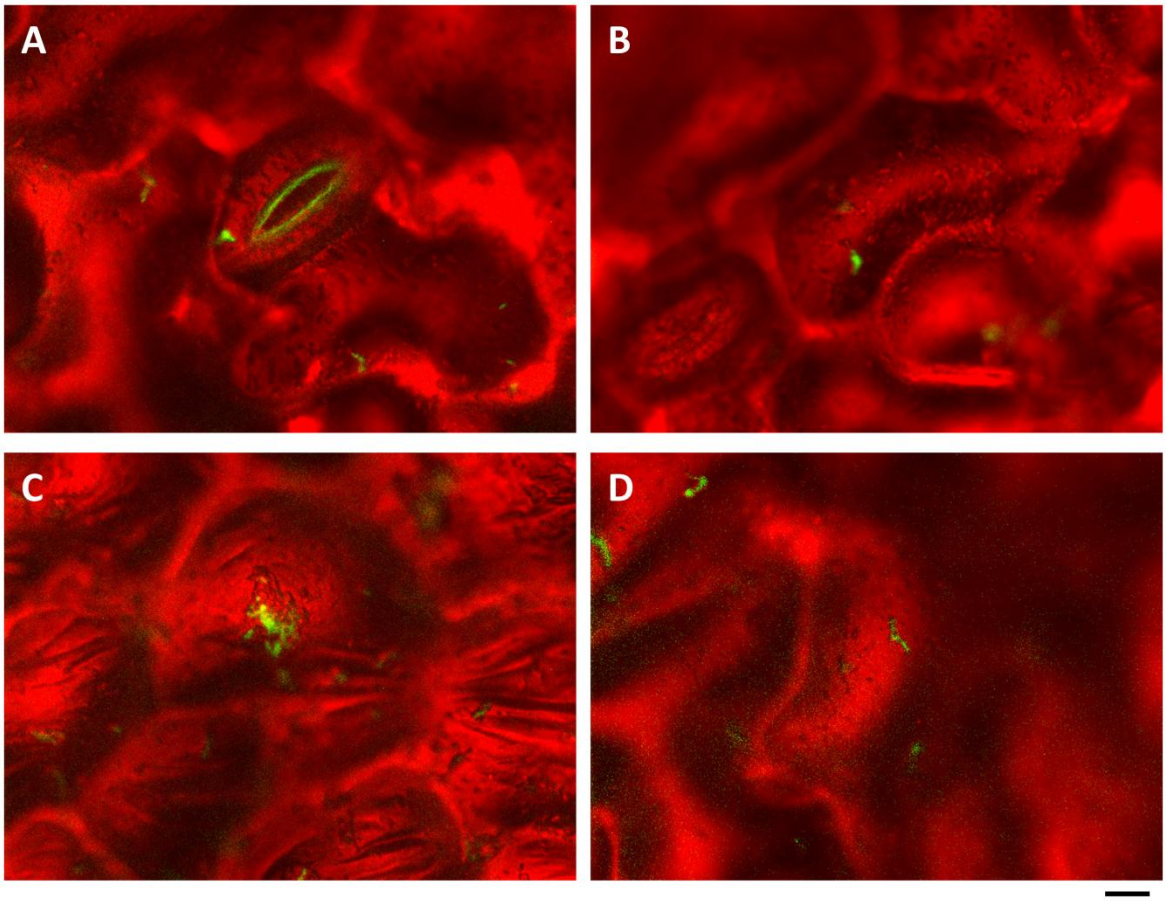
The effect of nitric oxide treatment on early colonisation of the spinach phylloplane by *Salmonella* Typhimurium (continued)

Upper surface



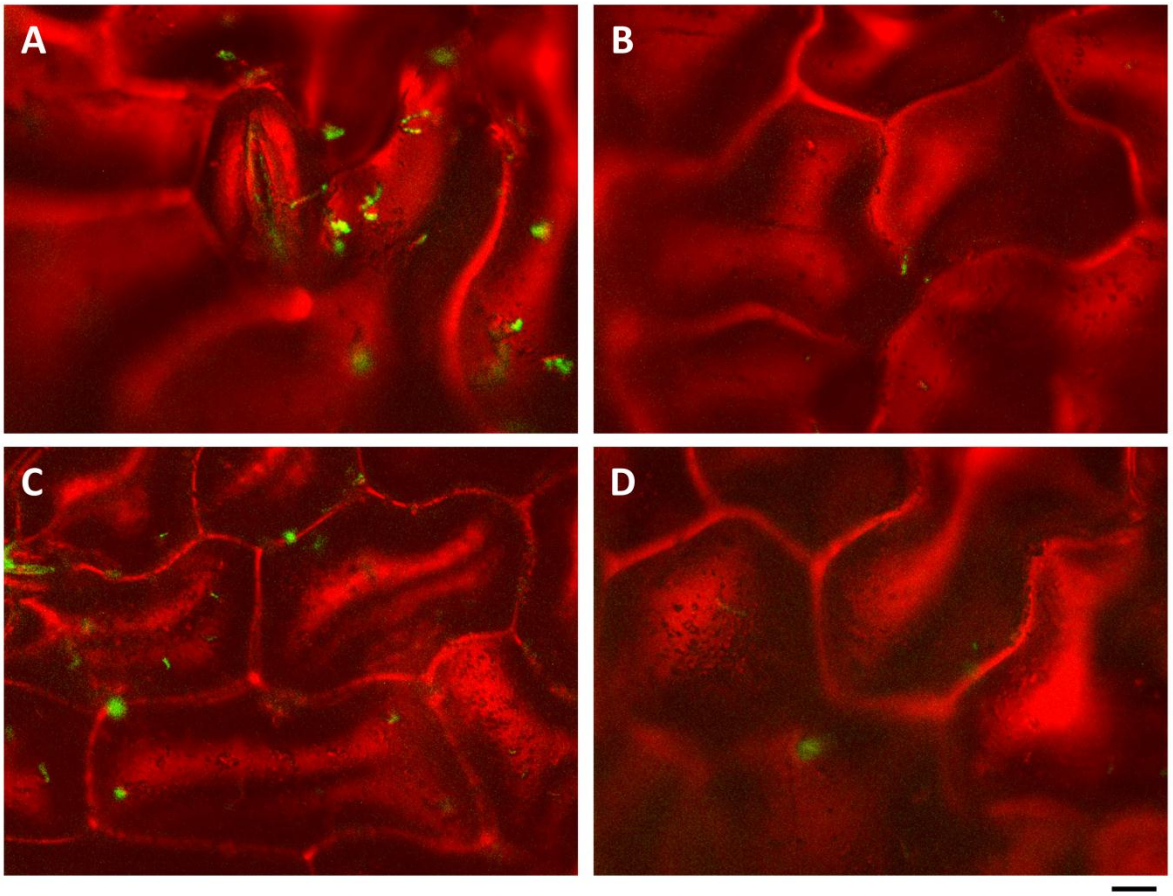
The effect of nitric oxide treatment on detachment of *Salmonella* Typhimurium from spinach leaves (1 hour nitric oxide treatment)

Lower surface



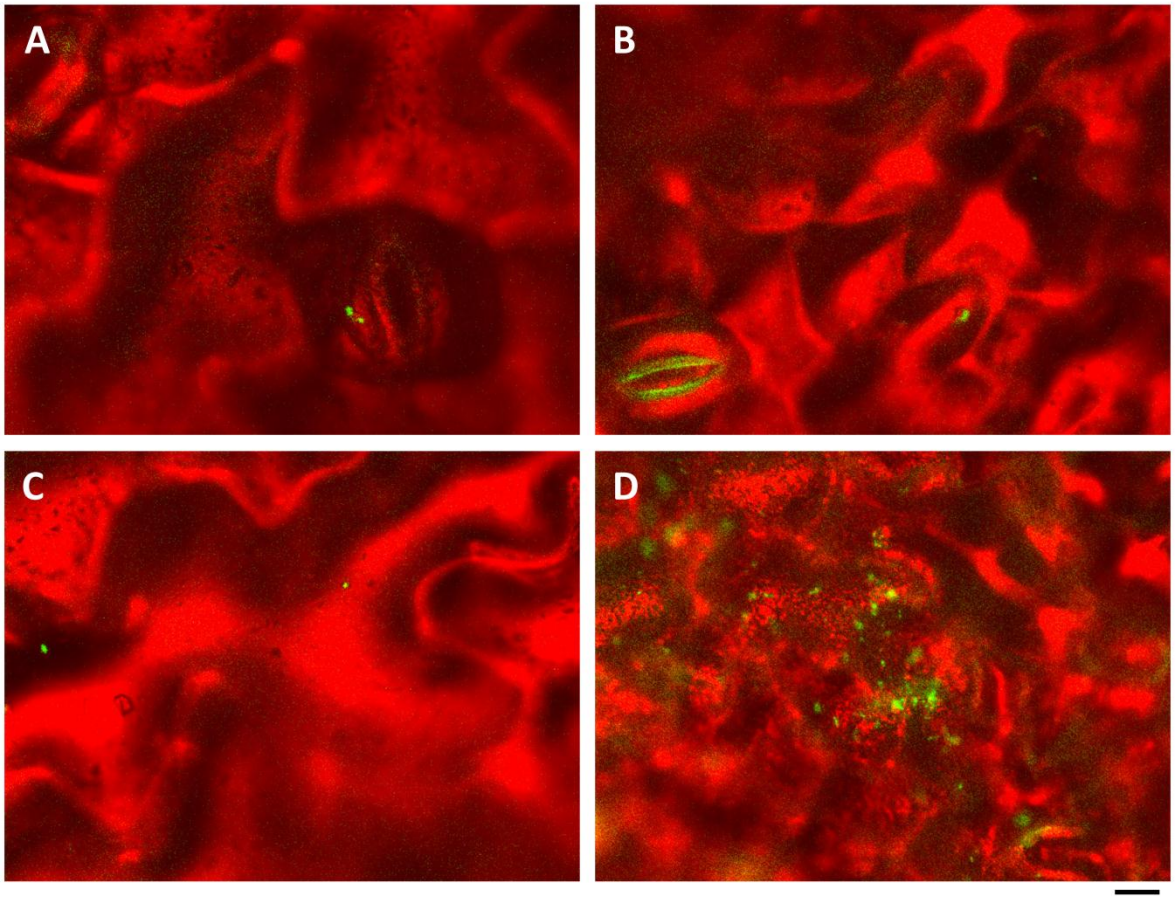
The effect of nitric oxide treatment on detachment of *Salmonella* Typhimurium from spinach leaves (1 hour nitric oxide treatment, continued)

Upper surface



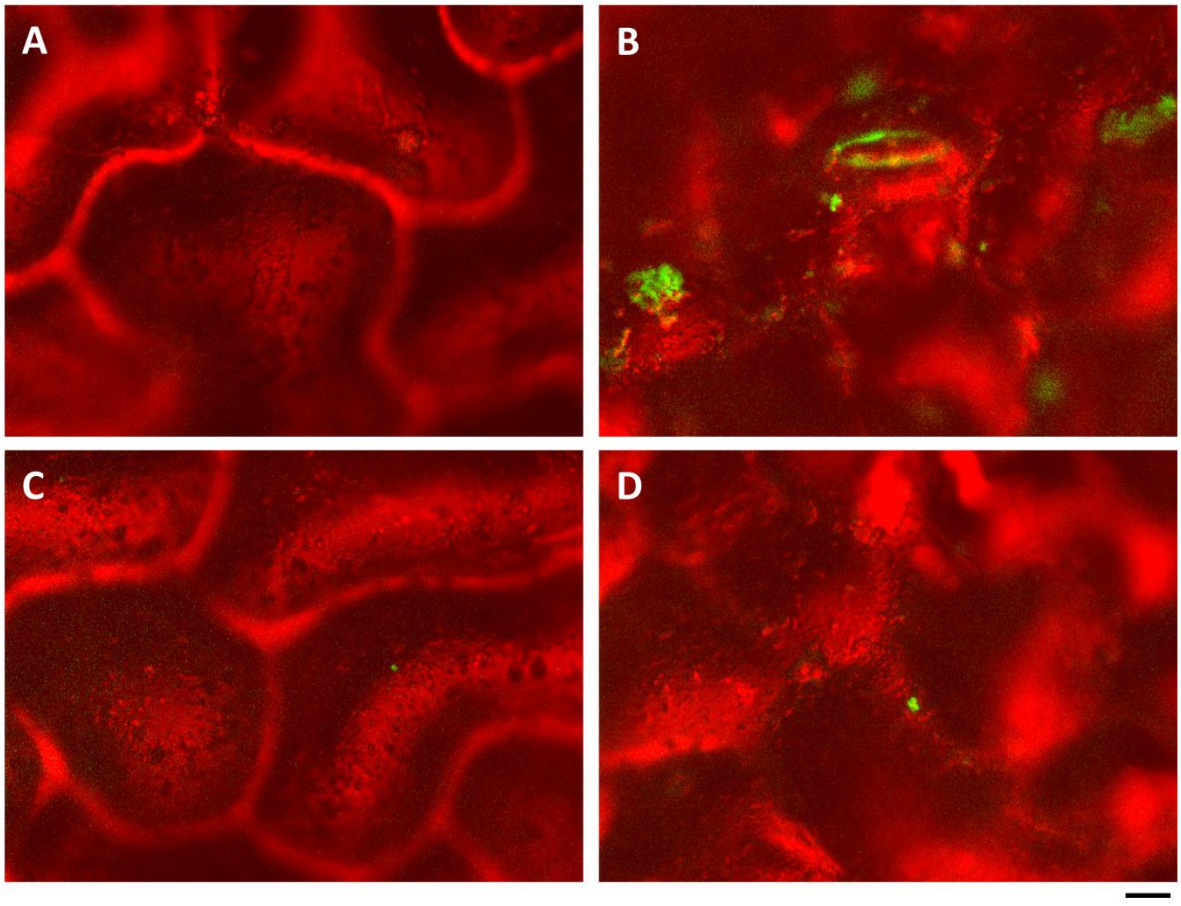
The effect of nitric oxide treatment on detachment of *Salmonella* Typhimurium from spinach leaves (24 hours nitric oxide treatment)

Lower surface



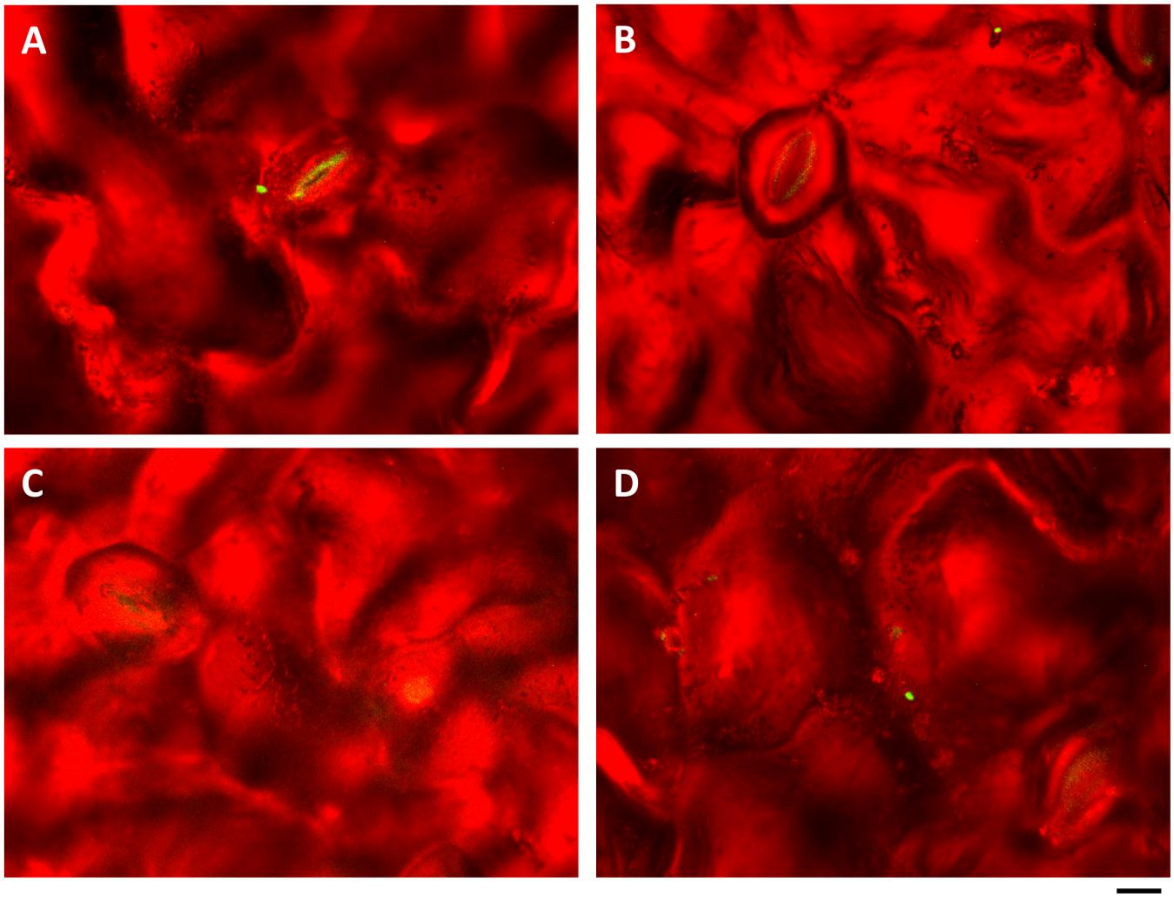
The effect of nitric oxide treatment on detachment of *Salmonella* Typhimurium from spinach leaves (24 hours nitric oxide treatment, continued)

Upper surface



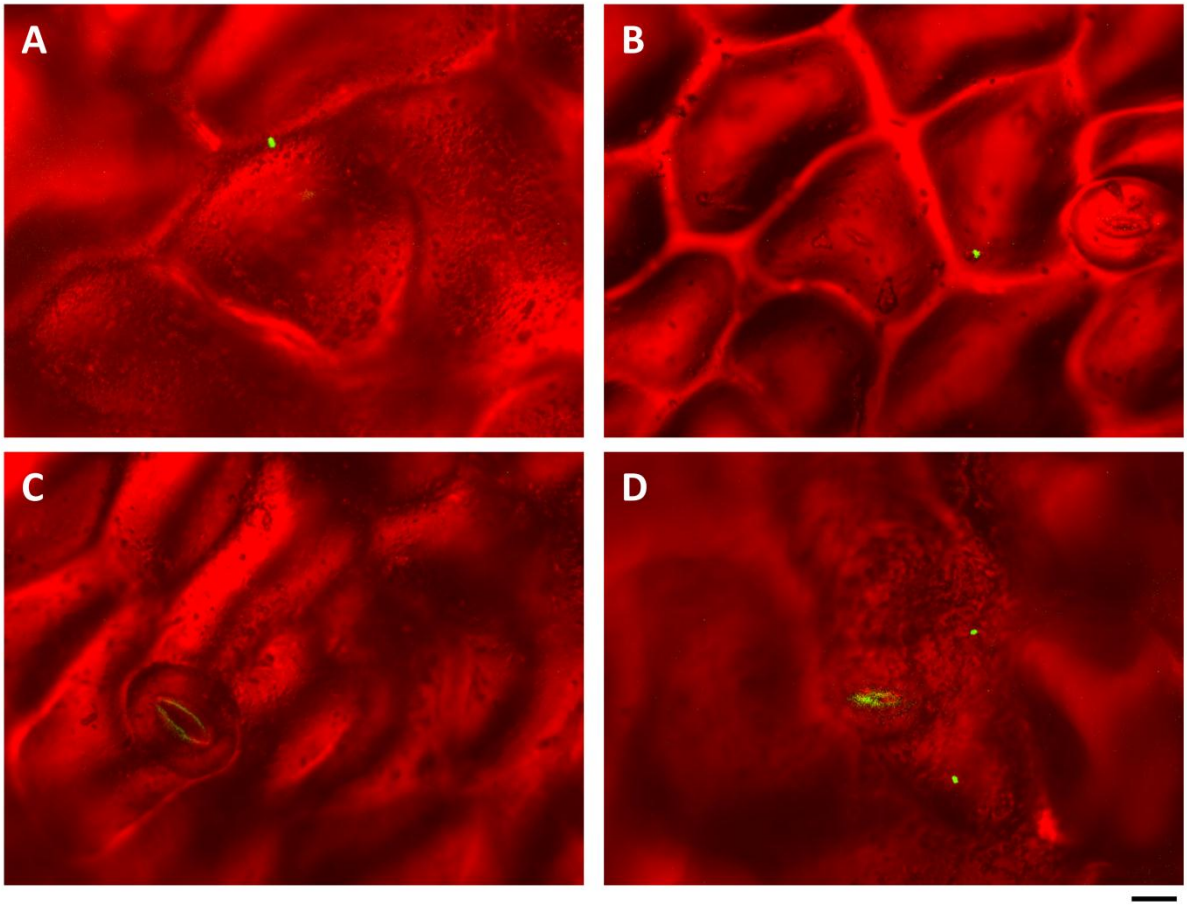
The effect of nitric oxide treatment on initial attachment of *Salmonella* Thompson to the spinach phylloplane

Lower surface



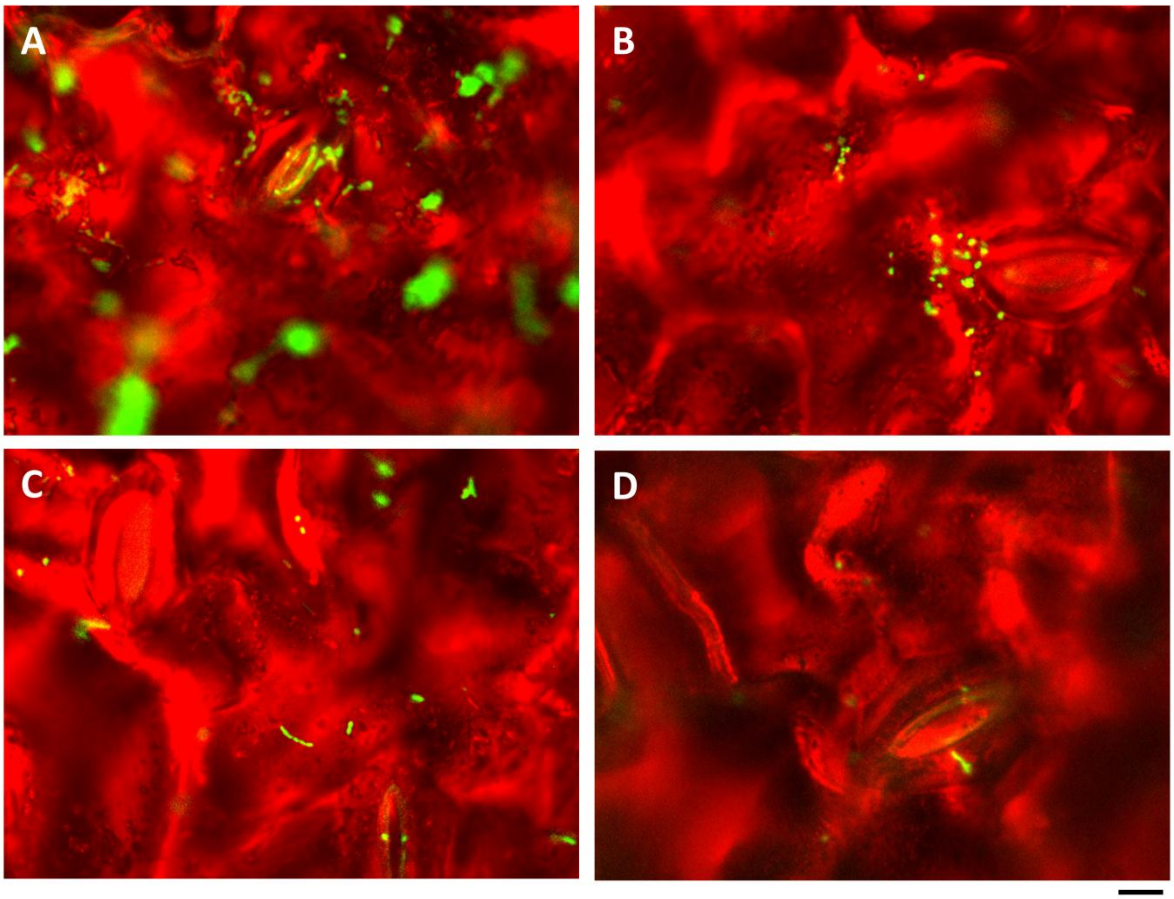
The effect of nitric oxide treatment on initial attachment of *Salmonella* Thompson to the spinach phylloplane (continued)

Upper surface



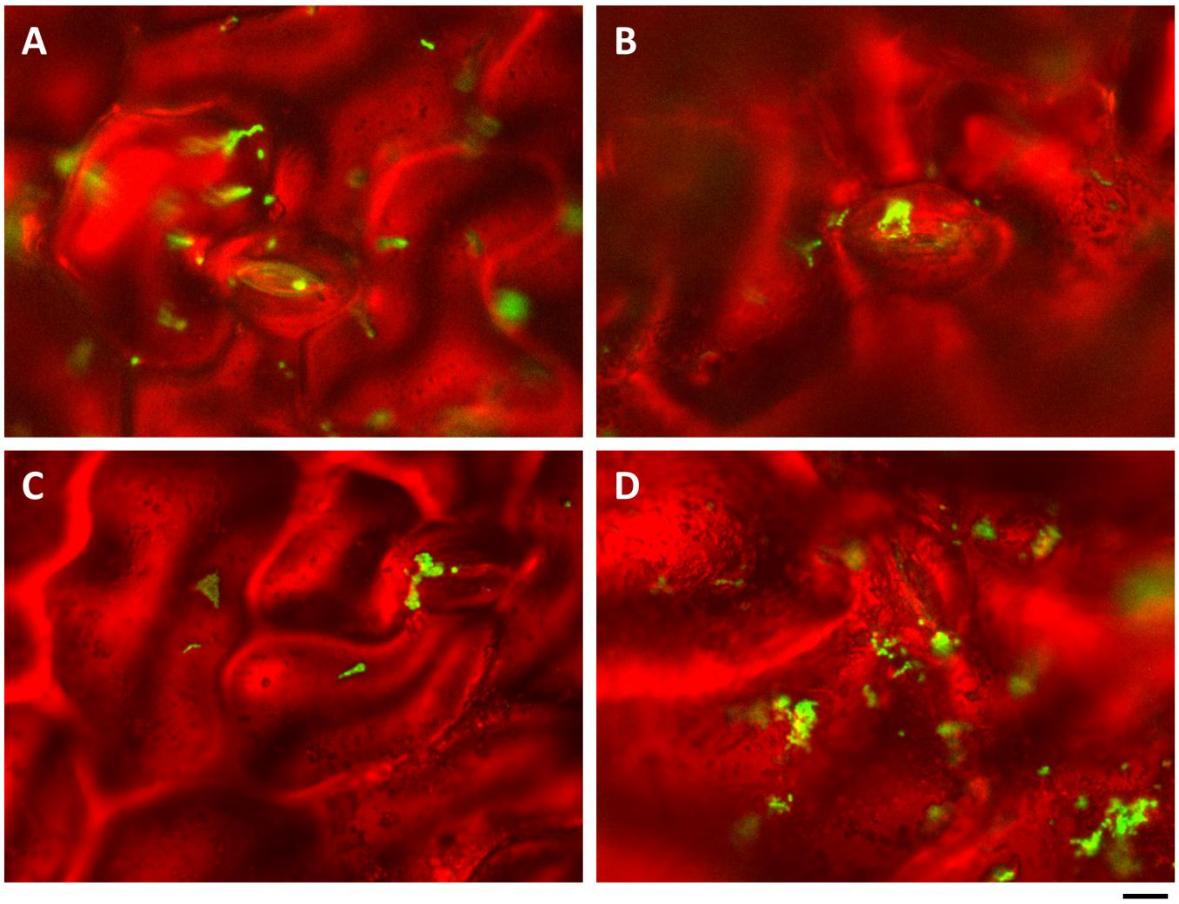
The effect of nitric oxide treatment on early colonisation of the spinach phylloplane by *Salmonella* Thompson

Lower surface



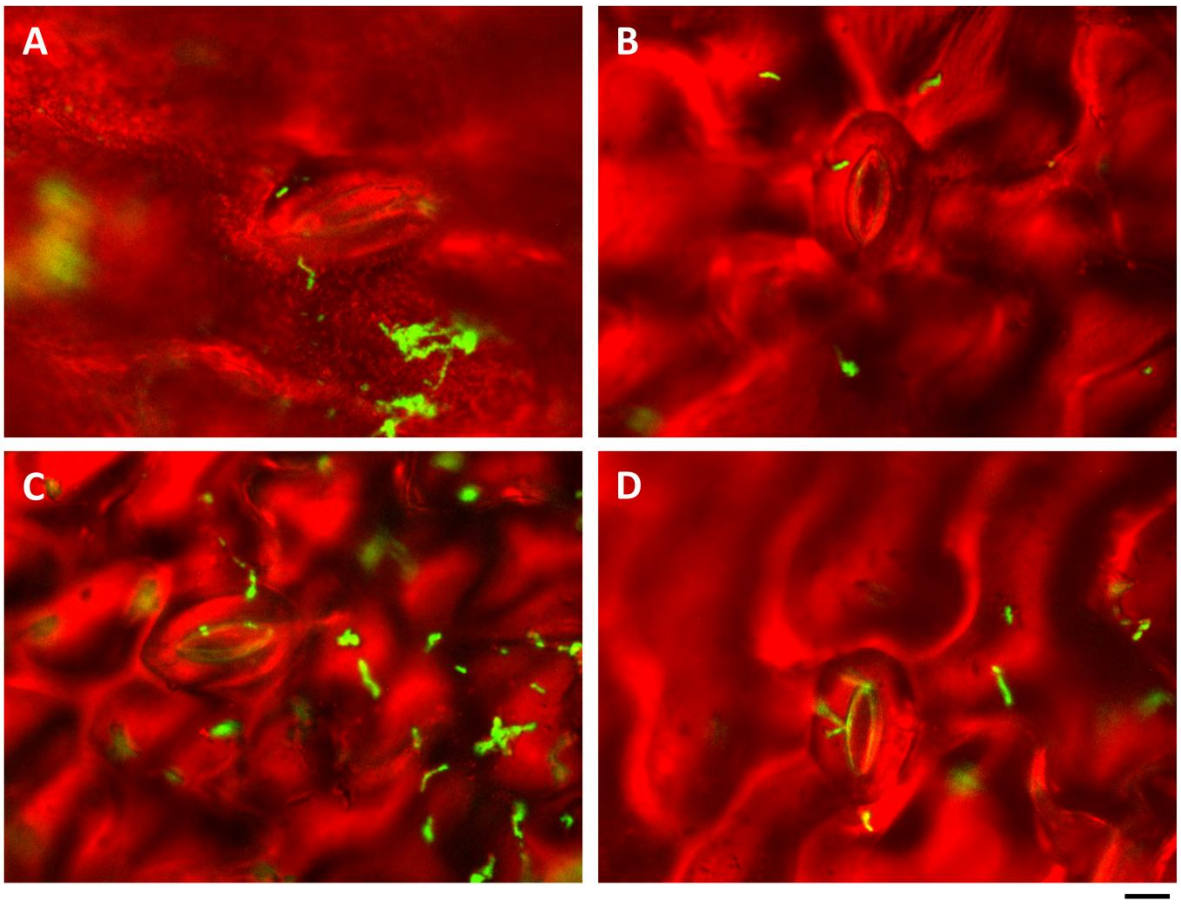
The effect of nitric oxide treatment on early colonisation of the spinach phylloplane by *Salmonella* Thompson (continued)

Upper surface



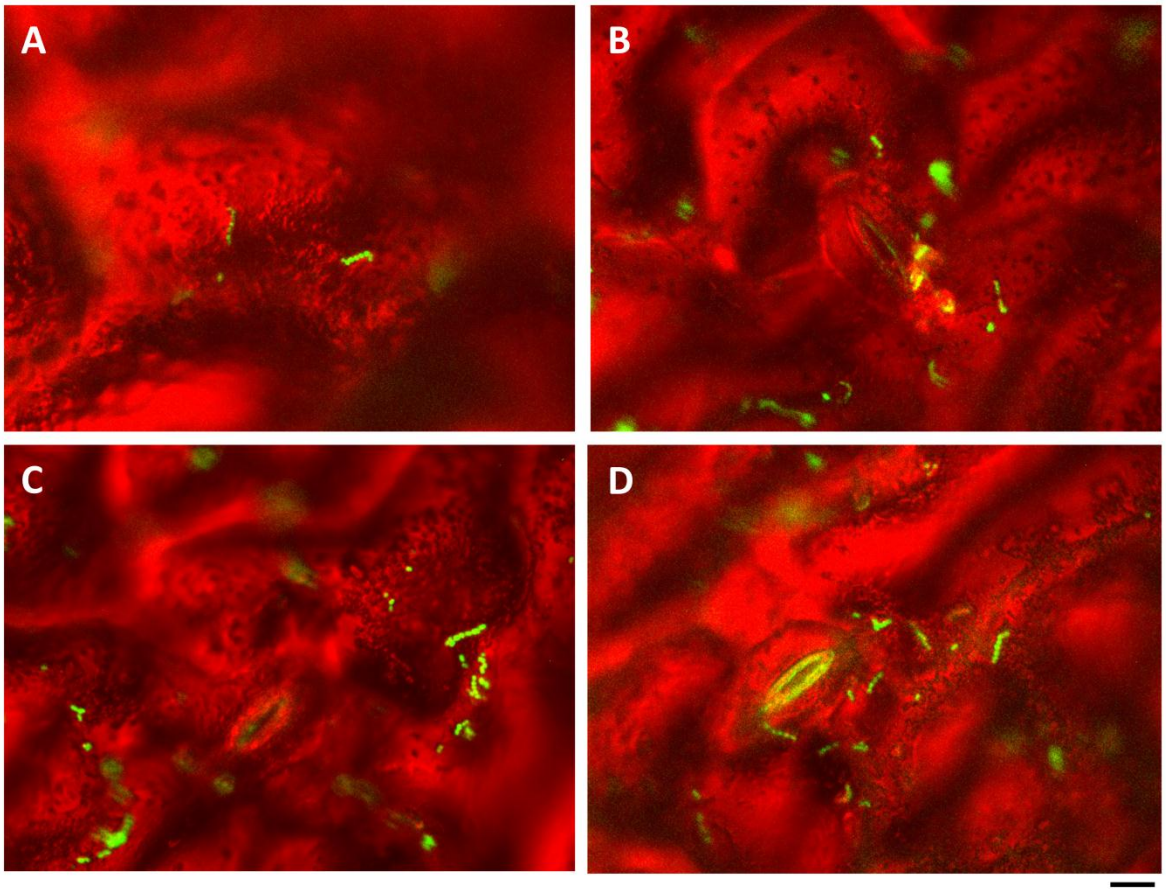
The effect of nitric oxide treatment on detachment of *Salmonella* Thompson from spinach leaves (1 hour nitric oxide treatment)

Lower surface



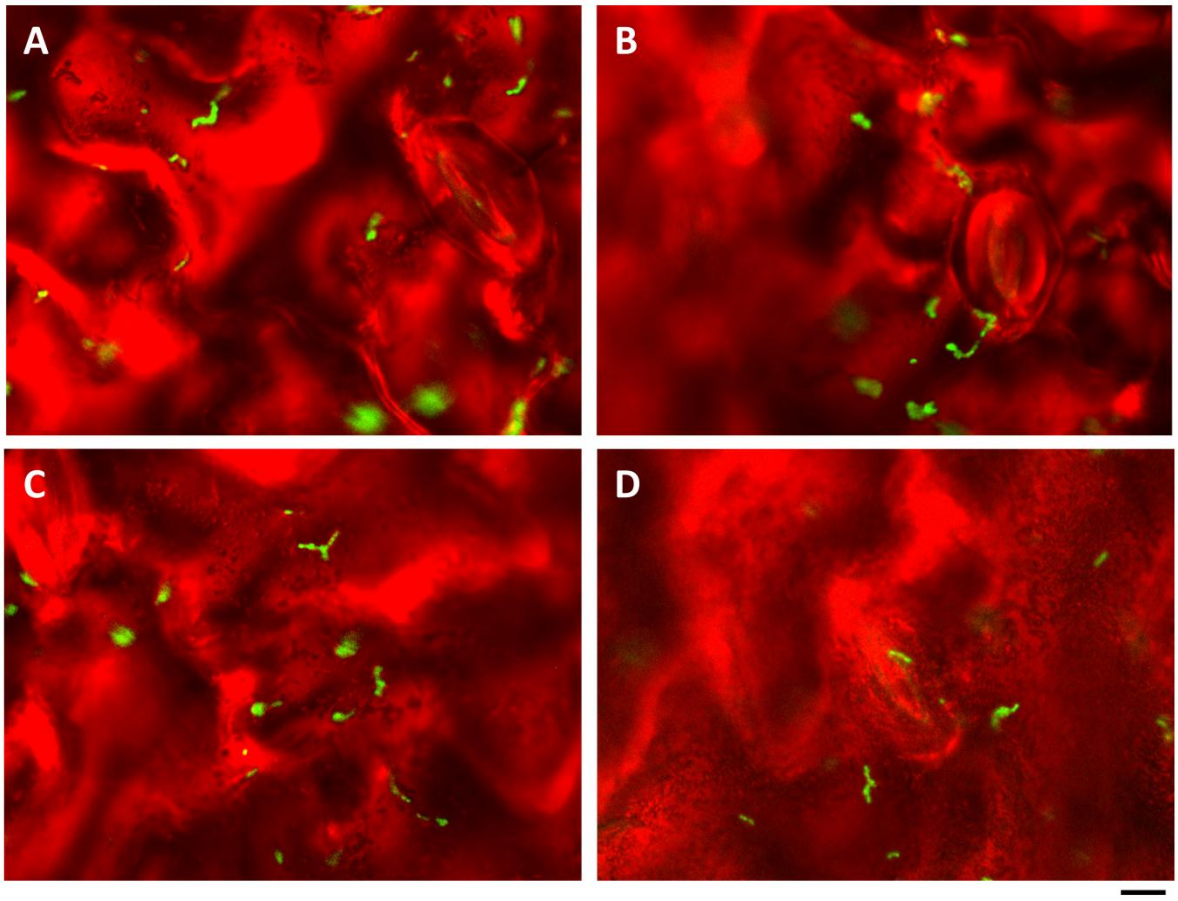
The effect of nitric oxide treatment on detachment of *Salmonella* Thompson from spinach leaves (1 hour nitric oxide treatment, continued)

Upper surface



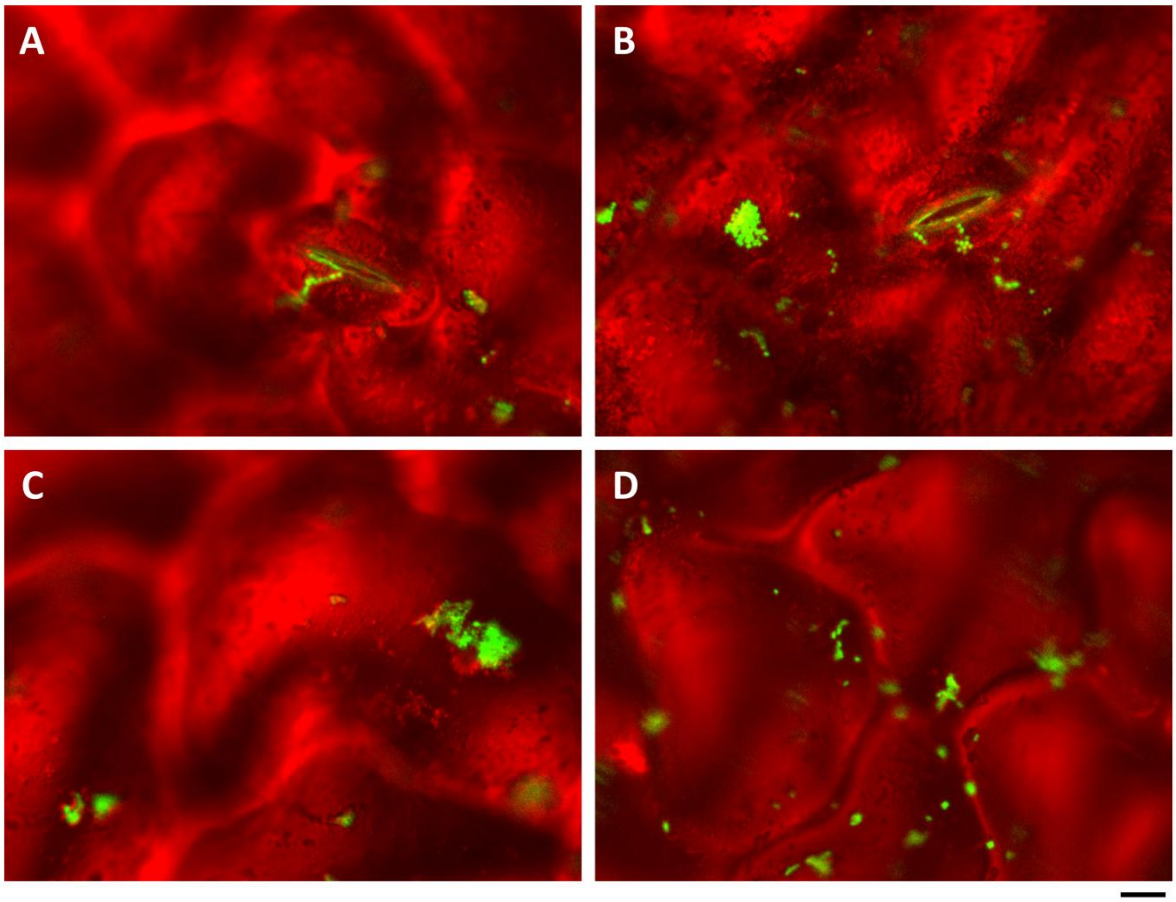
The effect of nitric oxide treatment on detachment of *Salmonella* Thompson from spinach leaves (24 hours nitric oxide treatment)

Lower surface



The effect of nitric oxide treatment on detachment of *Salmonella* Thompson from spinach leaves (24 hours nitric oxide treatment, continued)

Upper surface



APPENDIX 2

Microscopy images for the investigation of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane.

A & B Untreated Controls

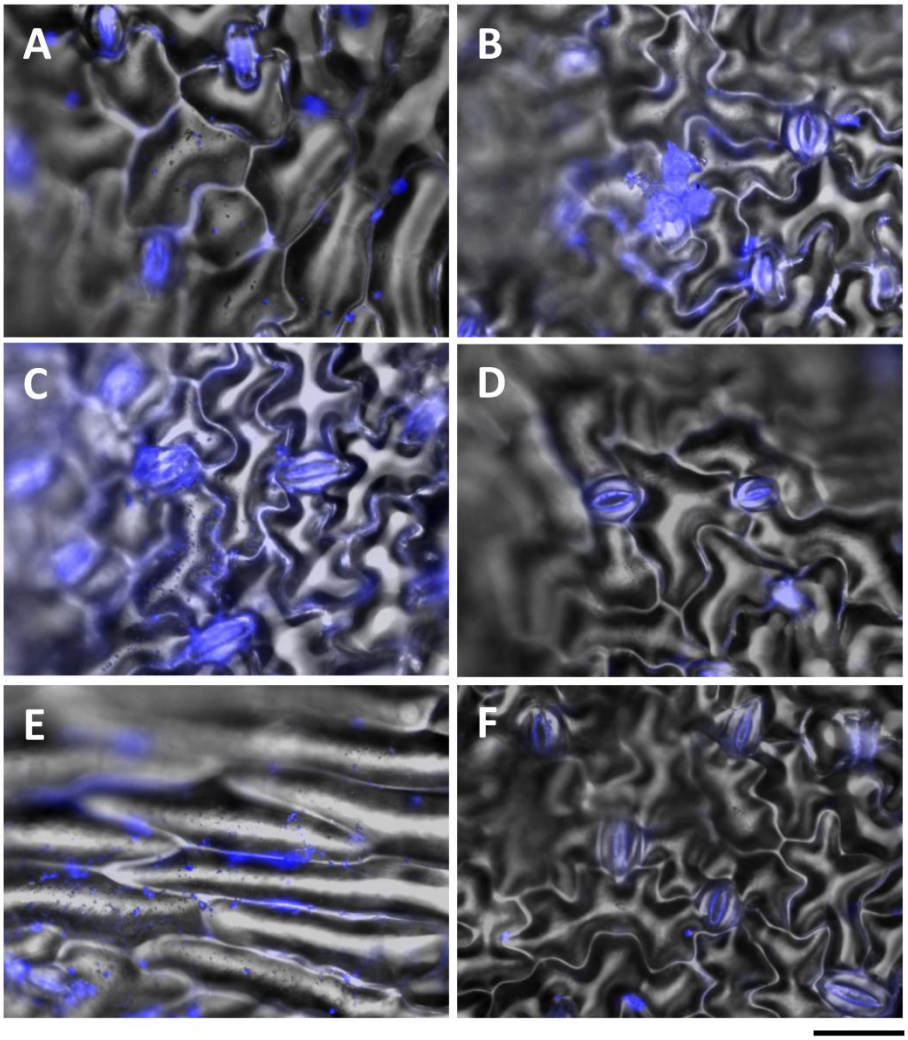
C & D 500 nM SNP

E & F 1 µM SNP

Bar = 50 microns

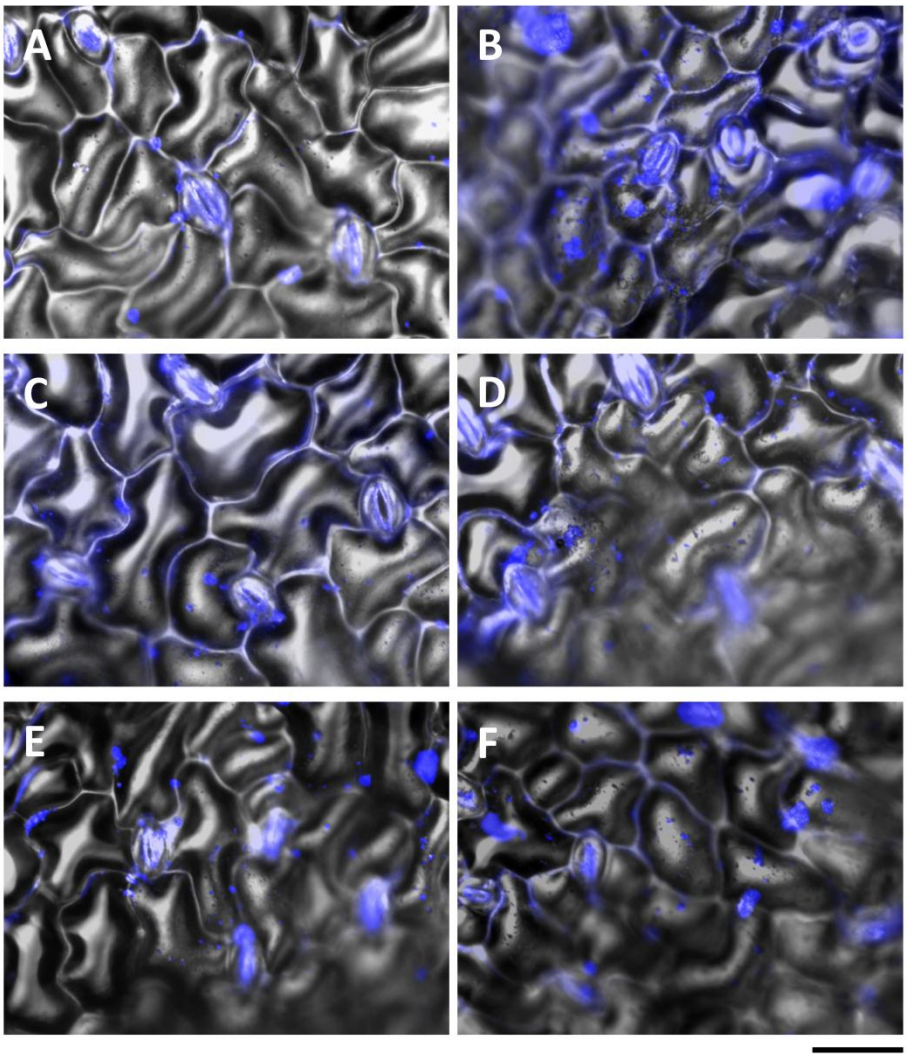
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 1

Lower surface



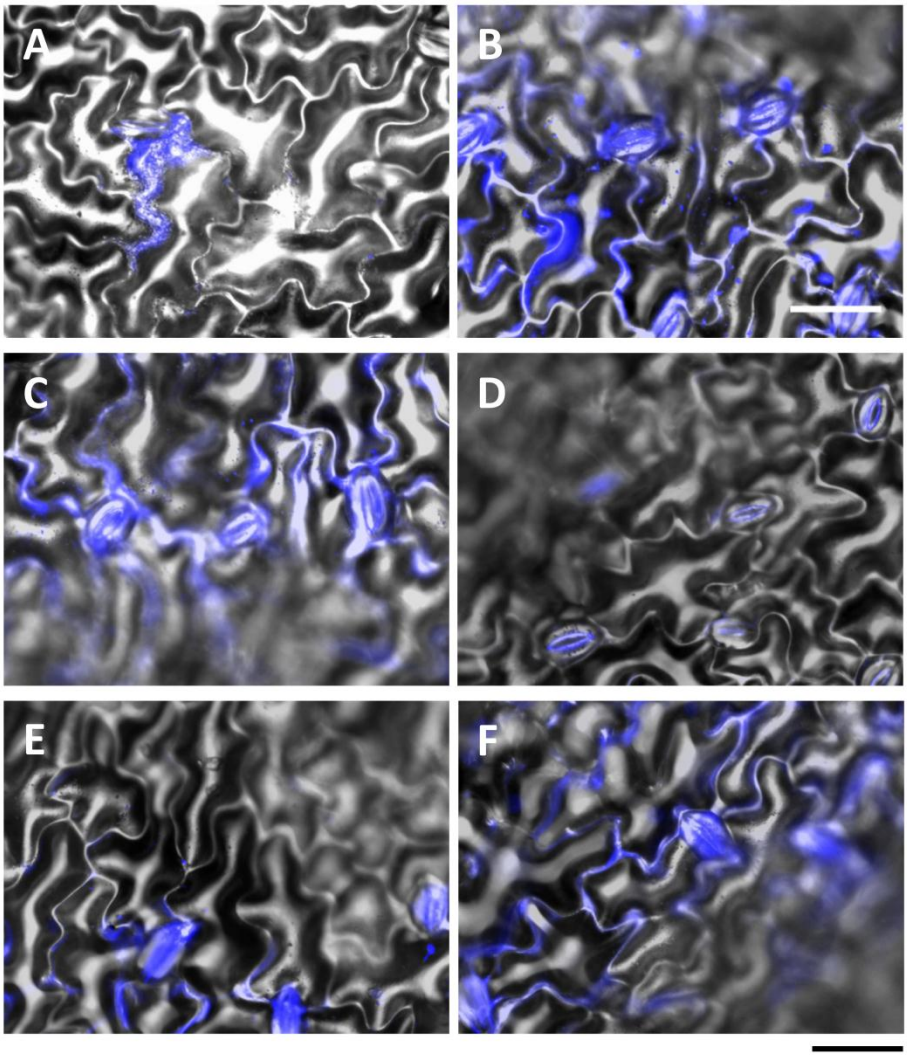
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 1

Upper surface



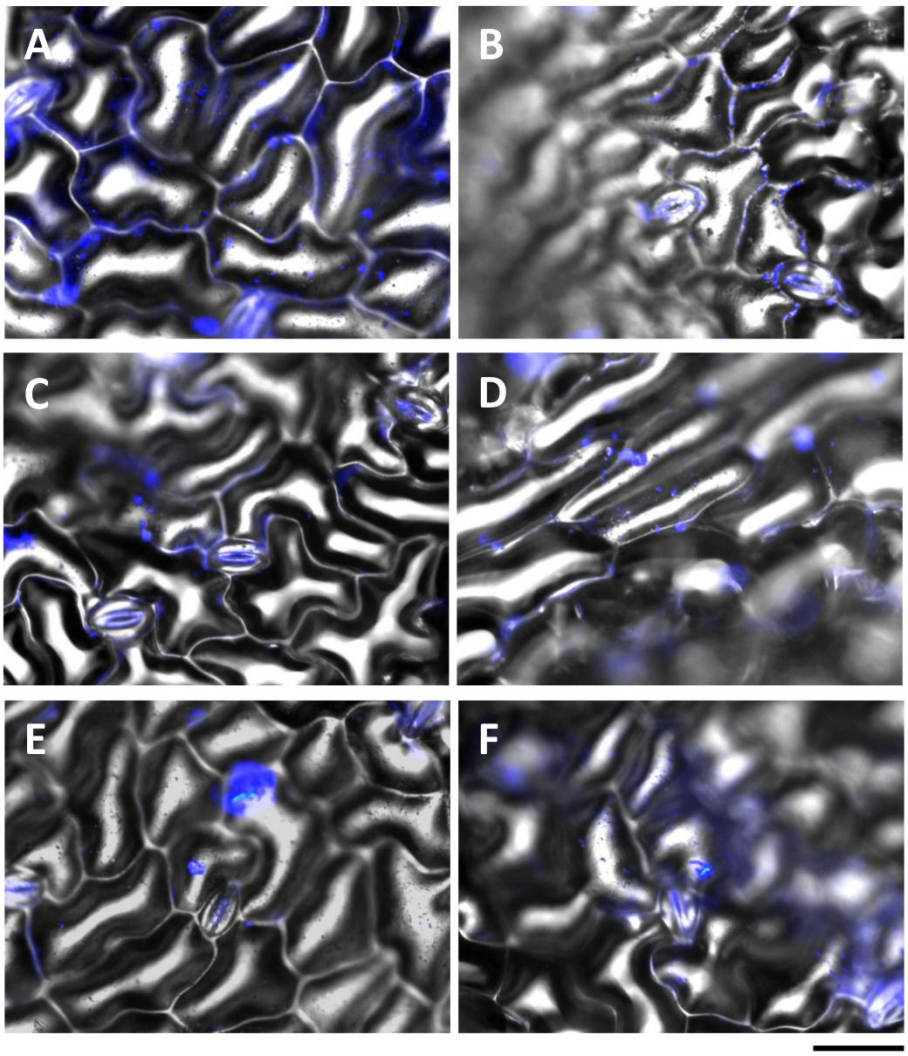
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 2

Lower surface



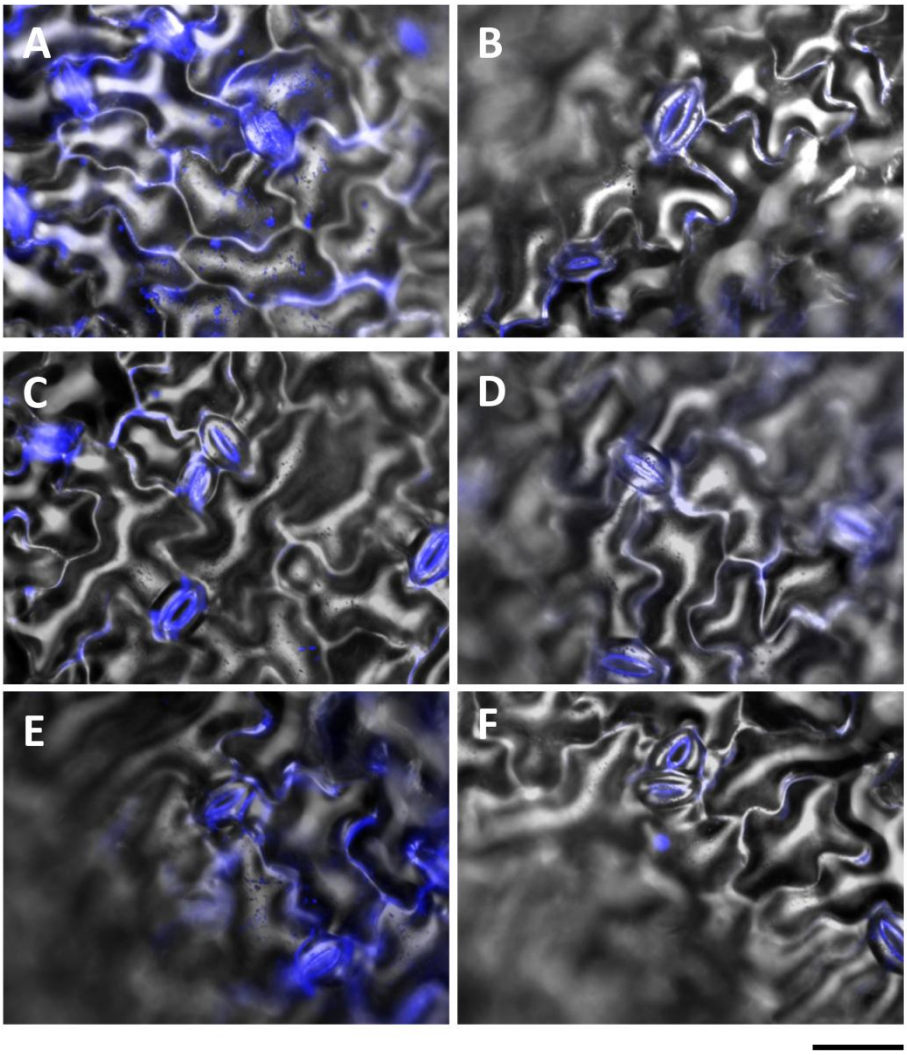
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 2

Upper surface



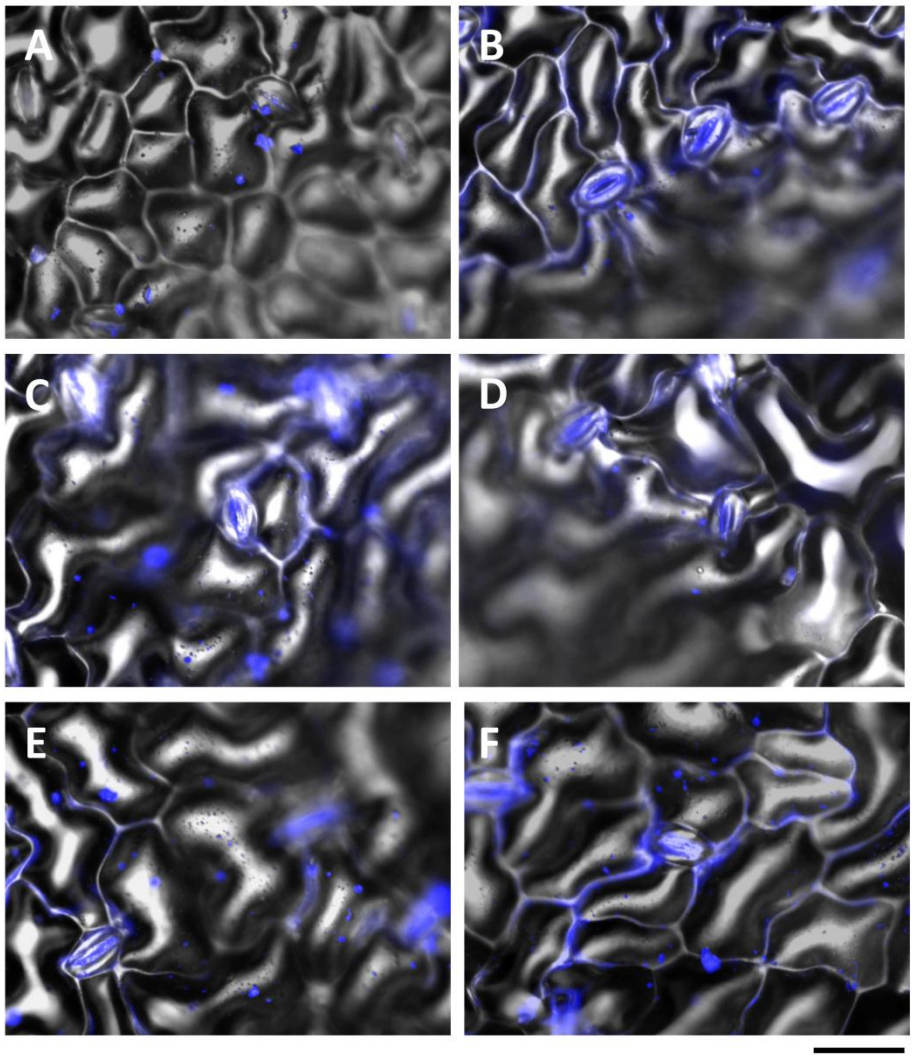
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 3

Lower surface



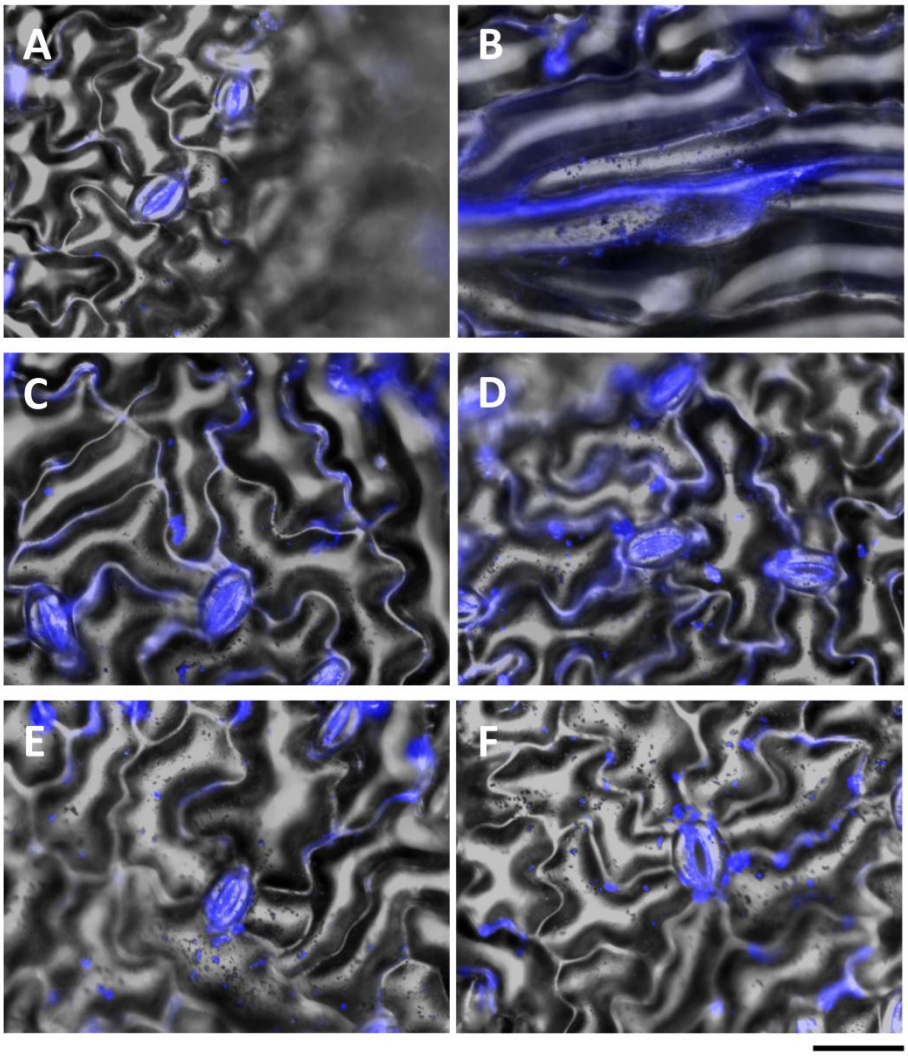
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 1 hour contact time on Day 3

Upper surface



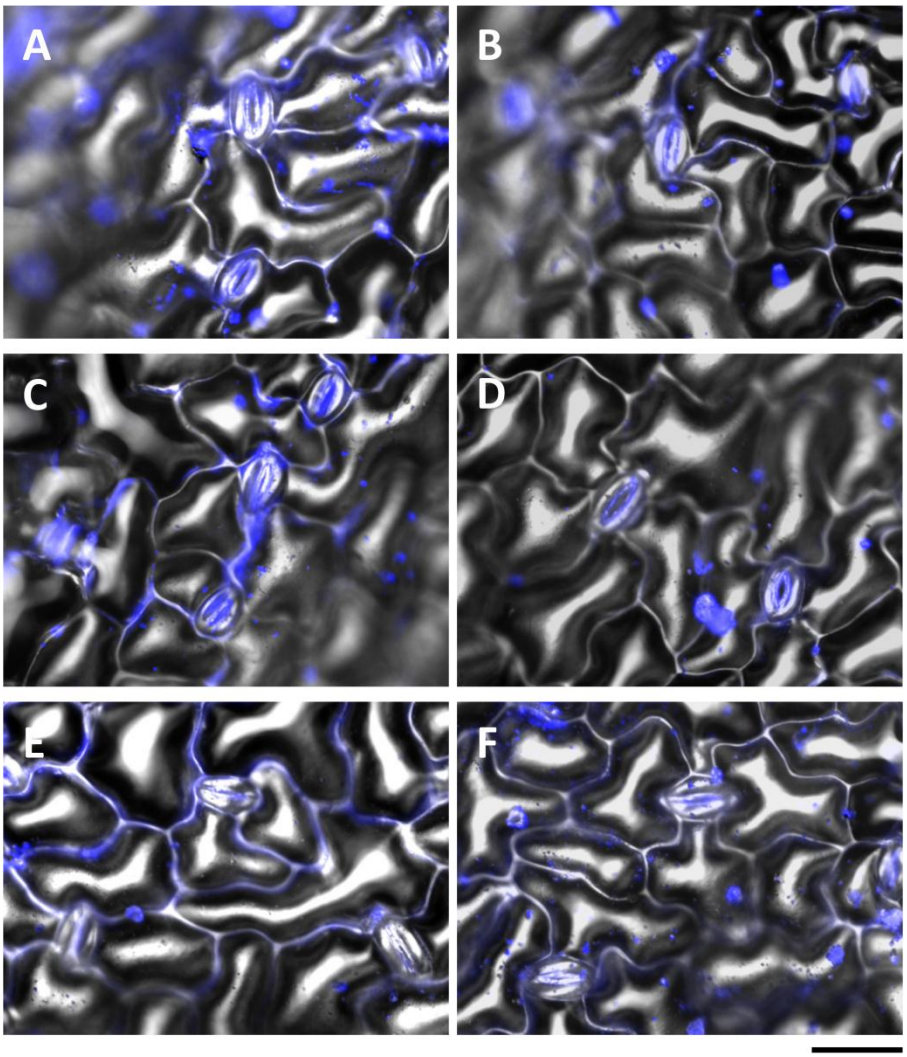
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 1

Lower surface



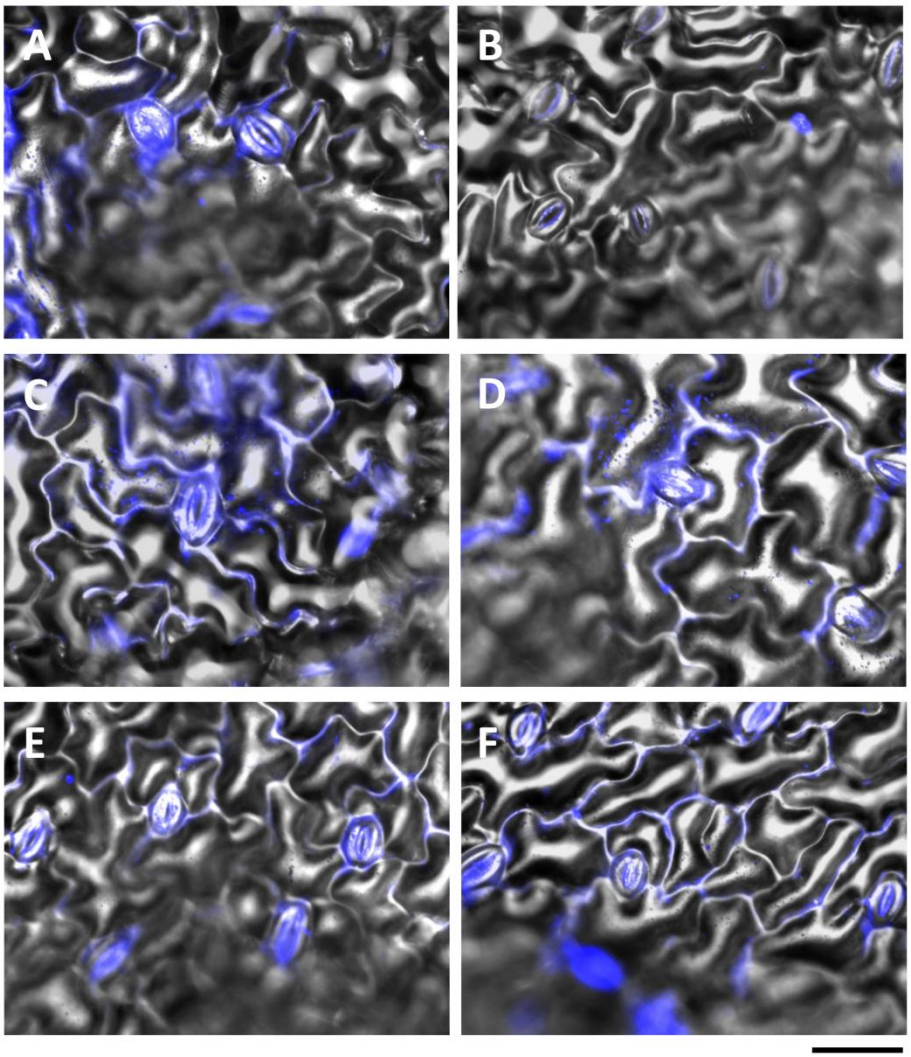
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 1

Upper surface



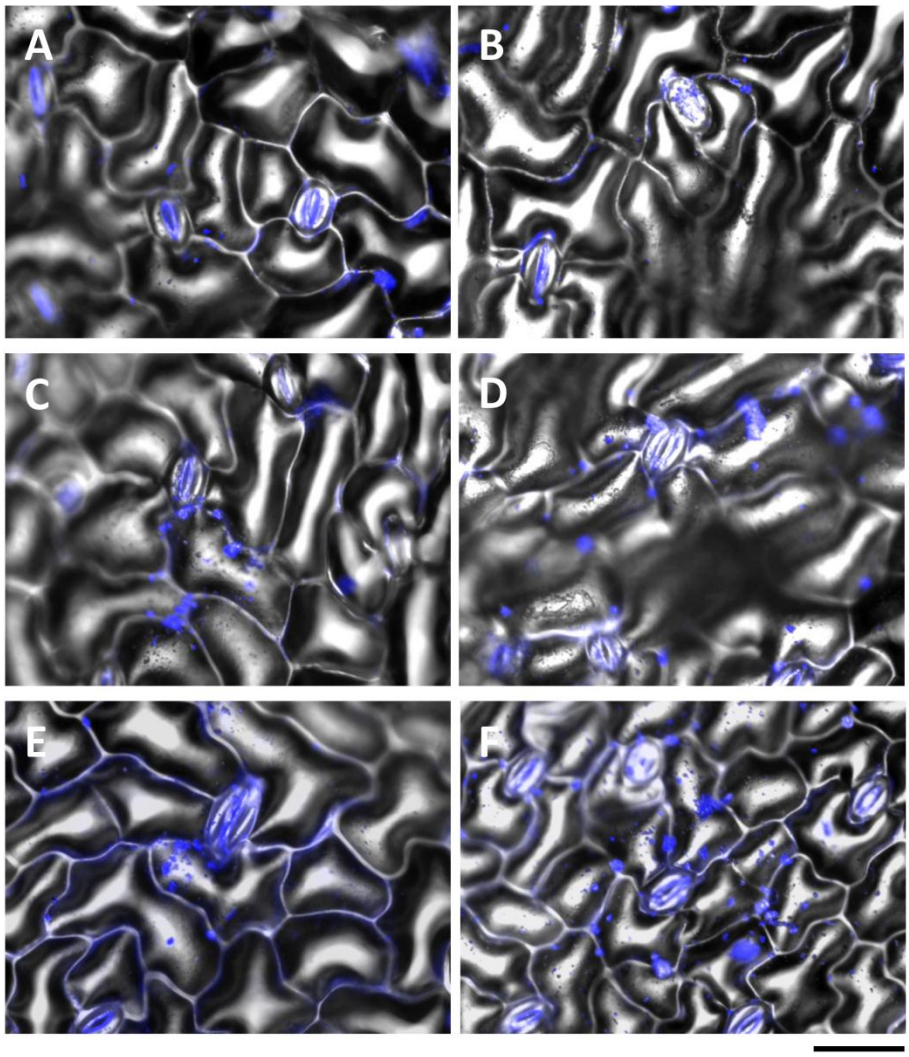
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 2

Lower surface



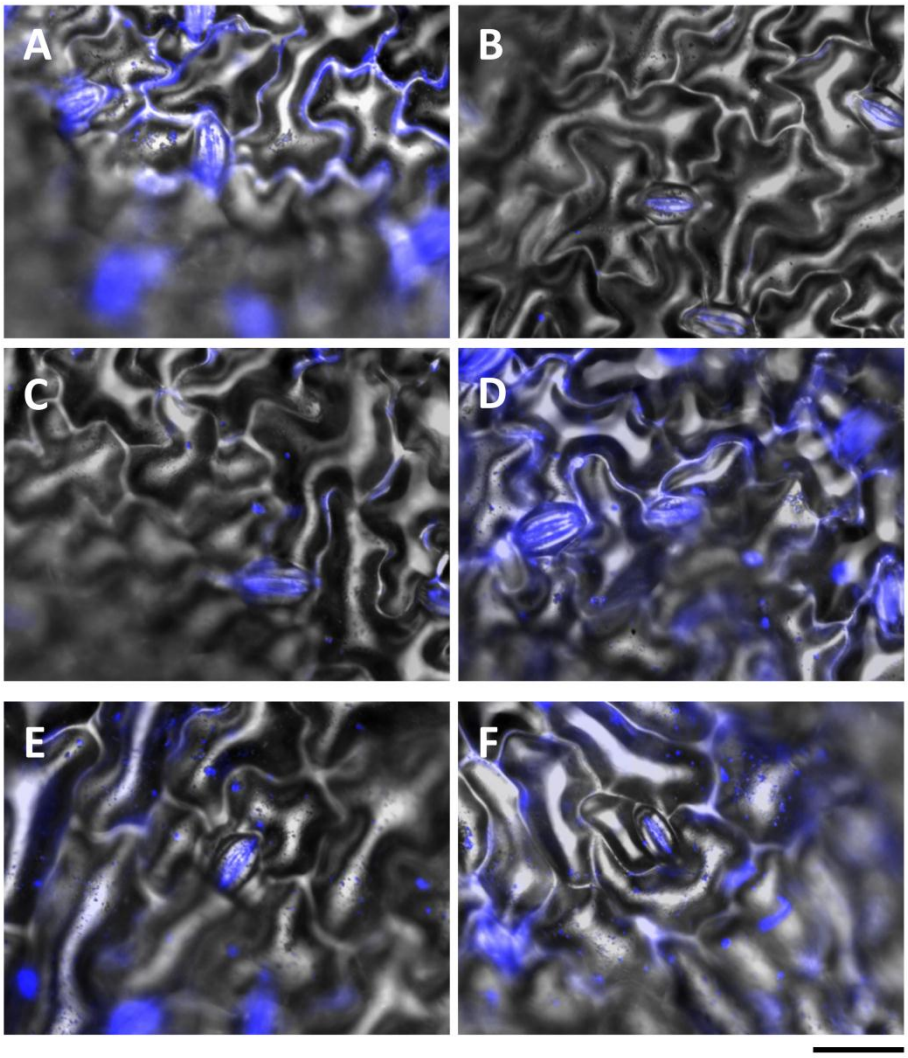
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 2

Upper surface



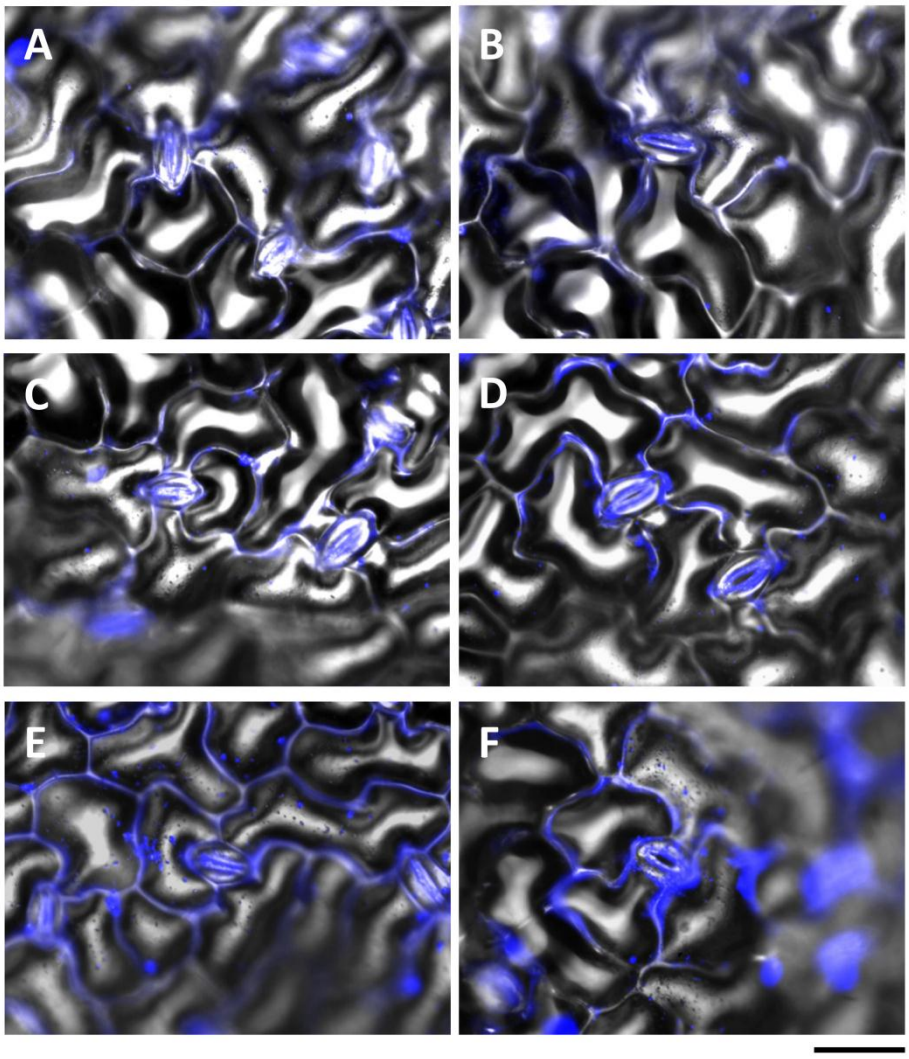
The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 3

Lower surface



The effect of nitric oxide treatment on indigenous bacterial biofilms at the spinach phylloplane after 24 hours contact time on Day 3

Upper surface



APPENDIX 3

Appendix 3

Proteomics tables

Key to tables		
Table description (sample description)	**Table number**	**Corresponding Figure number**
Proteins expressed only upon treatment with 500 nM SNP (proteins present in all 18 sample replicates for SNP treated samples, and absent from all 18 sample replicates for untreated Controls)	Appendix 3 Table A3.1	Chapter 5 Figure 40
Proteins up regulated in response to 500 nM SNP treatment (Proteins up-regulated in SNP treated samples by comparison to untreated Controls)	Appendix 3 Table A3.3	Chapter 5 Figure 41
Proteins absent from 500 nM SNP treated samples (not present in SNP treated samples by comparison to untreated Controls)	Appendix 3 Table A3.2	Chapter 5 Figure 42
Proteins down regulated in response to 500 nM SNP treatment (Proteins down-regulated in SNP treated samples by comparison to untreated Controls)	Appendix 3 Table A3.4	Chapter 5 Figure 43

Proteins involved in molecular signalling are highlighted in bold text.

Table A3.1 Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1W853	Pantothenate synthetase OS Salmonella typhimurium strain SL1344 GN panC PE 3 SV 1	Nucleotide binding; catalytic activity	Biosynthetic process
E1WDX1	Dihydroxy acid dehydratase OS Salmonella typhimurium strain SL1344 GN ilvD PE 3 SV 1	Binding; catalytic activity	Biosynthetic process
E1WAZ3	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN yjgF PE 4 SV 1	Hydrolase activity; catalytic activity	Biosynthetic process; biological process
E1WCR9	Pyridoxine kinase OS Salmonella typhimurium strain SL1344 GN pdxK PE 3 SV 1	Binding; nucleotide binding; kinase activity	Biosynthetic process; metabolic process
E1W8I6	Gamma glutamyl phosphate reductase OS Salmonella typhimurium strain SL1344 GN proA PE 3 SV 1	Nucleotide binding; catalytic activity	Biosynthetic process; metabolic process
E1WE45	Xaa Pro dipeptidase OS Salmonella typhimurium strain SL1344 GN pepQ PE 3 SV 1	Binding; hydrolase activity; peptidase activity	Biological process; protein metabolic process; catabolic process
E1WHJ8	Proline aminopeptidase II OS Salmonella typhimurium strain SL1344 GN pepP PE 4 SV 1	Binding; peptidase activity	Biological process; protein metabolic process; catabolic process
E1W8A4	Acetyl coenzyme A carboxylase carboxyl transferase subunit alpha OS Salmonella typhimurium strain S	Catalytic activity; nucleotide binding; transferase activity; protein binding	Biosynthetic process; lipid metabolic process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WIG2	Biotin carboxyl carrier protein OS Salmonella typhimurium strain SL1344 GN accB PE 4 SV 1	Catalytic activity	Biosynthetic process; lipid metabolic process
E1WH24	CDP glucose 4 6 dehydratase OS Salmonella typhimurium strain SL1344 GN rfbG PE 4 SV 1	Binding; nucleotide binding; catalytic activity	Biosynthetic process; carbohydrate metabolic process; lipid metabolic process
E1WFZ2	Glucose 1 phosphate uridylyltransferase OS Salmonella typhimurium strain SL1344 GN galU PE 4 SV 1	Binding; transferase activity	Biosynthetic process; carbohydrate metabolic process; lipid metabolic process; catabolic process; nucleobase-containing compound metabolic process; symbiosis
E1WBD7	Glutaredoxin 1 OS Salmonella typhimurium strain SL1344 GN grxA PE 4 SV 1	Electron carrier activity; catalytic activity	Biosynthetic process; nucleobase-containing compound metabolic process; cellular homeostasis; regulation of biological process; generation of precursor metabolites and energy
E1W9J1	Negative modulator of initiation of replication OS Salmonella typhimurium strain SL1344 GN seqA PE	DNA binding	Biosynthetic process; regulation of biological process; DNA metabolic process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WFH7	NH 3 dependent NAD synthetase OS Salmonella typhimurium strain SL1344 GN nadE PE 3 SV 1	Catalytic activity; nucleotide binding	Biosynthetic process; nucleobase-containing compound metabolic process; secondary metabolic process
E1WCX1	Phosphoribosylaminoimidazole succinocarboxamide synthase OS Salmonella typhimurium strain SL1344 G	Catalytic activity; nucleotide binding	Nucleobase-containing compound metabolic process
E1WJD8	Hypothetical sigma 54 modulation protein OS Salmonella typhimurium strain SL1344 GN yfiA PE 4 SV	-	Primary metabolic process
E1WBJ3	Hypothetical oxidoreductase OS Salmonella typhimurium strain SL1344 GN ydfG PE 3 SV 1	Nucleotide binding	Metabolic process
E1WB53	Hypothetical aldehyde dehydrogenase OS Salmonella typhimurium strain SL1344 GN SL1344 4450 PE 3 SV	Catalytic activity	Metabolic process
E1WFU5	Superoxide dismutase OS Salmonella typhimurium strain SL1344 GN sodB PE 3 SV 1	Binding; catabolic activity	Metabolic process
E1WHU5	Possible oxidoreductase OS Salmonella typhimurium strain SL1344 GN yghA PE 4 SV 1	Binding; catalytic activity; nucleotide binding	Metabolic process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WET5	Quinone oxidoreductase OS Salmonella typhimurium strain SL1344 GN qor PE 4 SV 1	Nucleotide binding; binding; catalytic activity	Metabolic process
E1WJ22	Extragenic suppressor protein SuhB OS Salmonella typhimurium strain SL1344 GN suhB PE 4 SV 1	Hydrolase activity; binding; protein binding	Lipid metabolic process
E1W9R4	UDP glucose 4 epimerase OS Salmonella typhimurium strain SL1344 GN galE PE 3 SV 1	Binding; nucleotide binding; catalytic activity	Metabolic process; carbohydrate metabolic process
E1W9H7	N acetylglucosamine 6 phosphate deacetylase OS Salmonella typhimurium strain SL1344 GN nagA PE 3 S	Hydrolase activity	Carbohydrate metabolic process
E1WI76	Phosphoglucosamine mutase OS Salmonella typhimurium strain SL1344 GN glmM PE 3 SV 1	Catalytic activity; binding	Carbohydrate metabolic process; biosynthetic process; nucleobase-containing compound metabolic process; cellular protein modification process
E1WCG3	NADH quinone oxidoreductase OS Salmonella typhimurium strain SL1344 GN nuoG PE 3 SV 1	Binding; election carrier activity; catalytic activity	Generation of precursor metabolites and energy
E1W9M9	2 oxoglutarate dehydrogenase E1 component OS Salmonella typhimurium strain SL1344 GN sucA PE 4 SV	Catalytic activity; binding	Generation of precursor metabolites and energy; catabolic process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WDU4	ATP synthase subunit delta OS Salmonella typhimurium strain SL1344 GN atpH PE 3 SV 1	Transporter activity; hydrolase activity	Generation of precursor metabolites and energy; nucleobase-containing compound metabolic process; ion transport; biosynthetic process
E1WHM6	Biosynthetic arginine decarboxylase OS Salmonella typhimurium strain SL1344 GN speA PE 3 SV 1	Binding; catalytic activity	Catabolic process; biosynthetic process
E1WCX5	Bacterioferritin comigratory protein OS Salmonella typhimurium strain SL1344 GN bcp PE 4 SV 1	Catalytic activity; antioxidant activity	Metabolic process; response to stress
E1W897	Chaperone protein skp OS Salmonella typhimurium strain SL1344 GN ompH PE 3 SV 1	DNA binding; binding; protein binding	Protein metabolic process; metabolic process; regulation of biological process; cellular component organisation
E1WJG8	Protein GrpE OS Salmonella typhimurium strain SL1344 GN grpE PE 3 SV 1	Protein binding; nucleotide binding; enzyme regulator activity	Protein metabolic process; response to stress
E1WF19	10 kDa chaperonin OS Salmonella typhimurium strain SL1344 GN groES PE 3 SV 1	Nucleotide binding	Protein metabolic process; response to stress; biological process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WBY3	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN ynaF PE 4 SV 1	Nucleotide binding	Response to stress
E1WEU6	Single stranded DNA binding protein OS Salmonella typhimurium strain SL1344 GN ssb PE 4 SV 1	DNA binding	Response to stress; DNA metabolic process; biosynthetic process
E1WD78	Cold shock protein OS Salmonella typhimurium strain SL1344 GN cspA PE 3 SV 1	DNA binding	Response to stress; regulation of biological process
E1WE53	Thiol disulfide interchange protein DsbA OS Salmonella typhimurium strain SL1344 GN dsbA PE 3 SV 1	Catalytic activity	Cellular homeostasis; regulation of biological process
E1WG74	Cold shock like protein CspC OS Salmonella typhimurium strain SL1344 GN cspC PE 3 SV 1	DNA binding	Regulation of biological process
E1W9I7	Ferric uptake regulation protein OS Salmonella typhimurium strain SL1344 GN fur PE 4 SV 1	Binding; sequence-specific DNA binding transcription factor activity; DNA binding	Regulation of biological process
E1WCJ4	Histidine binding periplasmic protein OS Salmonella typhimurium strain SL1344 GN hisJ PE 3 SV 1	Hydrolase activity; transporter activity; nucleotide binding; catalytic activity	Transport

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WG83	ProP effector OS Salmonella typhimurium strain SL1344 GN proP PE 3 SV 1	Transporter activity	Transport
E1W9R9	**Molybdate binding periplasmic protein OS Salmonella typhimurium strain SL1344 GN modA PE 4 SV 1**	**Hydrolase activity; transporter activity**	**Ion transport**
E1W6Z3	**Outer membrane lipoprotein carrier protein OS Salmonella typhimurium strain SL1344 GN lolA PE 3 SV**	**Transporter activity**	**Protein transport**
E1WGG2	**Chemotaxis protein CheA OS Salmonella typhimurium strain SL1344 GN cheA PE 4 SV 1**	**Protein kinase activity; signal transducer activity; nucleotide binding**	**Behaviour; response to external stimulus; signal transduction; cellular protein modification process**
E1WHZ8	RNA polymerase sigma factor OS Salmonella typhimurium strain SL1344 GN rpoD PE 3 SV 1	Sequence-specific DNA binding transcription factor activity; DNA binding; transcription regulator activity	Cellular component organisation; regulation of biological process
E1WI82	Transcription elongation factor GreA 1 OS Salmonella typhimurium strain SL1344 GN greA PE 3 SV 1	Translation factor activity, nucleic acid binding; DNA binding	Regulation of biological process; translation
E1W943	Peptidyl prolyl cis trans isomerase OS Salmonella typhimurium strain SL1344 GN ppiB PE 3 SV 1	Catalytic activity	Protein metabolic process; cellular protein modification process

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WIR4	Shikimate kinase 1 OS Salmonella typhimurium strain SL1344 GN aroK PE 3 SV 1	Kinase activity; nucleotide binding; binding	Carbohydrate metabolic process; metabolic process; biosynthetic process
E1WDD1	Glutaredoxin 3 OS Salmonella typhimurium strain SL1344 GN grxC PE 4 SV 1	Electron carrier activity; catalytic activity	Cellular homeostasis; regulation of biological process
E1W941	N5 carboxyaminoimidazole ribonucleotide mutase OS Salmonella typhimurium strain SL1344 GN purE PE	Protein binding; catalytic activity	Nucleobase-containing compound metabolic process, biosynthetic process
E1WFK4	Phenylalanine tRNA ligase alpha subunit OS Salmonella typhimurium strain SL1344 GN pheS PE 3 SV 1	Catalytic activity; nucleotide binding; binding; RNA binding	Translation; nucleobase-containing compound metabolic process
E1WB91	Peptide chain release factor 3 OS Salmonella typhimurium strain SL1344 GN prfC PE 3 SV 1	Translation factor activity, nucleic acid binding; hydrolase activity; nucleotide binding	Translation; nucleobase-containing compound metabolic process; catabolic process; cellular component organisation; regulation of biological process
E1WF54	Ribonuclease R OS Salmonella typhimurium strain SL1344 GN rnr PE 3 SV 1	RNA binding; nuclease activity	Nucleobase-containing compound metabolic process
E1WIJ5	30S ribosomal subunit protein S13 OS Salmonella typhimurium strain SL1344 GN rpsM PE 3 SV 1	Structural molecule activity	Biological process; translation

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WDF7	50S ribosomal protein L28 OS Salmonella typhimurium strain SL1344 GN rpmB PE 3 SV 1	Structural molecule activity	Translation
E1WIK9	50S ribosomal protein L29 OS Salmonella typhimurium strain SL1344 GN rpmC PE 3 SV 1	Structural molecule activity	Translation
E1WAS8	30S ribosomal protein S18 OS Salmonella typhimurium strain SL1344 GN rpsR PE 3 SV 1	Structural molecule activity; RNA binding	Translation
E1WI67	30S ribosomal protein S15 OS Salmonella typhimurium strain SL1344 GN rpsO PE 3 SV 1	Structural molecule activity; RNA binding	Translation
E1WIL1	30S ribosomal protein S3 OS Salmonella typhimurium strain SL1344 GN rpsC PE 3 SV 1	Structural molecule activity; RNA binding	Translation
E1WIK8	30S ribosomal protein S17 OS Salmonella typhimurium strain SL1344 GN rpsQ PE 3 SV 1	Structural molecule activity; RNA binding	Translation
E1WIL7	50S ribosomal protein L3 OS Salmonella typhimurium strain SL1344 GN rplC PE 3 SV 1	Structural molecule activity; RNA binding	Translation

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WIL5	50S ribosomal protein L23 OS Salmonella typhimurium strain SL1344 GN rplW PE 3 SV 1	RNA binding; structural molecule activity; nucleotide binding	Translation
E1WI87	50S ribosomal protein L21 OS Salmonella typhimurium strain SL1344 GN rplU PE 3 SV 1	Structural molecule activity; catalytic activity; RNA binding	Translation
E1WEF1	50S ribosomal protein L31 OS Salmonella typhimurium strain SL1344 GN rpmE PE 3 SV 1	Binding; structural molecule activity; RNA binding	Translation
E1WAS9	50S ribosomal protein L9 OS Salmonella typhimurium strain SL1344 GN rplI PE 3 SV 1	Structural molecule activity; transferase activity; RNA binding	Metabolic process; translation
E1WJG3	30S ribosomal protein S16 OS Salmonella typhimurium strain SL1344 GN rps16 PE 3 SV 1	Structural molecule activity; nuclease activity; DNA binding	Translation; organelle organisation
E1W858	Dosage dependent dnaK suppressor protein OS Salmonella typhimurium strain SL1344 GN dksA PE 4 SV 1	DNA binding; binding; protein binding	Cellular component organisation
E1WCR2	Cell division protein ZipA homolog OS Salmonella typhimurium strain SL1344 GN zipA PE 3 SV 1	-	Cell cycle; cellular component organisation

Table A3.1 (cont.) Proteins expressed upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WHJ4	Glycine cleavage system H protein OS Salmonella typhimurium strain SL1344 GN gcvH PE 3 SV 1	-	Catabolic process
E1WI81	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN yhbY PE 4 SV 1	RNA binding	-
E1WCH5	UPF0304 protein YfbU OS Salmonella typhimurium strain SL1344 GN yfbU PE 3 SV 1	-	-
E1WE51	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN yihD PE 4 SV 1	-	-
E1WDS6	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN yieF PE 4 SV 1	-	-

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Protein identities are given for all proteins that were present in all 18 replicates for SNP treated samples and absent from all 18 Control replicates.

Table A3.2 Proteins up-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1W6Z5	Serine tRNA ligase OS Salmonella typhimurium strain SL1344 GN serS PE 3 SV 1	Catalytic activity; nucleotide binding; binding	Biosynthetic process; translation; nucleobase-containing compound metabolic process	1.68
E1WIS7	Phosphoenolpyruvate carboxykinase ATP OS Salmonella typhimurium strain SL1344 GN pckA PE 3 SV 1	Kinase activity; catalytic activity; nucleotide binding	Biosynthetic process; carbohydrate metabolic process; metabolic process	1.87
E1WCH7	Acetate kinase OS Salmonella typhimurium strain SL1344 GN ackA PE 3 SV 1	Kinase activity; nucleotide binding	Biosynthetic process; metabolic process; catabolic process	1.66
E1WDD9	ADP L glycero D manno heptose 6 epimerase OS Salmonella typhimurium strain SL1344 GN rfaD PE 3 SV	Nucleotide binding; protein binding; catalytic activity	Biosynthetic process; carbohydrate metabolic process; lipid metabolic process; response to stress	1.73
E1W777	Superoxide dismutase Cu Zn OS Salmonella typhimurium strain SL1344 GN sodCI PE 3 SV 1	Binding; catalytic activity	Metabolic process	1.93
E1WIG3	Biotin carboxylase OS Salmonella typhimurium strain SL1344 GN accC PE 4 SV 1	Catalytic activity; binding; nucleotide activity	Metabolic process	1.72

Table A3.2 (cont.) Proteins up-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WFY9	Alcohol dehydrogenase OS Salmonella typhimurium strain SL1344 GN adh PE 4 SV 1	Catalytic activity; binding	Metabolic process	1.99
E1WHJ3	Glycine dehydrogenase decarboxylating OS Salmonella typhimurium strain SL1344 GN gcvP PE 3 SV 1	Binding; catalytic activity	Metabolic process	1.68
E1WG24	Hydrogenase 1 large chain NifE hydrogenase OS Salmonella typhimurium strain SL1344 GN SL1344 171	Catalytic activity; binding	Metabolic process	1.53
E1W9J2	Phosphoglucomutase OS Salmonella typhimurium strain SL1344 GN pgm PE 3 SV 1	Catalytic activity; binding	Carbohydrate metabolic process	1.98
E1WCR6	Phosphoenolpyruvate protein phosphotransferase OS Salmonella typhimurium strain SL1344 GN ptsI PE	Binding; kinase activity; transporter activity; transferase activity	Metabolic process; transport; signal transduction	1.53
E1WF33	Fumarate reductase flavoprotein subunit OS Salmonella typhimurium strain SL1344 GN frdA PE 4 SV 1	Nucleotide binding; catalytic activity	Generation of precursor metabolites and energy	1.89

Table A3.2 (cont.) Proteins up-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WJD4	**ClpB protein Heat shock protein f84 1 OS Salmonella typhimurium strain SL1344 GN clpB PE 3 SV 1**	**Peptidase activity; nucleotide binding; hydrolase activity**	**Protein metabolic process; catabolic process; response to stress; response to abiotic stimulus**	**1.53**
E1W7A2	**Outer membrane protein A OS Salmonella typhimurium strain SL1344 GN ompA PE 3 SV 1**	**Transporter activity; structural molecule activity**	**Biological process; ion transport**	**1.85**
E1WF52	Adenylosuccinate synthetase OS Salmonella typhimurium strain SL1344 GN purA PE 3 SV 1	Catalytic activity; binding; protein binding; nucleotide binding	Biosynthetic process; nucleobase-containing compound metabolic process	1.58
E1WGF7	**Chemotaxis protein CheY OS Salmonella typhimurium strain SL1344 GN cheY PE 4 SV 1**	**Transcription regulator activity; receptor activity; transferase activity; binding**	**Biological process; signal transduction; cellular protein modification process; regulation of biological process; behaviour; response to external stimulus**	**1.60**
E1W9W7	DNA protection during starvation protein OS Salmonella typhimurium strain SL1344 GN dps PE 3 SV 1	DNA binding; binding; catalytic activity	Cellular homeostasis; response to stress; organelle organisation; metabolic process	1.83

Table A3.2 (cont.) Proteins up-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WFW3	Tyrosine tRNA ligase OS Salmonella typhimurium strain SL1344 GN tyrS PE 3 SV 1	Catalytic activity; RNA binding; nucleotide binding	Translation; nucleobase-containing compound metabolic process	1.82
E1WB97	Deoxyribose phosphate aldolase OS Salmonella typhimurium strain SL1344 GN deoC PE 3 SV 1	Catalytic activity	Nucleobase-containing compound metabolic process; catabolic process; carbohydrate metabolic process	1.91
E1WIJ3	30S ribosomal protein S4 OS Salmonella typhimurium strain SL1344 GN rpsD PE 3 SV 1	RNA binding; structural molecule activity; translation regulator activity; nucleic acid binding; protein binding; hydrolase activity	Biological process; regulation of biological process; translation; cellular component organisation	1.57
E1WEJ4	50S ribosomal protein L1 OS Salmonella typhimurium strain SL1344 GN rplA PE 3 SV 1	Structural molecule activity; hydrolase activity; RNA binding	Regulation of biological process; translation	1.54
E1WFK1	**Translation initiation factor IF 3 OS Salmonella typhimurium strain SL1344 GN infC PE 3 SV 2**	**Protein binding; translation factor activity, nucleic acid binding**	**Translation; cellular component organisation; response to stress; response to abiotic stimulus**	**1.56**
E1WIJ9	50S ribosomal protein L30 OS Salmonella typhimurium strain SL1344 GN rpmD PE 3 SV 1	Structural molecule activity	Translation	1.86

Table A3.2 (cont.) Proteins up-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1W889	Elongation factor Ts OS Salmonella typhimurium strain SL1344 GN tsf PE 3 SV 1	Translation factor activity, nucleic acid binding	Translation	1.80
E1WBX8	D lactate dehydrogenase OS Salmonella typhimurium strain SL1344 GN ldhA PE 3 SV 1	Catalytic activity; nucleotide binding	Translation	1.74
E1WIJ1	50S ribosomal protein L17 OS Salmonella typhimurium strain SL1344 GN rplQ PE 3 SV 1	Structural molecule activity	Translation	1.58
E1WJ35	Lysine decarboxylase OS Salmonella typhimurium strain SL1344 GN cadA PE 4 SV 2	Binding; catalytic activity	-	1.94
E1WBP3	Hypothetical secreted protein OS Salmonella typhimurium strain SL1344 GN hdeB PE 4 SV 1	-	-	1.80

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Protein identities are given for all proteins that were up-regulated in all 18 replicates for SNP treated samples by comparison to Controls. Data are fold changes calculated from the mean of 18 replicates.

Table A3.3 Proteins lost upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1WF96	Peptidase T OS Salmonella typhimurium strain SL1344 GN pepT PE 3 SV 1	Metallopeptidase activity; Zinc ion binding; Tripeptide aminopeptidase activity	Peptide metabolic process; Proteolysis
E1WGC4	KHG KDPG aldolase OS Salmonella typhimurium strain SL1344 GN eda PE 4 SV 1	2-dehydro-3-deoxyphosphogluconate aldolase activity; oxaloacetate decarboxylase activity; 3-deoxy-8-phosphooctulonate synthase activity; 4-hydroxy-2-oxoglutarate aldolase activity	Metabolic process
E1WIC5	Stringent starvation protein A OS Salmonella typhimurium strain SL1344 GN sspA PE 3 SV 1	Actin binding; glutathione transferase activity	Pathogenesis
E1WED3	Hypothetical aldolase OS Salmonella typhimurium strain SL1344 GN yneB PE 4 SV 1	Lyase activity	Metabolic process
E1W9N1	Succinyl CoA ligase ADP forming subunit beta OS Salmonella typhimurium strain SL1344 GN sucC PE	Manganese ion binding; protein binding; ATP binding; magnesium ion binding; succinate-CoA ligase (ADP forming) activity	Tracarboxylic acid cycle

Table A3.3 (cont.) Proteins lost upon treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)
E1W879	5 methylthioadenosine S adenosylhomocysteine nucleosidase OS Salmonella typhimurium strain SL1344	Methylthioadenosine nucleosidase activity; adenosylohomocysteine nucleosidase activity	L-methionine biosynthetic process from S-adenosylmethionine; methionine salvage from methylthioadenosine; nucleosidase catabolic process
E1W9N2	Succinyl CoA ligase ADP forming subunit alpha OS Salmonella typhimurium strain SL1344 GN sucD PE	ATP binding; succinate-CoA ligase (ADP forming) activity; cofactor binding; ATP citrate synthase activity	Metabolic process

S. Typhimurium NCTC 12023 was cultured in the presence of 500 nM SNP; Control cultures were grown in the absence of SNP. Cell lysis was performed by homogenisation in 0.1 M TEAB for protein extraction, before preparation for analysis using label free mass spectrometry by the Centre for Proteomics Research, University of Southampton, UK. Protein identities are given for all proteins that were absent from all 18 replicates for SNP treated samples and present in all 18 Control replicates

Table A3.4 Proteins down-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1W8S7	6 7 dimethyl 8 ribityllumazine synthase OS Salmonella typhimurium strain SL1344 GN ribH PE 3 SV 1	Catalytic activity; transferase activity	Biosynthetic process	-1.95
E1W729	Aspartate aminotransferase OS Salmonella typhimurium strain SL1344 GN aspC PE 3 SV 1	Binding; transferase activity	Biosynthetic process	-1.51
E1W7M3	Malonyl CoA acyl carrier protein transacylase OS Salmonella typhimurium strain SL1344 GN fabD PE 3	Transferase activity	Biosynthetic process; lipid metabolic process	-1.83
E1WF66	3 oxoacyl acyl carrier protein synthase 2 OS Salmonella typhimurium strain SL1344 GN fabF PE 3 S	Transferase activity	Biosynthetic process; lipid metabolic process	-1.62
E1WDT7	Glucosamine fructose 6 phosphate aminotransferase isomerizing OS Salmonella typhimurium strain S	Transferase activity; carbohydrate binding	Biosynthetic process; carbohydrate metabolic process	-1.79
E1WGF6	**Protein phosphatase CheZ OS Salmonella typhimurium strain SL1344 GN cheZ PE 3 SV 1**	**Phosphoprotein phosphatase activity**	**Biological process; metabolic process; behaviour; response to external stimulus; regulation of biological process**	**-1.65**
E1WFZ1	H NS DNA binding protein Histone like protein Hlp II OS Salmonella typhimurium strain SL1344 GN	DNA binding	Regulation of biological process	-1.64

Table A3.4 (cont.) Proteins down-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WA48	DNA binding protein StpA OS Salmonella typhimurium strain SL1344 GN stpA PE 4 SV 1	Phosphoprotein phosphatase activity; protein binding; DNA binding	Regulation of biological process	-1.73
E1WD27	Glutathione reductase OS Salmonella typhimurium strain SL1344 GN gor PE 3 SV 1	Nucleotide binding; catalytic activity	Cellular homeostasis; regulation of biological process; metabolic process	-1.78
E1WGH4	Ferritin OS Salmonella typhimurium strain SL1344 GN ftnA PE 4 SV 1	Binding; catalytic activity	Cellular homeostasis; ion transport; metabolic process	-1.92
E1WEJ3	**50S ribosomal protein L11 OS Salmonella typhimurium strain SL1344 GN rplK PE 3 SV 1**	**RNA binding; structural molecule activity; protein binding**	**Response to external stimulus; cell communication; response to stress; cellular component organisation; translation**	**-1.98**
E1WEL4	Histone like DNA binding protein HU alpha NS2 HU 2 OS Salmonella typhimurium strain SL1344 GN	DNA binding; protein binding	Organelle organisation	-1.60
E1W9B5	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN ybdQ PE 4 SV 1		Response to stress	-1.63
E1W712	30S ribosomal protein S1 OS Salmonella typhimurium strain SL1344 GN rpsA PE 3 SV 1	RNA binding; structural molecule activity; catalytic activity	Metabolic process	-1.68

Table A3.4 (cont.) Proteins down-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WCR7	**Pts system glucose specific IIA component OS Salmonella typhimurium strain SL1344 GN crr PE 4 SV**	**Binding; kinase activity; transporter activity; transferase activity**	**Metabolic process; transport; signal transduction**	**-1.93**
E1WFF5	Uncharacterized protein OS Salmonella typhimurium strain SL1344 GN yeaD PE 4 SV 1	Carbohydrate binding; catalytic activity	Carbohydrate metabolic process	-1.76
E1WGC8	Pyruvate kinase OS Salmonella typhimurium strain SL1344 GN pykA PE 3 SV 1	Kinase activity; binding; nucleotide binding	Metabolic process; carbohydrate metabolic process; generation of precursor metabolites and energy; catabolic process	-1.61
E1WG17	Ribose phosphate pyrophosphokinase OS Salmonella typhimurium strain SL1344 GN prs PE 3 SV 1	Kinase activity; transferase activity; nucleotide binding; binding	Metabolic process; biosynthetic process; carbohydrate metabolic process; nucleobase-containing compound metabolic process	-1.76
E1WFP4	Pyruvate kinase OS Salmonella typhimurium strain SL1344 GN pykF PE 3 SV 1	Kinase activity; binding; nucleotide binding	Metabolic process; carbohydrate metabolic process; generation of precursor metabolites and energy; catabolic process	-1.68
E1WEB7	6 phosphofructokinase OS Salmonella typhimurium strain SL1344 GN pfkA PE 3 SV 1	Binding; kinase activity; nucleotide binding	Carbohydrate metabolic process; metabolic process; generation of precursor metabolites and energy; catabolic process	-1.57

Table A3.4 (cont.) Proteins down-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WH14	6 phosphogluconate dehydrogenase decarboxylating OS Salmonella typhimurium strain SL1344 GN gnd P	Nucleotide binding; catalytic activity	Carbohydrate metabolic process; nucleobase-containing compound metabolic process; catabolic process; secondary metabolic process	-1.65
E1WDU3	ATP synthase subunit alpha OS Salmonella typhimurium strain SL1344 GN atpA PE 3 SV 1	Transporter activity; nucleotide binding; protein binding; hydrolase activity	Generation of precursor metabolites and energy; nucleobase-containing compound metabolic process; ion transport; biosynthetic process	-1.98
E1WFA8	Isocitrate dehydrogenase NADP OS Salmonella typhimurium strain SL1344 GN icdA PE 3 SV 1	Nucleotide binding; catalytic activity; binding	Generation of precursor metabolites and energy; catabolic process; carbohydrate metabolic process	-1.94
E1W9M3	Citrate synthase OS Salmonella typhimurium strain SL1344 GN gltA PE 3 SV 1	Transferase activity	Generation of precursor metabolites and energy; catabolic process; carbohydrate metabolic process	-1.83
E1WIQ8	Tryptophan tRNA ligase OS Salmonella typhimurium strain SL1344 GN trpS PE 3 SV 1	Catalytic activity; nucleotide binding	Translation; nucleobase-containing compound metabolic process	-1.93

Table A3.4 (cont.) Proteins down-regulated in response to treatment of *S.* Typhimurium NCTC 12023 with 500 nM SNP

Protein Accession No.	Protein Identification	GO terms (function)	GO terms (biological process)	Fold change
E1WAS6	30S ribosomal protein S6 OS Salmonella typhimurium strain SL1344 GN rpsF PE 3 SV 1	Structural molecule activity; RNA binding	Translation	-1.82
E1WHZ6	30S ribosomal protein S21 OS Salmonella typhimurium strain SL1344 GN rpsU PE 3 SV 1	Hydrolase activity; structural molecule activity	Translation	-1.68
E1WIL2	50S ribosomal protein L22 OS Salmonella typhimurium strain SL1344 GN rplV PE 3 SV 1	Structural molecule activity; RNA binding	Translation	-1.96
E1WIK6	50S ribosomal protein L24 OS Salmonella typhimurium strain SL1344 GN rplX PE 3 SV 1	Structural molecule activity: RNA binding	Translation	-1.53
E1WEJ7	DNA directed RNA polymerase subunit beta OS Salmonella typhimurium strain SL1344 GN rpoB PE 3 SV 1	Transferase activity; binding; DNA binding	-	-1.85

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