UNIVERSITY OF SOUTHAMPTON
FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Decontamination of biofilm and VBNC zoonotic pathogens on the salad leaf phylloplane for enhanced food security and safety

By
Callum Highmore

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ABSTRACT

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Produce-associated outbreaks of foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* are rising in prominence among outbreaks of foodborne disease. Testing for foodborne pathogens by the agricultural industry relies heavily on culture-based techniques, excluding detection of viable but nonculturable (VBNC) pathogens. Here, a detection method is used that facilitates the use of qPCR on the complex environmental matrices of soil. Targeting the *tir* gene of *E. coli* O157, detection of the pathogen in peat-based compost and sand is achieved to a sensitivity of 10 CFU/g. When applied to pristine soil, 310 copies of the gene were detected. Further analysis using PNA-FISH and cell elongation determined the presence of 205 VBNC *E. coli* O157 cells per gram of soil sample. Resuscitation of the pathogen was achieved through prolonged enrichment in selective media. Decontamination of fresh produce using chlorine washes was simulated using *L. monocytogenes* and *S. enterica* serovar Thompson adhered to spinach leaves, resulting in complete VBNC induction of viable cells following two minutes exposure to 50 ppm and 100 ppm chlorine respectively. The infectivity of these chlorine induced VBNC pathogens was assessed *in vivo* using *Caenorhabditis elegans* as an animal model. VBNC *L. monocytogenes* retained its infectivity and caused a significant lifespan reduction ($p=0.0064$). Together, these data provide evidence of the presence and induction of VBNC foodborne pathogens throughout the food production chain, and determines that VBNC *L. monocytogenes* presents a threat to food safety.

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Academic Thesis: Declaration of Authorship

I, Callum Highmore, declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

Decontamination of biofilm and VBNC zoonotic pathogens on the salad leaf phylloplane for enhanced food security and safety

I confirm that:

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List of Abbreviations

Abbreviation	**Definition**
ABNC	Active but nonculturable
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHIB	Brain heart infusion broth
BLAST	Basic local alignment search tool
CAT-FISH	Capture antibody targeted fluorescence *in situ* hybridisation
C. elegans	*Caenorhabditis elegans*
CFU	Colony forming unit
cT	Cycle threshold
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DVC	Direct viable count
E. coli	*Escherichia coli*
EDIC	Episcopic differential interference contrast
eDNA	Extracellular deoxyribonucleic acid
EF	Epifluorescence
EHEC	Enterohaemorrhagic *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EMA	Ethidium monoazide
FISH	Fluorescence *in situ* hybridisation
GFP	Green fluorescent protein

HUS	Haemolytic uremic syndrome
L. monocytogenes	*Listeria monocytogenes*
MPN	Most probable number
mRNA	Messenger ribonucleic acid
mTSB	Modified tryptone soya broth
NCTC	National collection of type cultures
NGM	Nematode growth medium
PAMPs	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Propidium monoazide
PNA	Peptide nucleic acid
ppm	Parts per million
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPF	Resuscitation promoting factor
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
S. enterica	*Salmonella enterica*
SEM	Standard error of the mean
STEC	Shiga-toxin producing *Escherichia coli*
TTSS	Type three secretion system
UV	Ultraviolet
VBNC	Viable but nonculturable
VTEC	Vero cytotoxin-producing *Escherichia coli*

Chapter 1

General Introduction

1.1 Scope of research

The research carried out in this study is focused on the enhancement of food safety through the prevention of outbreaks of foodborne disease. It has previously been estimated that the burden of foodborne disease in the USA causes an annual loss of 61,166 quality-adjusted life years, and in 2009, a cost of $14.1 billion (Hoffmann et al., 2012). In particular, the bacterial pathogens *Escherichia coli* O157, *Listeria monocytogenes*, and *Salmonella enterica* were estimated to be responsible for the loss of 17, 817 quality-adjusted life years, and $6.1 billion. These pathogens are all known to enter a viable but nonculturable (VBNC) state (Besnard et al., 2002; Li et al., 2014) in response to a range of environmental stressors, which prevents their detection in the laboratory via colony formation on culture media. As these culture techniques are relied upon in industry to identify contamination, it is the hypothesis of this study that assurance of microbiological safety of food is dependent on the acknowledgement of the VBNC state of foodborne pathogens.

Despite its discovery in 1982 (Xu et al., 1982), the VBNC state has been studied relatively little. Inability to culture the organisms has led to difficulties in their isolation and manipulation, and as a result the biochemical pathways that constitute the state have not been established. In the discipline of microbiology, nonculturability can be mistaken for non-significance. For foodborne pathogens, this can lead to disease outbreaks.

While there is potential for VBNC mediated foodborne disease to be facilitated by any food vehicle, fresh produce is often sold as ‘ready-to-eat’, and so is reliant on the washing during food processing for the removal of foodborne pathogens. Chlorine is commonly used for this purpose; however several reports have previously shown that it is ineffective at killing bacteria (Ryu and Beuchat, 2005; Niemira and Cooke, 2010). There has also been evidence that chlorine exposure can induce the VBNC state in foodborne pathogens (Leriche and Carpentier, 1995; Oliver et al., 2005).

These factors together form the basis of the study. To assess the risk that VBNC foodborne pathogens pose to the public, the prevalence of VBNC pathogens entering the farm-to-fork chain

must be examined, as well as those that are induced into the state during food processing. Utilising this information, the pathogenicity of VBNC pathogens identified under these conditions must be determined, as previous studies have come to contrasting conclusions on the subject (Smith et al., 2000; Lindbäck et al., 2010; Liu et al., 2010). To alleviate the burden of foodborne disease and aid in the detection of VBNC contaminants, an assay will be developed for use in the agricultural industry to detect foodborne pathogens including those in the VBNC state, bypassing the need for culture techniques.

1.2 Background

Foodborne disease presents a consistent but frequently preventable threat to public health, and is responsible for an estimated 2.2 million deaths worldwide annually. In the UK, it is estimated that each year one million people suffer a foodborne illness, resulting in 500 deaths (Anon., 2011b). Of foodborne disease cases caused by bacterial pathogens, previous studies have attributed 27% of cases and 24.7% of deaths to outbreaks disseminated by fresh produce (Painter et al., 2013). The safety of fresh produce is particularly important due to a consumer focus on healthy eating leading to an increase in sales, and because unlike meat products, fresh produce such as leafy vegetables are frequently sold as ready-to-eat, meaning that effective decontamination of the food is reliant on agricultural industry practices.

Among the primary causative agents of bacterial foodborne disease are the pathogens *Escherichia coli* O157, *Listeria monocytogenes*, and *Salmonella enterica*. In 2010, these pathogens were responsible for over half of the total deaths in the UK caused by foodborne disease (Anon., 2011b). One UK study spanning 17 years determined that in foodborne outbreaks, *Salmonella* spp. were responsible for the highest number of people affected, *E. coli* O157 caused the highest proportion of hospitalisation, and the greatest proportion of deaths was caused by *L. monocytogenes* (Gormley et al., 2011). Figure 1 shows that *Salmonella* spp. were the dominant causative pathogens of foodborne disease outbreaks in 2009, both of those attributed to fruit and vegetables, and across all food

groups. However, a number of produce-associated enterohaemorrhagic *E. coli* outbreaks have since been reported in the UK and across the world.

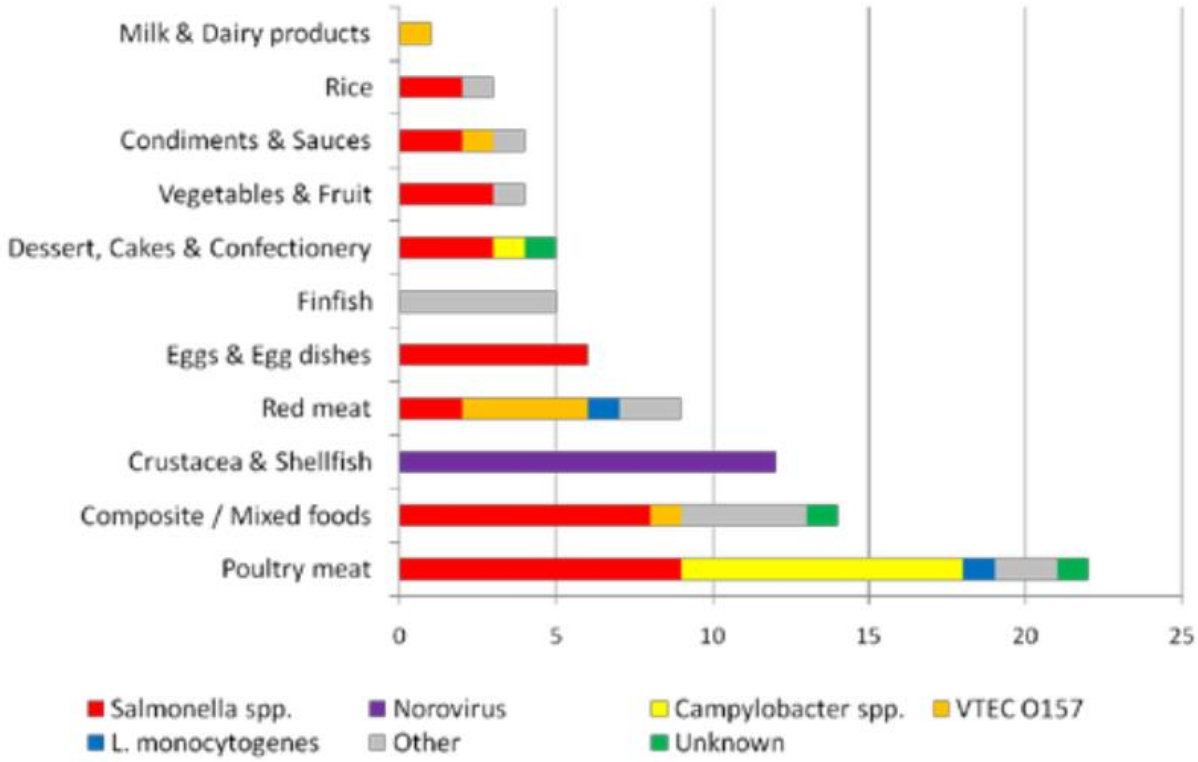

Figure 1. Foodborne pathogens and food vehicles responsible for outbreaks in England and Wales in 2009. Image taken from Foodborne Outbreaks in 2009, Food Standards Agency (eFOSS, 2010).

In addition to the increase in consumption of fresh produce, improved storage quality has allowed fresh produce to be distributed more widely, facilitating the dissemination of foodborne pathogens. For example, one production date in 2006 at one spinach processing plant in California led to an outbreak of *E. coli* O157:H7 spanning 26 states and Canada, causing 205 cases of illness and 3 deaths (Jay et al., 2007). Improved popularity and distribution range of fresh produce led to another international outbreak of an *E. coli* O157 strain, occurring in Europe the following year. 50 cases were diagnosed in Iceland and the Netherlands, where shredded lettuce was named as the most likely source of contamination (Friesema et al., 2008). In 2013, a further *E. coli* O157:H7 outbreak

caused by contaminated romaine lettuce took place causing 33 cases of illness across four US states (Anon., 2014), while an *E. coli* O157 outbreak linked to watercress in the UK resulted in 19 disease cases (Launders et al., 2013).

Other foodborne pathogens such as *L. monocytogenes* also contribute to disease outbreaks caused by contamination of fresh produce, an outbreak in 2016 in the USA spanned 9 states and caused 19 disease cases, where each resulted in hospitalisation (Anon., 2011a). An outbreak of *Salmonella enterica* serovar Saintpaul associated with peppers caused 1500 cases across 43 states of the USA, resulting in 315 hospitalisations and is thought to have caused two deaths (Barton Behravesh et al., 2011). *E. coli* O157 is not the only Shiga-toxin *E. coli* (STEC) serogroup responsible for produce-associated disease outbreaks. *E. coli* O104 was the causative agent of a large outbreak associated with sprout consumption that resulted in 3816 cases and 54 deaths across Germany (Frank et al., 2011), enunciating the current problem of contamination of ready-to-eat foods.

1.3 Foodborne pathogens

Escherichia coli O157:H7 is a serotype of the Gram-negative bacterium *E. coli*, able to produce Shiga toxin, a factor responsible for the bacterium's pathogenicity in humans. *E. coli* O157:H7 is particularly dangerous as a foodborne pathogen as it has a low infectious dose, often cited at 10-100 cells (Chart, 2000). The majority of cases consist of abdominal cramps and bloody diarrhoea, characteristic of haemorrhagic colitis, and are usually resolved without further treatment. However, approximately 5% of cases develop into haemolytic uremic syndrome (HUS), where a further 5% of those cases are fatal (Chart, 2000). This causes haemolytic anaemia and acute renal failure in up to 70% of patients. HUS associated with the Shiga toxin gene is estimated to affect 2.1 individuals per 100,000 a year, primarily affecting children under the age of 5; 70% of these cases are preceded by infection with *E. coli* O157:H7. The effects of HUS are severe, 70% of patients require blood transfusions while 25% have neurologic involvement, which could manifest as stroke or coma (Noris and Remuzzi, 2005).

Listeria monocytogenes is a Gram-positive bacterium that is ubiquitous in both nature and in domestic environments (Beumer et al., 1996; Swaminathan and Gerner-Smidt, 2007). Across different countries, the incidence of listeriosis (infection by *L. monocytogenes*) ranges between 0.01 and 1.3 cases per 100,000 people. Despite its prevalence in the environment, the bacterium rarely causes disease in humans. This doesn't undermine the severity of listeriosis when infection does occur. Up to 30% of cases result in death, while the disease itself ranges in severity, ranging from mild flu-like symptoms to septicaemia, bacterial meningitis or encephalitis (McLauchlin et al., 2004; Swaminathan and Gerner-Smidt, 2007). Listeriosis is known to manifest more severely in pregnant women, where colonisation of placenta may lead to preterm labour and complications of miscarriage or stillbirth (Abram et al., 2003). The principal route of infection by *L. monocytogenes* is through food, including minimally processed vegetables, making the understanding of this pathogen extremely relevant to enhancing food safety (McLauchlin et al., 2004).

Salmonella enterica is a Gram-negative bacterial species categorised into six known subspecies with 2500 serovars. Of these, serovars Typhi and Paratyphi cause enteric fever in humans, whereas serovar Enteritidis was the most common cause of all bacterial foodborne pathogen outbreaks between 1998 and 2002 (Coburn et al., 2007; Linam and Gerber, 2007). Salmonellosis is usually characterised by a self-limiting period of vomiting, diarrhoea and muscle aches, although infection leading to enteric fever has the potential to cause encephalitis or intestinal haemorrhage. Approximately 40,000 cases of salmonellosis are reported in the USA each year, with 7585 in the UK in 2013 and 1.3 billion cases worldwide (Darwin and Miller, 1999; Coburn et al., 2007; England, 2014). *Salmonella* spp. were responsible for 41% of outbreaks linked to fresh produce in the UK between 1992 and 2000, and as the consumption of minimally processed vegetables is only increasing, effective methods of decontaminating vegetables of the pathogen have become more important than ever (Heaton and Jones, 2008).

1.4 The viable but nonculturable state

1.4.1 Biology of VBNC cells

It has been discovered that in response to a range of physical and chemical stresses, many bacterial species are capable of entering a VBNC state. The state is typically characterised by a decrease in cell size and increased tolerance to stresses such as antibiotic pressure (Lleo et al., 2007), but is defined by the inability of these cells to grow on culture media.

Previous research has focused on the factors that induce the VBNC state in bacteria, in particular the response to starvation and low temperatures (Biosca et al., 1996; Mizunoe et al., 1999; Dinu and Bach, 2011). This is an established topic of research, partially because *Vibrio vulnificus* is frequently studied as a model VBNC organism, and is known to enter the state at temperatures below 15^{o}C (Wolf and Oliver, 1992). Under these conditions, bacteria can persist for years in the VBNC state. Viable *Vibrio fluvialis* has been detected in VBNC samples following starvation lasting 6 years (Amel et al., 2008).

Temperature and starvation may be the most common survival challenges to bacteria in their environments; however other fundamental environmental factors such as pH and osmotic stress can induce the state. This has been observed in *E. coli* (Darcan et al., 2009), where incubation at pH 8.3 showed a more pronounced difference between culture-based detection and viable cell counts, than incubation at pH 7.2 or 5.5. The salinity of seawater was also found to induce the VBNC state in a portion of *E. coli* cells. Exposure to ultraviolet radiation is a survival challenge to bacteria that exist in environments in direct sunlight, such as on the phylloplane of crop plants. It is also used as a method by which water can be decontaminated, and so its role in VBNC induction has been previously investigated. *S. enterica* was induced into the state as a means of modelling solar disinfection of water (Smith et al., 2000). Using UV A irradiation and a water temperature recorded in the Kenyan Rift Valley, culturability decreased by 6 log over 8 hours, compared with a control that was not irradiated. VBNC cells were identified using the Kogure cell elongation method (Kogure et al., 1979),

finding 5% of the total cell count had entered into the state. Another study induced the VBNC state in *Pseudomonas aeruginosa* and *E. coli* using UV light (Zhang et al., 2015), a dose of UV at 100 mJ/cm^2 produced a significant difference between *E. coli* culture data and that obtained by reverse transcription (RT) qPCR, relative to an untreated control.

In addition to stressors likely to be encountered naturally, human activity brings bacteria into contact with chemical agents intended to kill them, such as antibiotics and chemical sanitisers that cause oxidative stress, such as chlorine. Following treatment with vancomycin or quinupristin/dalfopristin, VBNC cells were identified in *Staphylococcus aureus* biofilms through the use of RT-qPCR and epifluorescence microscopy (Pasquaroli et al., 2013). The authors hypothesise that VBNC induction by antibiotic stress could be due to the inhibition of protein translation causing oxidative stress by failed production of catalase and superoxide dismutase. This supports other research, where oxidative stress has been investigated in its role in VBNC induction and resuscitation. One study used a *V. vulnificus* mutant lacking catalase to determine that the inability to break down H_2O_2 prevents the culture of the bacterium on agar (Kong et al., 2004).

VBNC cells are considered to have common attributes regardless of the variety in environmental stressors that induce the state. A reduction in physical size of cells is often observed (Linder and Oliver, 1989; Du et al., 2007b), which has been attributed to the conservation of resources by the VBNC bacterium. The cell membrane is also altered by VBNC induction through its fatty acid composition (Linder and Oliver, 1989). The dwarfing of the VBNC cell is consistent with its lower metabolic rate. The continued metabolism of VBNC cells has previously been established (Lleo et al., 2000; Morishige et al., 2015), and using cell staining techniques such as SYTO9 and Rhodamine-123, Shleeva et al. recognised a low metabolism in VBNC *Mycobacterium smegmatis* distinct from both culturable and dead cells (Shleeva et al., 2004).

The reactive nature of the VBNC state leads to an increased tolerance against environmental stressors. Nowakowska and Oliver subjected VBNC *V. vulnificus* to a heat challenge of 50^{o}C, ethanol

exposure, high salinity, pH changes, H_2O_2 exposure, and antibiotic exposure (Nowakowska and Oliver, 2013). The bacterium was induced into the state by chemical or physical challenges, or by incubation at 5^{o}C. Despite the difference between induction method and experimental challenge, VBNC *V. vulnificus* was found to endure high temperature more effectively than cells challenged while in the logarithmic phase. This corroborates with similar data previously obtained for *Vibrio parahaemolyticus* (Wong and Wang, 2004). Some protection was also observed against ethanol, high salinity, pH changes, and H_2O_2 exposure. The effect was more pronounced in VBNC cell populations exposed to ampicillin or chloramphenicol for 4 hours, where previously culturable cells could not be recovered, but VBNC cells were resuscitated to initial levels (Nowakowska and Oliver, 2013).

The characteristics of VBNC cells have been well studied, but the mechanisms that underpin them are still poorly understood. Analyses have been conducted that show VBNC *Enterococcus faecalis* possess distinct cell membrane lipids (Signoretto et al., 2000) and protein profiles (Heim et al., 2002) while in the VBNC state. One factor that has been implicated in the regulation of the VBNC state is the stress response RNA polymerase factor RpoS. It mediates gene expression for the response to starvation, osmotic stress, and oxidative stress, among others (Li et al., 2014), all of which are known to induce the VBNC state. Through disruption of RpoS using different *Salmonella* spp., it was determined that levels of RpoS were negatively correlated to VBNC induction (Kusumoto et al., 2012). The mechanism of action of VBNC induction is not yet known, but the authors note that gene products essential for cell division are under the regulation of RpoS. These results support the work of Boaretti et al. where RpoS deficient *E. coli* cells were not able to maintain the VBNC state and died in response to low temperature stress (Boaretti et al., 2003). OxyR is another transcriptional regulator, associated with protection against oxidative stress. Required for the production of catalase, deficient *V. vulnificus* mutants were unable to grow on agar plates, due to the presence of H_2O_2 in the medium (Kong et al., 2004).

Studies involving deficient mutants have been extremely valuable in identifying genes involved in the state. When carried out in *E. coli* on the environmental sensor EnvZ, bacteria were found to be unable to enter the VBNC state because the sensor could not react to the environmental challenges of starvation or high salinity (Darcan et al., 2009). Another protein associated with the outer membrane of *E. coli* has been implicated with the VBNC state, OmpW. *E. coli* O157 passaged through a mouse was exposed to H_2O_2 to induce oxidative stress causing the VBNC state, and using a Western blot OmpW expression was upregulated in comparison to a H_2O_2 resistant strain which remained culturable (Asakura et al., 2008). As with other factors implicated in the state, the biochemical basis of OmpW in the VBNC state is unknown. However, the authors propose that in *E. coli*, as the gene sequence of OmpW indicates a PDZ domain in the protein which could link it to the stress response mediated by RpoE. The continuing identification of genes involved in the VBNC state will lead to characterisation of biochemical pathways, which will accelerate the research into their manipulation.

Some links have been made between the VBNC cells and persister cells, which represents another type of microbial dormancy (Ayrapetyan et al., 2015). Persister cells comprise a sub-population of non-growing cells within a growing population, and are thought to occur stochastically within a bacterial culture or biofilm (Maisonneuve et al., 2013). The toxin-antitoxin systems that have a role in this modulation have also been implicated in the VBNC state, linking the two phenotypes. These systems are comprised of a protein toxin and RNA antitoxin, where in response to stress toxin production can outpace antitoxin production, and the toxin can disrupt membranes preventing cell growth (Page and Peti, 2016). By inducing toxins RelE and ChpAK in *E. coli*, the cells were reduced to a non-culturable state (Pedersen et al., 2002; Ayrapetyan et al., 2015). Similar results were obtained in *M. smegmatis*, where overexpression of the VapC toxin caused cell growth arrest, and overexpression of VapB antitoxin prevented the bacterium from entering the VBNC state where it normally would be able to following exposure to limited potassium media (Demidenok et al., 2014). The novelty of this research highlights the poor understanding of the VBNC state, its role in nature and the depth of connections to other supposedly discrete physiological states.

The return of VBNC cells to a culturable state is referred to as resuscitation. The notion of VBNC resuscitation is contentious, as the state has previously been described as irreversible (Barer and Bogosian, 2004), however several studied have reported its occurrence in response to a number of stimulating factors. It has been argued that the VBNC state can be reversed by removing the inducing factor, for example a temperature upshift for cells exposed to low temperature. This has been achieved in several bacterial species, including *S. enterica* (Gupte et al., 2003), and *V. vulnificus* (Rao et al., 2014), but others argue that requirements for resuscitation are more demanding. Additional factors that have been added to growth media to aid resuscitation include amino acids (Pinto et al., 2011), Tween 20, and catalase (Zeng et al., 2013). The influence of bacterial messaging upon resuscitation has also been noted; autoinducer signalling molecules produced by culturable *E. coli* O157:H7 cause resuscitation of VBNC *E. coli* O157:H7 induced by starvation at low temperature or chlorine treatment, when incubated in liquid medium (Liu et al., 2009). Without the presence of these autoinducers, resuscitation was not achieved by otherwise identical treatment.

At a molecular level, resuscitation promoting factor (Rpf) has been identified as an agent of resuscitation. Rpf is an extracellular protein with homologues in *Mycobacterium*, *L. monocytogenes*, and *S. enterica* (Ramamurthy et al., 2014), and it has not been determined whether it has an autocrine or paracrine effect. There are currently three proposed mechanisms for Rpf activity. Rpf contains a conserved lysozyme-like domain which it may utilise to cleave cell wall components which can act as signalling molecules for the exit of the VBNC state. (Li et al., 2014; Ramamurthy et al., 2014; Pinto et al., 2015). Another model related to the lysozyme-like domain suggests that Rpfs cleave the peptidoglycan layer of their parent cell. The resulting released components bind to receptors on a different cell surface which initiate resuscitation. The third model asserts that Rpf binds to receptors directly on the VBNC cell walls to trigger the same effect.

Studies that explore resuscitation of VBNC cells have often previously induced the state in their target bacteria, so that the timeframe in which VBNC cells are able to resuscitate has not been

exhaustively explored. Fera et al. induced the VBNC state in *Arcobacter butzleri* by incubating in seawater over 20 days (Fera et al., 2008). Every 3 months, viability was assessed using a *Bac*Light™ LIVE/DEAD stain, which determined that viability remained consistent throughout the 270 day experiment. Cells could also be resuscitated with the addition of nutrients and an increase in temperature; this was carried out on VBNC cells aged for 270 days, which was the duration of the experiment. Another study showed that *E. coli* O157 induced into the VBNC state by river water could be resuscitated 10 months later using prolonged incubation with exposure to autoinducers (Liu et al., 2009). The purpose of this study was not to identify the extent of the resuscitation window, so the mechanics and parameters behind resuscitation are yet to be fully understood.

1.4.2 Infectivity of VBNC foodborne pathogens

There is a wealth of research defining the conditions by which the VBNC state is induced in bacterial cells, with evidence that it can be caused by environmental factors such as a decrease in temperature, deprivation of nutrients, low pH and NaCl concentration, as well as light exposure (Besnard et al., 2002; Cook and Bolster, 2007). Many of these conditions are common to the salad leaf phylloplane, where the VBNC state might help contaminating foodborne pathogens endure the decontamination procedures used in the agricultural industry before reaching the public.

Further research has explored this risk; studies have verified the propensity of *E. coli* O157:H7 to enter the VBNC state on the lettuce phylloplane. The pathogen was inoculated onto lettuce leaves and incubated at 8^{o}C for 2 weeks under starvation conditions to induce a VBNC state. While culturable cells were undetectable on trypticase soy agar and sorbitol-MacConkey agar plates after as few as 6 days, *E. coli* O157:H7 tagged with green fluorescent protein (GFP) could be detected on the phylloplane after 16 days using confocal microscopy (Dinu and Bach, 2011). This discrepancy indicates that VBNC *E. coli* O157:H7 induced by conditions potentially occurring *in situ* can be present on lettuce leaves, which could impact on the safety of consuming minimally-processed vegetables.

There is evidence that despite the VBNC cells' inability to be cultured, they still retain their pathogenicity. The enterohaemorrhagic *E. coli* O78:H11 was found to produce its enterotoxin while in the VBNC state. VBNC cells were induced by light irradiation and inoculated into a rabbit intestinal loop. The enterotoxin was detected using ELISA (Pommepuy et al., 1996), showing that while foodborne pathogens are unable to be cultured in a laboratory, they may be able to cause disease if ingested. Similar results have been obtained using RT-qPCR to detect expression of the Shiga-toxin genes *stx1* and *stx2* of VBNC *E. coli* O157 induced by incubation in chloraminated and river water (Liu et al., 2010). The cytotoxicity of these toxins was confirmed by exposing them to Vero cells and observing a reduction in total cell number.

Conversely, there is evidence against the pathogenicity of *L. monocytogenes* while in the VBNC state. Cells induced into the VBNC state by starvation was determined avirulent when introduced to human adenocarcinoma cells and a mouse model (Cappelier et al., 2005). *Salmonella enterica* serovar Typhimurium was also unable to cause infection by injection into a mouse model, following VBNC induction by ultraviolet irradiation (Smith et al., 2000). However, it is accepted that foodborne pathogens can resuscitate to a culturable state under favourable conditions and regain virulence, often once ingested by a suitable host. *L. monocytogenes* regained its pathogenicity after resuscitation in embryonated chicken eggs, where unfertilised eggs were not sufficient to cause resuscitation (Baffone et al., 2003; Cappelier et al., 2007). Contrary to *Salmonella* Typhimurium research, *Salmonella* Oranienburg was capable of resuscitation and causing diarrhoea in mice following VBNC induction via exposure to high NaCl (Asakura et al., 2002). These conflicting results seemingly dependent on bacterial species, serovar, and method of VBNC induction reveal the complexity of the state. A comprehensive understanding of VBNC cells is currently inhibited by the challenges of their detection and subsequent manipulation.

1.4.3 The philosophical challenge of the VBNC state

The VBNC state was first identified in 1982 (Xu et al., 1982), noting the limitations of culture-based methods in the detection of *E. coli* and *Vibrio cholerae* in aquatic environments. Subsequent research into the VBNC state has generated little consensus and caused considerable debate regarding its most basic defining parameters. It has been argued that cells described as VBNC are undergoing a transition toward cell death (Nystrom, 2003) rather than enduring external stressors. A common criticism of studies that claim to observe resuscitation of VBNC cells is that any successful culture may be due to the detection of a low concentration of consistently culturable cells (Kell et al., 1998). As explored previously, there is also no consensus on the infective potential of VBNC and resuscitated pathogens.

Bacteria may exist in other nonculturable states, such as sporulation and persistence (Pinto et al., 2015). Sporulation has been most extensively studied in *Bacillus subtilis*, and similarly to the VBNC state, is a response to harsh environmental conditions such as starvation (Driks, 2002). Unlike the VBNC state, bacterial spores are characterised by a lack of metabolic activity and distinct morphological characteristics when compared to culturable cells (Pinto et al., 2015). Since sporulation was first described in the 19th century, it is far better defined than the VBNC state (Higgins and Dworkin, 2012). Upon spore formation, cell components are localised to an asymmetric division septum and a peptidoglycan layer is assembled between proteinaceous layers to compose the spore cortex. The enveloping membrane of the parent cell degrades to release the mature spore. The spore is a separate entity from its parent cell, whereas VBNC cells alter the composition of their membranes, but remain intact.

Persister cells are typically a subpopulation of non-growing cells within a growing population (Pinto et al., 2015). Two models have been proposed as to the formation of persisters, they may develop as a result of misfolded proteins inducing the dormant state, or it may be a controlled occurrence modulated by epigenetic factors so that populations of cells can endure potential changes to their environment, such as exposure to antibiotics. This is in opposition to the current understanding of

the VBNC state, where it is induced in response to unfavourable conditions. However, it has been noted that persister cells can form in response to amino acid starvation (Gerdes and Maisonneuve, 2012), blurring the lines between the two states. One study differentiates between persister and VBNC cells by defining persister cells by their antibiotic tolerance and culturability following antibiotic exposure (Orman and Brynildsen, 2013), although paradoxically once the cell is cultured it can no longer be considered a persister. VBNC cells were enumerated by flow cytometry and fluorescent *Bac*Light™ LIVE/DEAD staining, and the study determined that an *E. coli* culture treated with ampicillin contained VBNC cells at a concentration of 100 times the persister population. While there are quantifiable differences between the two states, a better understanding of the VBNC state would support these distinctions.

The VBNC state has been described in *V. vulnificus* following exposure to temperatures below 15^{o}C in its aquatic environment (Wolf and Oliver, 1992; Nowakowska and Oliver, 2013). Whitesides, 1997, has shown that its resuscitation is achieved by a temperature increase to room temperature (Whitesides and Oliver, 1997), and provided evidence for true resuscitation of the bacteria, in place of exposing already culturable organisms to suitable growth conditions. This was carried out by comparing plate counts of VBNC *V. vulnificus* to plate counts of a sample that had been incubated for 24 hours at room temperature prior to plating. Cells that were not incubated prior to plating produced no colonies, despite incubation at room temperature for 30 days. Those that underwent the preliminary incubation produced colonies indicating a concentration of 10^{6}CFU/ml *V. vulnificus*.

Despite this research, the argument remains that resuscitation of VBNC cells is the result of satisfying growth requirements of bacteria, following injury by a stressor such as low temperature. One study used nalidixic acid to inhibit cell division and instead cause cell elongation, to show replication and potential culturability in starved *V. parahaemolyticus* that produced no colonies using culture-based techniques (Jiang and Chai, 1996). Similar conclusions were drawn by Weichart

(Weichart et al., 1992), where a temperature upshift was thought to induce cell growth of a few culturable *V. vulnificus* cells among an otherwise nonculturable population.

The challenge of discriminating true resuscitated cells from stressed or fastidious but culturable cells has been addressed in other studies, that claim the addition or removal of particular reagents can stimulate growth in VBNCs. It has been hypothesised that nonculturable cells may be inhibited from returning to a culturable state by free radicals (Mukamolova et al., 2003), and reagents that degrade peroxide, such as catalase (Mizunoe et al., 1999) and sodium pyruvate (Mizunoe et al., 1999; Pinto et al., 2011; Pasquaroli et al., 2013) have been used to resuscitate populations of different bacterial species made VBNC by different environmental stressors. One study extended the exploration of resuscitation promoting reagents (Pinto et al., 2011), using the supernatants of *E. coli* cultures in late logarithmic phase to cause resuscitation of VBNC *E. coli* induced by starvation at low temperature. The supernatant was treated with proteinase K, determining that the supernatant factor responsible for resuscitation was not proteinaceous. This corroborates with previous work that exposed VBNC *E. coli* and *S. enterica* to an enterobacterium autoinducer signalling molecule to stimulate resuscitation (Reissbrodt et al., 2002). The identification of bacterial signalling molecules that play a role in resuscitation is valuable, and may assist in the overall definition of the VBNC state.

More recently, Tween 80 was used to aid the resuscitation of *Listeria innocua* from the VBNC state (Trinh et al., 2015). The studies reviewed here have each determined a set of parameters for VBNC resuscitation in accordance with their observations, but in the absence of a fully characterised VBNC state, there can be no answer to the question of true resuscitation.

While it is tempting to attribute resuscitation of VBNC cells to the addition or removal of environmental factors, it is important to acknowledge the experimental context. In nature, the acclimation of bacteria to a new environmental condition via the VBNC state may be bound to a host of other factors that are beyond replication by current laboratory techniques.

As Kell proposes (Kell et al., 1998), the conflicting hypotheses of VBNC research can frequently be attributed to the semantics of the state. The term 'viable but nonculturable' is a term used to categorise bacteria in response to laboratory techniques; it is not defined by any particular biochemical activity or unified through a standardised assay. Due to the difficulties in manipulating VBNC cells, and perhaps due to the connotations of its nomenclature, the state is insufficiently studied. The resulting lack of understanding precludes the current perspective of the VBNC state; it is referred to as a single physiological state, when in nature it remains a phenotypic abstraction.

1.5 Sources of contamination

1.5.1 Wastewater

As infection by foodborne pathogens is frequently linked with diarrhoea, through which the responsible bacteria are shed, contamination of fresh produce is made possible by the application of contaminated faecal matter used as fertiliser. Worldwide, the primary vehicle for pathogen introduction into farmland is through the application of contaminated matter used as fertilisers, including slurries, sewage sludge, abattoir waste and manure (Heaton and Jones, 2008). Contaminated growing media has been named as the source of previously mentioned outbreaks (Launders et al., 2013).

The use of sewage sludge as fertiliser is frequently used in the developing world; in Iraq, one study detected the presence of *L. monocytogenes* in this material which was then found to successfully transmit in low numbers to the alfalfa plants grown in it (al-Ghazali and al-Azawi, 1990). While the use of untreated sewage as agricultural fertiliser is illegal in the USA and throughout Europe (Avery et al., 2005), it has been found that even treated wastewater has the potential to bear foodborne pathogens. Beaudette et al. (2007) used qPCR to detect a number of bacterial pathogens following different stages of wastewater treatment. While a 3-4 log reduction was observed in pathogen survival following wastewater treatment, *E. coli* was still detected in the final effluent with a gene copy number of 5.75×10^3 (Shannon et al., 2007). While this study does not directly document *E. coli*

O157:H7 cells surviving the wastewater treatment process, it demonstrates the ability of faecal coliforms to do so. However, the use of qPCR to detect microorganisms in wastewater effluents doesn't account for the presence of DNA from dead cells. Thus, the viable bacterial load in treated wastewater may be overestimated in this study. Another study used effluent from a conventional wastewater treatment plant in the UK to irrigate plots of lettuce. While it was found that covered plots of lettuce would attain an acceptable concentration of faecal coliforms 3 days after irrigation cessation (10^3 faecal coliforms per 100 g), lettuce on uncovered plots could become re-contaminated by the contaminated soil following rainfall (Blumenthal et al., 2000).

The use of contaminated wastewater for crop irrigation poses a risk to both the consumers of the crops, and the farmers growing them. Despite this, outbreaks of enteric infections attributed to wastewater irrigation are relatively rare, and primarily associated with the use of untreated wastewater (Blumenthal et al., 2000).

1.5.2 Abattoir wastes

Cattle are recognised as a cardinal reservoir of *E. coli* O157:H7, and are utilised by farmers in several ways to fertilise agricultural land. Untreated abattoir wastes are regularly used to fertilise soil for crop growth (Hepburn et al., 2002), for example up to 26000 wet tonnes of blood and gut contents were applied to land in Scotland in 1997 (SEPA, 1998). In this way, enteric pathogens such as *E. coli* O157:H7 and *Listeria monocytogenes* can be introduced to soil. These pathogens can be found in the intestinal tracts of cattle and during the slaughtering process are released into abattoir waste (Mittal, 2004). It has been shown that while *E. coli* O157:H7 numbers decrease within 24 hours following inoculation into fresh bovine blood, results indicate that the pathogen is able to proliferate in blood that had been aged 24 hours prior to inoculation (Hepburn et al., 2002). In industry, *E. coli* O157:H7 would be exposed to blood following the slaughter of the animal, when any antimicrobial quality has been lost. This supports the notion that the pathogen is able to contaminate soil through the use of abattoir wastes in agriculture. A further study took place, in which the survival of *E. coli* O157:H7

was studied in abattoir wastes over 2 months. It was found that there was little decrease in viable counts of the pathogen over the first 2 weeks, with viable cells detected in a range of abattoir wastes for the duration of the study (Avery et al., 2005). By day 64 most abattoir wastes displayed *E. coli* O157:H7 concentrations in the order of 10 CFU/cm^3, which despite seeming sufficient to spread infection due to the low infectious dose of the pathogen, may be an underestimation. The study used culture-based techniques to enumerate the bacteria, which is ineffective in detecting VBNC cells. The induction of a VBNC state in *E. coli* O157:H7 exposed to abattoir wastes appears to have gone unstudied, and may provide an additional dimension to the survival of foodborne pathogens in organic wastes used for agriculture.

The potential for *E. coli* serotype O157:H7 to be disseminated throughout farmland via the use of abattoir wastes is feasible as the pathogen is prevalent among beef carcasses. One study isolated the organism from 4% of 2103 cattle sourced to a single abattoir from several counties in the UK, and further detected the pathogen on 30% of the carcasses of the infected cattle following processing at the abattoir (Chapman et al., 1993). Another study investigating the prevalence of STEC of serogroup O157 in cattle carcasses produced data from 210 abattoirs across the UK. Using immunomagnetic separation, they found that 0.47% of beef carcasses tested possessed the pathogen. The sampling was carried out 'blind', so that the exact geographical distribution of STEC O157 is unknown (Richards et al., 1998). In conjunction, these studies may indicate the propensity of *E. coli* O157:H7 to spread throughout an abattoir. While it was found that where one abattoir was positive for the pathogen in 18.75% of tested carcasses (Chapman et al., 1993), only a 0.47% prevalence was documented across 210 abattoirs (Richards et al., 1998). While this discrepancy could be attributed to a difference in detection assays or mass of each quantity sampled, it is possible that contamination of *E. coli* O157:H7 could spread throughout an abattoir, for example through the use of contaminated knives on previously uncontaminated carcasses.

1.5.3 Bovine manure

The potential for outbreaks of foodborne pathogens caused by the use of abattoir waste as fertiliser for agricultural land has been established; however in Scotland abattoir waste only constitutes approximately 0.17% of organic waste spread on land. The major component (at 96%) is agricultural waste, comprising mainly of manure and slurries. These are categorised by their consistencies, as solid waste is generally considered to be manure, while semi-solid and liquid wastes are categorised as slurries (Anon., 1997b). Naturally, the potential for agricultural waste to be a vehicle by which foodborne pathogens can contaminate agricultural soil relies on the capacity of the organism to be shed in the faeces of cattle.

E. coli serogroup O157 has previously been found in cattle of all ages, and regardless of age, the pathogen appears to cause no disease (Anon., 1997a). One study used traditional culture techniques to examine the presence of *E. coli* O157:H7 in 3570 dairy cattle across 60 herds. It was found that 10 cattle were positive for the bacterium (0.28%), across 5 herds (8.3%) (Hancock et al., 1994). Another study compared the prevalence of the pathogen between a number of herds associated with *E. coli* O157:H7 outbreaks in humans. Within herds, prevalence ranged from 1.3% to 9.5%, whereas in herds of cattle not associated with any *E. coli* O157:H7 cases in humans, it was found that 0% to 6.1% of cattle were infected with the pathogen (Anon., 1997a). While these figures highlight the ability of foodborne pathogens to reach agricultural land through cattle manure, they may underrepresent the true prevalence of *E. coli* O157:H7 in cattle; culture methods overlooking the possibility for VBNC cells were used exclusively, a state which may enhance the survival of pathogens in manure when applied to agricultural land.

A further factor influencing the prevalence and detection of *E. coli* O157:H7 is the fluctuations in shedding between individual cattle and in individual cattle over time. When seasonal changes in *E. coli* O157:H7 were examined, 6 of 7 cattle herds displayed the greatest proportion of shedding between the months of April and August. Across 7 herds, all of which had tested positive for the pathogen, no positive samples were collected on 63% of sampling visits (Hancock et al., 1997). This indicates a temporal separation of periods of *E. coli* O157:H7 shedding in cattle, where long periods of undetectable excretion are punctuated by short periods of high prevalence shedding of the pathogen. A further study observed a six-fold increase in shedding of *E. coli* O157 from cattle in the summer months relative to winter, supporting the influence of seasons on shedding the pathogen (Ogden et al., 2004). One model generated suggests that the distribution of *E. coli* O157 shedding is caused by individual 'super-shedding' cattle. This minority of cattle maintain and shed a higher population of the pathogen than other cattle; the model dictates that it is likely that 11% of cattle are super-shedders (Matthews et al., 2006). While the nuances of faecal shedding of *E. coli* O157 are yet to be fully discerned in cattle, its significance in the introduction of the pathogen to agricultural soil is undisputed.

Manure used to irrigate crops is increasingly being stored in lagoons prior to its application, as a consequence of larger herds of cattle. One study assessed the ability of *E. coli* O157:H7 to survive in microcosms simulating these lagoons, finding that an inoculum of up to 10^7 CFU/ml decreased to undetectable levels within 14 days. The study did not rule out the possibility that the pathogen could persist in a VBNC state until exposure to more favourable conditions (Ravva et al., 2006). Other studies have examined the survival of foodborne pathogens in manure. The survival of *E. coli* O157:H7 in bovine manure was monitored for 12 months, finding that the pathogen persisted in aerated manure for 47 days (Kudva et al., 1998). Other reports have detected *E. coli* O157:H7 in manure over 200 days following inoculation, granting it the time to transmit to agricultural crops (Ongeng et al., 2013). Aeration of manure may affect survival of some foodborne pathogens. One investigation recorded the maximum survival of *L. monocytogenes* and *Salmonella* Typhimurium at

only 4 days in both aerated and non-aerated manure, whereas *E. coli* O157:H7 was able to survive for 8 days in non-aerated manure (and 4 days in aerated manure) (Nicholson et al., 2005a). The length of time in which *E. coli* O157:H7 can survive in manure is not inconsequential; a child had become infected by the pathogen following exposure to soil recently fertilised with bovine manure (Mukherjee et al., 2006), emphasising the risk posed by the introduction of foodborne pathogens onto agricultural land.

Slurries have also been investigated for their ability to harbour foodborne pathogens. Different studies have documented *E. coli* O157:H7 survival in bovine slurries following one month (Fenlon et al., 2000), 64 days (Avery et al., 2005) and when incubated at 4-10^{o}C, 100 days (Kudva et al., 1998). Of course the difference in survival time could be due to a number of factors, such as initial concentration of bacterial inoculum, temperature of incubation and duration of study. Again, these studies neglected molecular technique in favour of culture methods, so survival of VBNC cells would not be accounted for.

1.5.4 Other livestock

Cattle are the primary source of *E. coli* O157:H7 in agricultural land but not the only one. An alternate reservoir of the pathogen is found in sheep, with studies in ovine abattoir waste documenting its survival in blood and gut contents is increased when incubated at higher temperatures (Hepburn et al., 2002). Another study has shown that *E. coli* O157:H7 is able to survive much longer in ovine manure than in bovine manure, at 4 months and 47 days respectively. It was also found that in non-aerated manure, *E. coli* O157:H7 could be recovered 21 months from the start of the study (Kudva et al., 1998). Similarly, cases of listeriosis have been linked with increased shedding of *L. monocytogenes* by sheep and goats. The study found that levels of shedding of the pathogen by cattle were similar in farms both with and without links to listeriosis cases (Nightingale et al., 2004).

The infectivity of foodborne pathogens shed by farm animals has been documented; there are examples of humans becoming infected following exposure to contaminated ovine faeces. In 2000, twenty scouts acquired *E. coli* O157 at a meeting before which sheep grazed on the same land. The subsequent investigation found that the bacterium could survive *in situ* for 105 days. It was hypothesised that heavy rainfall reduced the amount of time *E. coli* O157 could survive in the soil (Ogden et al., 2002).

In more developed countries, regulated treatment of wastes could mean that the ingress of wild animals into agricultural land is a more pertinent cause of contamination. A major outbreak of *E. coli* O157:H7 in the USA caused by feral swine led to the consideration of pigs as a more prominent source of the pathogen. 205 reports of illness and 3 deaths were associated with the outbreak, spread across the continent by bagged spinach. Upon analysis of the feral swine populating the area, it was found that 14.9% of samples taken tested positive for *E. coli* O157 (Jay et al., 2007). The viability of pigs becoming a major reservoir of the pathogen was assessed; the infectious dose for 3-4 month old pigs was characterised as 6×10^3 CFU, and transmission of *E. coli* O157:H7 to naïve swine was established through faecal shedding by infected swine (Cornick and Helgerson, 2004). Pigs are also responsible for the dissemination of other foodborne pathogens. One pig farm in Denmark linked with multiple infections of *S. enterica* applied contaminated slurry to agricultural land, isolating the pathogen 2 weeks later (Baloda et al., 2001).

1.5.5 Wild animals

The spread of foodborne pathogens to agricultural land may be facilitated by other wild animal carriers. Rats infected with 10^9 CFU of *E. coli* O157:H7 shed the pathogen for up to 11 days, whereas similarly infected pigeons shed for up to 29 days (Cizek et al., 2000). These animals are able to contaminate agricultural land through defecation, after which the bacteria may spread to crops. While the presence of *E. coli* O157 is rare in pigeons, it has been documented. One study recorded that one of 99 birds tested were infected in one farm (Shere et al., 1998). A further study in

Denmark isolated STEC from a starling and a brown rat, identical to isolates found in infected cattle in separate farms (serogroups O2 and O136 respectively). This demonstrates the capacity for farm animals to transmit foodborne pathogens to wild animals and vice versa (Nielsen et al., 2004).

The contribution of birds to the contamination of agricultural land has been explored more recently. Should they possess enteric populations of STEC, they could distribute foodborne pathogens over wide tracts of land through faecal shedding while airborne. Several studies have gathered evidence supporting this possibility, isolating *E. coli* O157 from avian faeces. Through the use of selective culture media, 0.9% and 2.9% of 349 and 342 avian faecal samples, primarily belonging to gulls, possessed *E. coli* O157 at a landfill site and on intertidal sediments respectively. The author observes that although the proportion of contaminated samples is low, there is a large population of gulls and they have been known to roost on nearby farmland (Wallace et al., 1997). Another study using similar techniques found that of 231 faecal samples collected over 36 months, only one was found positive for *E. coli* O157, although the infected bird in this case was likely to be a chaffinch, greenfinch, blackbird, or house sparrow (Foster et al., 2006).

A study carried out in Canada further revealed the potential for carriage of STEC by a range of birds. The *stx2* gene was detected by qPCR in 23% of 412 *E. coli* isolates collected across 15 avian species, where the positive samples originated from 8 species. The species in which the gene was most prevalent were raven, turkey and pigeon, where isolates also originated in mallard ducks and pheasants. The *eae* gene, indicative of enteropathogenic *E. coli*, was also detected in 15% of samples across 11 bird species. It was found that five strains isolated from pheasant were enterohaemorrhagic *E. coli* other than O157, due to the absence of the rfb_{O157} gene (Chandran and Mazumder, 2014). The presence of the *stx2* gene suggests that the corresponding *E. coli* isolate is STEC, and the range of birds that it is present in supports the idea that wild birds can disseminate the pathogen unchecked across farmland.

In addition to the contamination of agricultural land through their own defecation, research has been carried out investigating the role of wild birds in the contamination of livestock feed, causing the introduction of foodborne pathogens to agricultural land through the faecal shedding of infected cattle. One study compared the number of sightings on different farms of European starlings, known to eat livestock feed, with the incidence of *E. coli* O157:H7 in faecal samples from cattle belonging to different farms. It was found that the prevalence of the pathogens in bovine faeces was correlated to the number of starlings seen on site relative to the number of milking cows present on the farm. Farms containing infected cows have a mean of 1 starling seen per visit per milking cow, whereas uninfected farms had a mean of 0.5 starlings seen per visit per milking cow (Cernicchiaro et al., 2012). The impact of contaminated wild birds on contamination of agricultural soil has not yet been fully elucidated, although these studies provide mounting evidence that they are able to transmit STEC to fresh produce in a number of ways.

While invertebrates might prove inefficient at transmitting foodborne pathogens to farms, their carriage may expedite the introduction of pathogens to the crops themselves. The common agricultural pest, the grey field slug (*Deroceras reticulatum*) was assessed as a vector of *E. coli* O157. The prevalence of *E. coli* O157 was estimated at 0.21%, or 1.44/m^2 infected slugs. Experiments confirmed that the pathogen could remain in the slug for several days, granting it enough time to transfer the pathogen from contaminated soil to crop plant (Sproston et al., 2006). Worms have also been realised as capable vectors of *E. coli* O157:H7. Ingestion of an inoculum by earthworms resulted in movement of the pathogen in the horizontal and vertical planes, depending on the species of worm. In the long-term, the *E. coli* O157:H7 survival was unaffected by passage through the earthworm intestine, even being augmented by the worms' effect on the surrounding soil (Williams et al., 2006). Foodborne pathogens could reach their plant hosts through adhesion to the surface of plant-parasitic nematodes, such as *Meloidogyne javanica. E. coli* strain TG1 was able to persist on the nematodes for the 4 weeks tested, even multiplying between weeks 2 and 4, however

this may have been due to the bacteria growing on dead nematodes (Maghodia et al., 2008). Even protozoa have been found to aid the survival of foodborne pathogens in soil. *E. coli* O157 has been found in *Acanthamoeba polyphaga*, after being internalised into protozoan vacuoles, some containing over ten bacterial cells evading digestion. It was suggested that *E. coli* could use the protozoan vector to spread itself about the environment as the amoeba cysts can be carried by the wind (Barker et al., 1999).

1.6 Survival of foodborne pathogens in soil

The contamination of plants by foodborne pathogens depends on their ability to survive in soil, providing them with more time to come into contact with a suitable plant host. Studies have shown *E. coli* O157:H7 is able to survive in different soils for different lengths of time. For example, one study detected *E. coli* O157:H7 in a range of soils: up to 31 days in soil from Salinas Valley and only up to 15 days in soil collected from Yuma, AZ (Ma et al., 2012). Another study could detect the pathogen for 21 days in soil maintained in greenhouse conditions, compared with 17 days in soil collected from an open field (Yao et al., 2013). A wider study examining 36 different soil types could detect the pathogen for 54 – 104 days (Franz et al., 2008), while there have been reports claiming *E. coli* O157:H7 can persist for over one year in soil (Wang et al., 2014a). *L. monocytogenes* is abundant in soil, where one survey isolated the pathogen in 8.3% of cultivated field samples and 30.8% of uncultivated field samples. The author proposed that the difference was due to the association of *L. monocytogenes* to the rhizosphere, which is less frequently disturbed in uncultivated fields. The pathogen was cultured for a month following inoculation to soils, but the experiment ended before it lost culturability (Dowe et al., 1997). These studies primarily focus on detection by culture techniques, there is the possibility that VBNC pathogens may persist much longer.

Aside from the obvious discrepancies in *E. coli* O157 survival time caused by differences in experimental design, the varying times have been attributed to the organic carbon and nitrogen contents in soils (Ma et al., 2012), in addition to high pH and indigenous microorganisms affecting

the establishment of the pathogen in soil (Yao et al., 2013). Further evidence supporting this was obtained, where survival time of *E. coli* O157:H7 in soil was positively and negatively correlated with the relative abundances of *Acidobacteria* and *Chloroflexi* populations respectively (Wang et al., 2014b).

The common practice of amending agricultural soil with manure may significantly enhance the survival time of foodborne pathogens. Where *E. coli* O157:H7 was detected for only around several weeks in soil, there have been reports of it persisting for up to 92 and 231 days in manure-amended soil. Survival of *Salmonella* Typhimurium in bovine manure-amended soil has been similarly reported at 231 days (Mukherjee et al., 2006; Ongeng et al., 2013). A study comparing the longevities of foodborne pathogens in both liquid slurries and following application of solid manure to agricultural land found that *E. coli* O157, *Salmonella* Typhimurium and *C. jejuni* survived for 3 months in liquid but only up to one month after application to land. *L. monocytogenes* was detected in slurry for 6 months, and for longer than one month after application to land (Nicholson et al., 2005b).

The complexity of pathogen survival in soil deepens as it has been suggested that the diet of cattle affects survival of pathogens in its manure; ingestion of tanniferous forage plants by cattle could impede the survival of shed *S. enterica* and *E. coli* O157:H7 (Ongeng et al., 2013). A further study showed that *E. coli* O157:H7 survival was reduced by a higher fibre diet, although *Salmonella* Typhimurium survival was unaffected (Franz et al., 2005).

The movement of pathogens through soil has also been assessed; their distribution throughout soil enhances their ability to contaminate crops in agricultural land. Macropores and tubular pores caused by earthworms, insects and plant roots have been cited as passages for microbial movement (Mittal, 2004). One study concluded that *E. coli* O157:H7 is able to survive for two months if allowed to travel beneath the top layers of soil by rainfall. It was also indicated that soluble nitrogen may aid transport of the pathogen through soil (Gagliardi and Karns, 2000).

1.7 Transmission of foodborne pathogens to plants

Agricultural land provides a vast area across which foodborne pathogens can be deposited and can survive. Despite the previously mentioned occurrences of infection directly from contaminated soil and manure, the primary threat of foodborne pathogens arises following their transmission to a food vehicle. Transmission of foodborne pathogens to crop plants from soil can be as complex as carriage by nematodes (Maghodia et al., 2008) or as simple as splashing of soil particles onto the phylloplane by rainfall or water gun irrigation (Heaton and Jones, 2008). Colonisation of plants by the pathogens may even occur from the germination of seeds in contaminated soil.

One study showed the effect of the seasons on colonisation of vegetables. *S. enterica* and *E. coli* O157:H7 were isolated more frequently on the phylloplane of plants grown after a June application of contaminated manure than those from March (Natvig et al., 2002). Alternatively, contaminating bacteria can be introduced to established plants. A study examined routes of contamination of lettuce by *E. coli* O157:H7, finding that over 9 weeks in compost inoculated with 10^6 CFU/g, 62.5% of samples were contaminated with the pathogen on their outer leaves, and 18.8% were contaminated on their inner leaves. While the population decreased over the 5 weeks examined, viable *E. coli* O157:H7 cells were still culturable at the end of the experiment. Lettuce contaminated by sprinkling with contaminated water at 10^7 CFU/ml harboured *E. coli* O157:H7 cells that could only be detected by enrichment after 4 weeks, as direct plating was ineffective (Oliveira et al., 2012). The negative result generated by direct plating may be indicative of VBNC cells, due to the hostile nature of the phylloplane environment.

Foodborne pathogens can be deposited directly onto the phylloplane by insect vectors. It has been shown that *E. coli* is able to adhere to the Mediterranean fruit fly (*Ceratitis capitata*) and contaminate apples. The bacterium is also able to survive for 7 days on the fly (Sela et al., 2005). This allows for greater dissemination of a pathogen from a contaminated source, such as manure, which insects commonly visit. However, foodborne pathogens do not necessarily require a vector to

colonise the phyllosphere; from contaminated soil it is possible for them to be internalised at the rhizosphere and travel through the plant's vasculature to reach the interior of the leaf. Solomon et al showed that using a high concentration inoculum in manure, the edible portion of lettuce plants can become contaminated by *E. coli* O157:H7 (Solomon et al., 2002).

The wide range of ways by which pathogens can reach the phyllosphere is negated by the relatively harsh conditions of the leaf surface. Contamination of the plant is not assured solely by the presence of the pathogen. One study detected *E. coli* O157:H7 in 8 of 10 manure samples and 10 of 10 slurry samples in which lettuce was grown. Despite this, the pathogen was not detected in any lettuce sample at harvest (Johannessen et al., 2004). Another study found that when inoculated via droplets of aqueous manure, *E. coli* O157:H7 survival had decreased to undetectable levels in fewer than 14 days. Soil inoculated at an equal concentration maintained detectable numbers of the pathogen throughout the 28 day study (Patel et al.). However, the detection method used was the culture-based MPN (most probable number) method, so any cells in a VBNC state were left unexamined. There is evidence of a positive correlation between inoculum concentration and the association of *E. coli* O157:H7 with lettuce roots (Fremaux et al., 2008), which could account for the failure of the pathogen to effectively colonise the phyllosphere in these discussed studies. The efficacy of transmission of foodborne pathogens to the plant phylloplane is dependent on a wide range of factors, although under the right circumstances they are able to persist on the leaf surface for considerable periods of time.

1.8 Survival on the phylloplane

For foodborne pathogens, the phyllosphere is a relatively inhospitable environment. It undergoes dramatic changes in temperature and humidity, with moisture only transiently available in the form of dew, rain and through the use of spray irrigation. The leaf surface also exposes its inhabitants to UV radiation, while providing them with few nutrients (Lindow and Brandl, 2003). In addition, some plants' innate defences have evolved to recognise pathogen-associated molecular patterns (PAMPs)

of foodborne pathogens. For example, *Salmonella* Typhimurium flagella and type-three secretion-system (TTSS) elicits an immune response in a number of plants (Iniguez et al., 2005), however is able to evade these defences and establish itself as an endopathogen (Schikora et al., 2008).

These conditions do not completely prohibit the survival of foodborne pathogens on the phyllosphere; successful pathogens are able to survive on the leaves of crop plants for months. *Salmonella* Typhimurium was detected on lettuce leaves for up to 161 days in one study (Islam et al., 2004b), whereas another found *Salmonella* Typhimurium present on arugula leaves 17 weeks after contaminated manure was applied (Natvig et al., 2002). It has been observed that *S. enterica* may be better suited to life on the phylloplane than *E. coli* O157:H7, a hypothesis that is supported by the greater prevalence of produce-associated *S. enterica* outbreaks. A study tested multiple strains of each pathogen to find that *S. enterica* grew to higher levels than *E. coli* O157:H7 on 2-day old alfalfa sprouts (Charkowski et al., 2002). Despite the short time period of this study, its results are reflected in other studies of *E. coli* O157:H7 survival on the phylloplane. Compared with the considerable number of months during which *S. enterica* was detected on arugula leaves, *E. coli* O157:H7 inoculated onto 2 week old lettuce survived for fewer than 28 days (Moyne et al., 2011). A further study found that *E. coli* O157:H7 was only detectable on spinach shoots for fewer than 14 days (Patel et al.). While these studies do indicate the relative fitness of different foodborne pathogens on the phylloplane, they are not directly comparable due to the difference in plant hosts and experimental conditions. Furthermore, the presence of VBNC *E. coli* O157:H7 was not tested for, which could supplement the population of the pathogen on the leaf surface.

Despite the great range of factors affecting survival of foodborne pathogens on the phylloplane, some have been extensively characterised. For example, damage to the leaf surface can abate the dearth of nutrients that otherwise restrict the growth of foodborne pathogens on the phylloplane. The survival of *E. coli* O157:H7 was examined on lettuce leaves which had been either mechanically injured or infected with the plant pathogen *Xanthomonas campestris*. Over ten days, *E. coli* O157:H7

populations decreased much less on damaged leaves than on healthy ones, at averages of 2.1 x 10^5 and 1.9 x 10^3 CFU per leaf respectively (Aruscavage et al., 2008). It has also been found that molecules expressed by *E. coli* O157:H7 can induce plant defence pathways, reducing survival of the pathogen on plants. One study generated *E. coli* O157:H7 mutants lacking either curli (Δ*csgD*) or flagellin (Δ*fliC*), to find that on *Arabidopsis* plants, Δ*fliC* mutants maintained a population approximately 30 times greater than the wild type pathogen after 5 days. Results also indicated that the wild type *E. coli* O157:H7 induced twice the immune response compared with the Δ*fliC* mutant, and thrice that of the Δ*csgD* mutant. Together, these results suggest that protein structures on the surface of *E. coli* O157:H7 can be recognised by plants to raise a defence response, influencing their survival on the phylloplane (Seo and Matthews, 2012).

L. monocytogenes has been isolated from a range of salad vegetables and several studies have assessed its ability to survive upon the phylloplane. The pathogen flourished on lettuce leaves, increasing in number over one week following inoculation by 2 orders of magnitude (Li et al., 2002). Another study shows *L. monocytogenes* growing on lettuce over two weeks, plateauing by day 12 at 10^7 CFU/g (Carrasco et al., 2008). These results demonstrate the ability of *L. monocytogenes* to thrive on the phylloplane; however both studies were conducted using cut lettuce, so previously discussed factors such as damage to the leaf surface and the lack of a plant defence response could enhance the survival of the pathogen.

1.9 Internalisation into the phyllosphere

Due to the hostile nature of the phylloplane, internalisation into the salad leaf can yield a number of benefits to foodborne pathogens. Some of the stresses induced by irradiation and fluctuating humidity are alleviated, as well as the paucity of nutrients as found on the leaf surface (Lindow and Brandl, 2003). The most prominent opening into the leaf mesophyll is through the stomata. While usually open to allow gas exchange, it has been documented in having an innate immune function as it closes in response to plant and human pathogens. Within 2 hours of infection by with *E. coli*

O157:H7, the average *Arabidopsis* stomatal aperture widened from 3 µm to under 1.5 µm, and only decreased over the following 6 hours (Melotto et al., 2006). The existence of this defence mechanism emphasises the value of internalisation to the pathogen, particularly when considering the prevalence at which it does so. Internalisation of pathogens into leaf tissues of fresh produce creates a further challenge for the agricultural industry when preparing to sell the plants as food.

Despite plant defences, many studies have investigated the ability of foodborne pathogens to internalise through the stomata. One study found that *S. enterica* is unable to enter lettuce leaves from the phylloplane while stomata are closed, however open stomata are not sufficient to induce internalisation. Stomata chemically forced open in darkness yielded no internalisation of the pathogen, where internalisation in the light was observed; it is likely that this is due to light inducing taxis toward stomata in *S. enterica*. Mutants deficient in either motility or chemotaxis were generated to better elucidate the processes involved in internalisation of *S. enterica*, and entry into the lettuce leaf was greatly impaired in both, showing *S. enterica* is actively drawn towards stomata in order to internalise into the lettuce leaf (Kroupitski et al., 2009). Furthermore, internalisation of *S. enterica* was observed within 2 hours of inoculation, indicating that the pathogen does either not possess the recognition patterns that cause stomata to close when exposed to *E. coli* O157:H7, or it has evolved a method to subvert the plants' defences (Melotto et al., 2006; Kroupitski et al., 2009). The difference in response across these studies could also be due to the difference in plants used in the studies, although internalisation of *S. enterica* has been documented in a number of salad plants. A survey was carried out estimating the relative distribution of the pathogen on and inside salad plants, including iceberg lettuce, romaine lettuce, red lettuce and arugula. In all cases, a higher number of *S. enterica* cells were found in the internal tissues of the plants than on the surface (Golberg et al., 2011). Figure 2 shows the colonisation of the spinach phylloplane by *Salmonella enterica* serovar Thompson localised primarily at the stomata, facilitating internalisation (Warner et al., 2008).

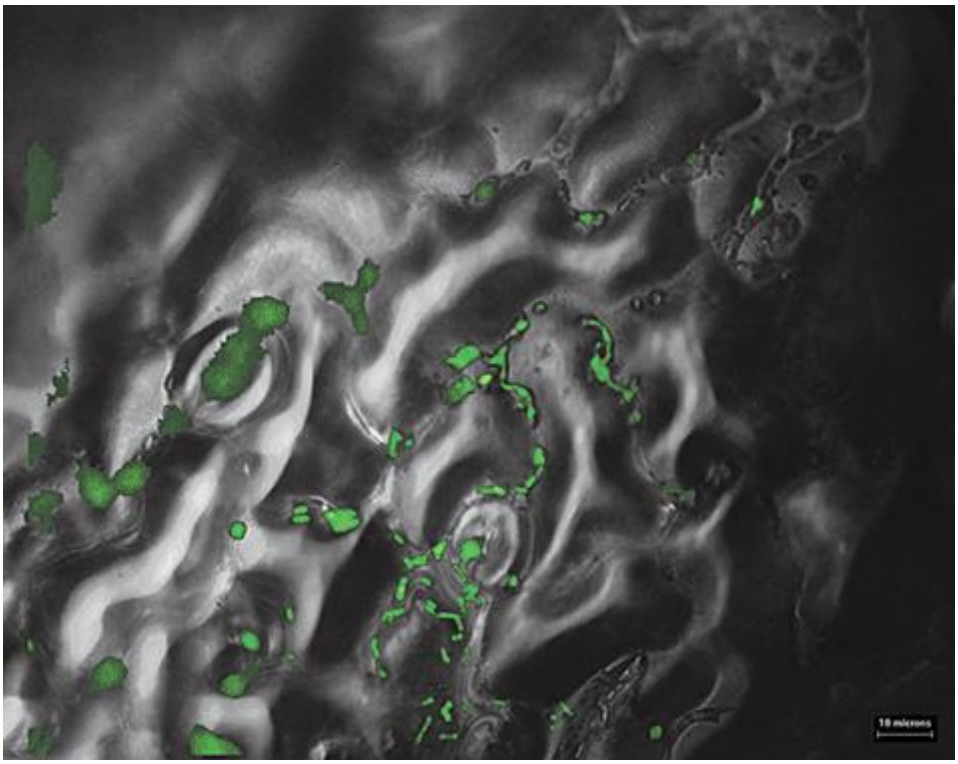

Figure 2. *Salmonella* Thompson expressing green fluorescent protein on the spinach phylloplane, using episcopic differential interference contrast microscopy and epifluorescence microscopy. Scale bar represents 10 micrometres. Image taken from Warner et al. 2008 (Warner et al., 2008).

E. coli O157:H7 is also able to contravene plant innate immunity to colonise internal tissues of the phyllosphere. Contaminated lettuce and spinach leaves were treated with gentamicin for 2 hours and homogenised so that any bacteria surviving within the leaf could be detected. *E. coli* O157:H7 was detected, though culture methods were used for detection so any VBNC cells were not represented, suggesting the ability of the pathogen to internalise within the leaf. This does not necessarily mean that *E. coli* O157:H7 is capable of penetrating the plant through its stomata; while the pathogen was concentrated on the stomatal guard cells (visualised using immunofluorescence microscopy), the stomata remained closed. Δ*fliC* and Δ*escN* mutants were less able to invade

spinach leaves, which implies a role for flagella and the TTSS in *E. coli* O157:H7 internalisation (Xicohtencatl-Cortes et al., 2009). The stomata are not the sole gateway to the internal tissues of the phyllosphere, wounds on the leaf surface could also provide an entrance for the foodborne pathogen.

Internalisation into the tissues does not only occur at the phylloplane. There is evidence that foodborne pathogens can internalise at plant roots and travel through the plant vasculature to the phyllosphere. Lettuce seeds were planted in manure contaminated with GFP-tagged *E. coli* O157:H7 and internal tissues of the resultant seedlings were examined for the presence of the pathogen, finding *E. coli* O157:H7 at up to 45 µm beneath the plant surface (Solomon et al., 2002). As there was no phylloplane through which the bacterium could enter, it is likely that it was internalised through the roots. The inessentiality of the phyllosphere in *E. coli* O157:H7 internalisation was verified by irrigating mature plants with contaminated water, keeping the aerial portion of the plant separate from the soil. Within 5 days of irrigation, *E. coli* O157:H7 was found within the edible leaf tissues of the lettuce (Solomon et al., 2002), providing evidence that exposure to the rhizosphere is sufficient for *E. coli* O157:H7 contamination of the internal tissues of the lettuce leaf. Later experiments concluded that *E. coli* O157:H7 was not taken up by lettuce seedlings from contaminated manure, having tested at 3 and 7 weeks following planting (Johannessen et al., 2005). However, these studies are not directly comparable, having a number of differences including a lower *E. coli* O157:H7 concentration in the latter study, and in the former study introducing the pathogen to the plant at an earlier stage in its life (Solomon et al., 2002; Johannessen et al., 2005). The inability to detect *E. coli* O157:H7 could also be due to the length of time passed before testing (3 weeks), as survival time of the pathogen within the leaf has not yet been examined. Contaminating growing media with *L. monocytogenes* also led to internalisation of the pathogen in leaves of several edible plants, including in lettuce for up to 80 days, however internalisation was only observed after incubating the plant at 24^{o}C and not at 30^{o}C (Chitarra et al., 2014).

The effect of time at which plants are exposed to contaminated soil has since been investigated. *E. coli* O157:H7 or *S. enterica* was exposed to cabbage seedlings at different time-points over 105 days and at different concentrations of inocula. It was found that the pathogens were only present inside the leaf when exposed to manure inoculated with the high pathogen concentration of 10^7 CFU/g, and when inoculation took place at the point of seedling transplantation (Ongeng et al., 2011). Not only does this show that internalisation at the root occurs at exclusively high concentrations of contamination, but that seedlings' roots are more susceptible to internalisation than mature plants'. The 120 day time span of the experiment also gives an indication of the length of time the bacterium can persist within the plant. Both *E. coli* O157:H7 and *S. enterica* were detected in 100% of cabbages under these conditions, showing that *S. enterica* is also able to internalise through plant roots and travel to the phyllosphere (Ongeng et al., 2011).

There is some question as to whether *E. coli* O157:H7 is able to internalise in plant roots without there being some root damage. It was hypothesised that as *E. coli* O157:H7 internalisation through roots is more common in plants grown in soil than hydroponically, root damage caused by soil could be facilitating colonisation of the interior of the plants. Lettuce plants grown in hydroponic media contaminated with *E. coli* O157:H7 led to only 3.7% samples having internalised the pathogen; however after damaging the roots by pruning, 64% samples were found to have *E. coli* O157:H7 present, detected by direct plating (Macarisin et al., 2014). While root damage may aid internalisation of foodborne pathogens, there is currently no evidence of it being necessary for the process.

Foodborne pathogens may be able to gain entry to the inner leaf tissues through damage to the leaf surface. Seo and Frank determined through confocal microscopy that *E. coli* O157:H7 would adhere preferentially to cut edges of lettuce leaves, and was found up to 100 µm below the surface at cut edges (Seo and Frank, 1999). While cut edges could benefit pathogens on the leaf surface due to the increase in nutrients, the presence of bacterial cells internalised beneath the cut edges suggest that

E. coli O157:H7 is localised there so that it can persist within the lettuce tissues. Regardless of the passage into the leaf tissues, internalisation of foodborne pathogens drastically affects food safety. It has been stated that decontamination of salad leaves using the current 200 ppm chlorine is ineffective against internalised pathogens, and ozone may be unable to reach bacteria within the leaf (Gomes et al., 2011).

1.10 The antimicrobial effect of chlorine

Currently, decontamination of fresh produce in the UK relies heavily on the use of chlorine, with the aim of removing spoilage bacteria and foodborne pathogens. However, recent studies coupled with persisting outbreaks of foodborne disease question the efficacy of this treatment. It has been determined that formation of biofilms on the leaf surface impedes the efficacy of chemical treatments, rendering current sanitisation practices inadequate. On stainless steel, it was discovered that 50 ppm chlorine could render *E. coli* O157:H7 cells in the planktonic phase unculturable within one minute, however when 200 ppm was applied to cells in a biofilm, a population persisted for the entire 5 minutes tested (Ryu and Beuchat, 2005). This protection extends to the phylloplane. A study on *E. coli* O157:H7 cells attached to spinach and lettuce leaves found that a 3 minute wash using 600 ppm chlorine only reduced bacterial populations by up to 1.77 log CFU/g. Furthermore, this reduction was only achieved when chlorine treatment immediately followed bacterial inoculation. The antimicrobial effect of chlorine was found to be lessened when used on bacterial cells that had been allowed to develop biofilms on the leaf surface (Niemira and Cooke, 2010).

Other studies have corroborated the resistance of biofilms to sanitisation using chlorine. One recorded both *E. coli* O157:H7 and *L. monocytogenes* populations decrease by over 3 log CFU/g following exposure to 100 ppm chlorine 6 hours after inoculation; a 48 hour incubation time yielded a 1 log reduction in *E. coli* O157:H7 and a 1.5 log reduction in *L. monocytogenes*. This is attributed to the pathogens forming biofilms on the lettuce phylloplane during incubation (Ölmez and Temur, 2010). While these studies demonstrate the potency of chlorine following a short incubation time, in

a factory setting, pathogens on contaminated leaves would have had time to establish biofilm communities some time prior to the sanitisation step (depending on the source of the contamination), so the utility of chlorine in the decontamination of salad leaves may be limited. VBNC cells were also undetected by this study as culture methods were used, so their tolerance to chlorine treatment was not examined.

Washing vegetables in chlorinated water has led to some health concerns due to its production of trihalomethanes, so chlorine dioxide has been proposed as a safer alternative (Kim et al., 2008). Its efficacy in the removal of *E. coli* O157:H7 from lettuce leaves has been compared with that of chlorine, showing that chlorine dioxide is also a more effective sanitiser. After 18-24 hours incubation on lettuce, a reduction of 0.91 log CFU/g was observed in *E. coli* O157:H7 populations following exposure to chlorine, whereas chlorine dioxide of the same concentration (200 ppm) caused a 1.13 log CFU/g reduction. Despite this reduction, 200 ppm chlorine dioxide affected the lettuce quality by bleaching the cut edges, so perhaps this treatment is also unsuitable for sanitisation of salad leaves in a commercial setting (Keskinen et al., 2009). Lower concentrations of chlorine dioxide have been tested on several foodborne pathogens to assess its use, circumventing the damage caused to the plant product. The results collected from this study observe a much more potent effect of chlorine dioxide on the pathogens, with 50 ppm reducing *E. coli* O157:H7 by 1.38 log CFU/g. Similarly, *Salmonella* Typhimurium decreased by 1.95 log and *L. monocytogenes* by 1.2 log (Kim et al., 2008), all greater reductions than that which was achieved by chlorine dioxide on *E. coli* O157:H7 in the previously discussed study. This may have been due to the incubation time following inoculation, this study only mentions a 30 minute period to allow the inocula to air dry, as opposed to the 24 hours of the former study (Kim et al., 2008; Keskinen et al., 2009). The increased resistance to sanitisers as a consequence establishment on the phylloplane corroborates with studies on decontamination of biofilms from salad leaves (Ölmez and Temur, 2010). It was also found that storing the leaf samples for five days after treatment caused the continued decrease in populations

of *E. coli* O157:H7 and *Salmonella* Typhimurium, but not in *L. monocytogenes*, which steadily increased in population (Kim et al., 2008). For this foodborne pathogen, these results indicate that 50 ppm chlorine dioxide is insufficient for decontamination as surviving bacteria are able to re-colonise the phylloplane.

While bacterial response to oxidative stress has been extensively studied, the mechanisms beneath the response to chlorine stress are relatively unexplored. Research that has taken place indicates that reactive chlorine species mediate bacterial cell death through interfering with processes at the inner membrane, such as protein and metabolite transport across the inner membrane, inhibition of DNA replication, and loss of ATP (Albrich et al., 1986; Barrette et al., 1989; Rosen et al., 1990; Gray et al., 2013). This conclusion has been reached due to hypochlorous acid (HOCl) concentration required for the inhibition of these processes correlating with the concentration required for cell death, while the required concentration for the inactivation of sensitive cytoplasmic enzymes is greater than the lethal dose (Albrich et al., 1986; Gray et al., 2013). Figure 3 shows the current understanding of the cytotoxic effect of reactive chlorine species and the bacterial mechanisms of defence.

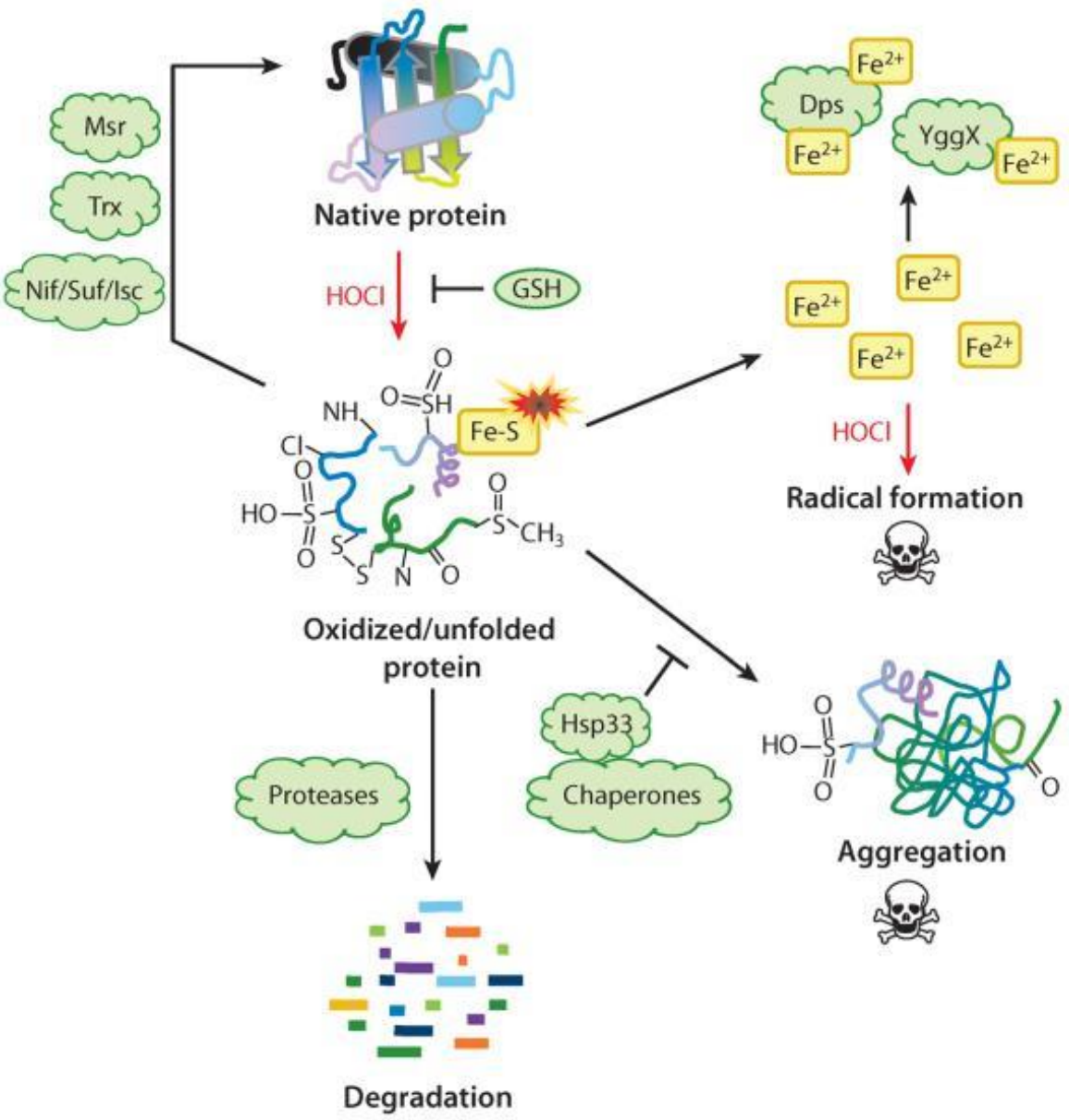

Figure 3. Bacterial response to damage by reactive chlorine species. Image taken from Gray et al. 2013 (Gray et al., 2013).

HOCl causes side chain oxidation and unfolding by reacting with proteins. Enzymes Msr and Trx, and Fe-S repair systems (Nif/Suf/Isc) reverse this damage, and thiols such as GSH react with reactive chlorine species to reduce their total concentration in the cell. Damaged proteins that release metal ions can catalyse oxidative damage, so these ions are sequestered (Dps and YggX). Unfolded proteins

can also lead to aggregation and cell death, so this effect is impeded by the action of chaperone proteins, such as Hsp33. Irreversibly damaged proteins are degraded by proteases (Gray et al., 2013).

1.10.1 VBNC induction by chlorine

In addition to multiple studies finding chlorine an ineffective treatment for decontamination of produce, there is also evidence that chlorine treatment can induce the VBNC state in bacteria. This extends to foodborne pathogens such as *Salmonella* Typhimurium. Culture techniques were coupled with direct viable counts (DVC) to determine that exposure to 5.5 ppm chlorine for 5 minutes resulted in 50% of *Salmonella* Typhimurium cells becoming VBNC in a 4 day old biofilm. The tolerance to chlorine was increased when *Salmonella* Typhimurium was co-cultured with *P. fluorescens* (Leriche and Carpentier, 1995). This study was designed to mimic the disinfection practices of the food industry, highlighting the issues surrounding chlorine disinfection.

Chlorination of wastewater and drinking water has also led to research being carried out assessing VBNC induction. *E. coli* and *Salmonella* Typhimurium were subjected to a combination of free chlorine and sodium hypochlorite totalling 1.7 ppm chlorine for up to one hour. It was determined that while 99% cells were killed, 10^4 per ml persisted in the VBNC state (Oliver et al., 2005). A study assessing the sensitivity of *Legionella pneumophila* to chlorine found that after 30 minutes of exposure to 0.7 ppm free chlorine, the number of culturable cells fell beneath the detection limit. The total number of cells remained stable relative to the negative control (Gião et al., 2008). Although there has been research carried out assessing the potential for VBNC formation of foodborne pathogens in response to chlorine, there is little that puts this research into the context of the phylloplane; the physical protection attained by bacterial adherence to leaf cells, and the risk that VBNC pathogens pose to the public following chlorination.

1.11 Detection of VBNC cells

As VBNC cells by definition cannot be detected by culture techniques, their enumeration requires alternate methods. The quantitation of VBNC cells most often compares culture techniques to DVC, citing the method of Kogure *et al*, which causes the elongation of bacterial cells, inhibiting action of DNA gyrase by incubating with nalidixic acid (Kogure et al., 1979). Using this method allows for the easy enumeration of bacteria, particularly when analysing complex environmental samples, and confirms viability of cells through their elongation. Incubation with pipemidic acid can be used to the same end (Juhna et al., 2007).

While enumeration under the microscope is the most direct method to quantify VBNC cells, in most cases a fluorescent stain is required to aid visualisation or for the selection of certain species. The *Bac*Light™ LIVE/DEAD stain is frequently used to determine viability of bacterial cells, where the fluorescent green stain SYTO9 will stain viable cells with intact membranes green. The larger counterstain propidium iodide will displace SYTO9 in cells with compromised membranes, staining them red to indicate dead cells (Gião et al., 2008; Lindbäck et al., 2010). However, this selection method relies on damage to bacterial cell membrane to define non-viability, so should be used with caution. To select for specific bacteria in conjunction with DVC by cell elongation, fluorescence *in situ* hybridisation (FISH) can be used. In complex samples with the potential for multiple bacterial species, DNA or peptide nucleic acid (PNA) probes can be used to target specific sites within cells. PNA-FISH has been used to target a conserved region of the *E. coli* O157 23S rRNA to facilitate its detection in food samples (Almeida et al., 2013).

Molecular methods of detection can also be used to detect VBNC cells. Quantitative polymerase chain reaction (qPCR) uses primers either tagged with a fluorescent protein, or fluorescent dyes that intercalate between DNA strands, to measure the quantity of specific bacterial DNA in a sample, which can be correlated to a total number of cells (Wittwer et al., 1997). While this completely

avoids any need for culture techniques, detection of total DNA can result in detection of dead cells. To remedy this, PCR and qPCR can be coupled with the reverse transcription of mRNA, which in human cells has been found to have a half-life of only 1 hour (Schmittgen et al., 2000), ensuring the source of the genetic material would not have been a long dead cell. This technique has been used to determine expression of virulence genes in VBNC *V. parahaemolyticus* (Coutard et al., 2005). Viability can also be selected for by treating samples with ethidium monoazide (EMA) or propidium monoazide (PMA) prior to quantitation by qPCR. This occurs via the intercalation of EMA or PMA between DNA strands of cells with compromised bacterial membranes, which prevent replication during qPCR. This method has previously been used to differentiate between heat-killed and VBNC *L. pneumophila* (Slimani et al., 2012).

The question that drives a substantial portion of VBNC research is whether VBNC pathogens pose any threat to humans. To this end, several experimental avenues have been explored to measure the infectivity of pathogens in this state. Mouse models are often used, particularly for foodborne pathogens, as they can replicate the symptoms of foodborne disease in humans, and go some way to replicating conditions of the human digestive tract (Asakura et al., 2002; Cappelier et al., 2005; Zeng et al., 2013). A study has also used embryonated chicken eggs to determine the capacity of resuscitated VBNC *L. monocytogenes* to cause infection (Cappelier et al., 2007). Animal cell lines lack the features of a digestive tract but can provide a simple model to measure cytotoxicity caused by foodborne pathogens. Vero cells have been used to assess Shiga-toxin activity of VBNC *E. coli* O157, which causes the lysis of cells (Liu et al., 2010). Human adenocarcinoma cells have also been used in plaque forming assays, to assess the infectivity of *L. monocytogenes* while in the VBNC state (Cappelier et al., 2005).

Caenorhabditis elegans is a suitable animal model for studying foodborne pathogens (Labrousse et al., 2000), as it has a defined digestive tract, but is free from the ethical constraints that come with animal models such as mice. It can also model infection by ingestion, where mouse models often

require intravenous inoculation of pathogens. However, as bacterial inocula to be assessed using *C. elegans* are commonly grown on agar plates, they have not been explored as models for VBNC pathogens.

1.12 Experimental overview

The research reviewed here exhibits the potential for contamination of fresh produce by foodborne pathogens, and the inability of chlorine to effectively decontaminate the products before they reach consumers. This, coupled with the potential for VBNC induction of foodborne pathogens prior to and due to food production practices presents a clear challenge to the food industry; how can VBNC contamination be detected in agricultural materials, and are produce-associated VBNC pathogens a risk to public health?

In the first paper, 'Improved sample preparation for direct quantitative detection of *Escherichia coli* O157 in soil using qPCR without pre-enrichment', an assay is developed to detect and quantify contamination of agricultural soil that does not exclude VBNC cells. To bypass culture techniques, qPCR is used, where it was determined that the assay would act as a pre-screen, and further analysis would discriminate between positive results caused by living and dead cells. The assay focuses on the preparation of complex environmental samples, where analysis of soil samples by qPCR would either be restricted by sample size or inhibited by the presence of humic acids (Tsai and Olson, 1992). The assay was specific for the detection of *E. coli* O157 as it is a particularly relevant foodborne pathogen responsible for recent outbreaks of disease (Anon., 2014), however as the specificity is dependent entirely on the primer used, any foodborne pathogen or bacterial species could potentially be detected.

This paper aims to improve the current culture-based detection method for contamination of agricultural soil by foodborne pathogens, and addresses the absence of VBNC pathogen detection in an environment where its presence is highly likely. Following recent outbreaks of *E. coli* serogroup O55, no source could be identified (McFarland et al., 2017). This work could help to identify

contamination sources in the wake of foodborne disease outbreaks, and confirm lack of contamination of agricultural material to prevent its unnecessary destruction when an outbreak source has not been confirmed. Beyond the direct applications to the food industry, this research provides the necessary tools for the investigation of complex environmental matrices regarding contamination by VBNC pathogens.

The paper was published in the journal Microbial Biotechnology. Author Callum Highmore carried out all experiments, determined the experimental direction, analysed the data, and prepared the paper. Author Dr Steve Rothwell provided an industrial perspective for the applications of the work, and author Professor Bill Keevil managed the research and reviewed the submitted manuscript.

The second paper, 'Survival of viable but nonculturable *Escherichia coli* O157 in horticultural growing media', utilises the findings of the first paper to detect VBNC *E. coli* O157 *in situ* in horticultural growing media, realising the threat of VBNC pathogens in soil. The assay was applied to pristine horticultural soil to reveal *E. coli* O157 genetic material in the soil, and PNA-FISH in conjunction with cell elongation and culture techniques was used to determine the presence of VBNC cells in the soil sample. The pathogen was then resuscitated through prolonged incubation in enriching media, affirming the potential danger of the presence of VBNC pathogens in an agricultural environment.

Previously, VBNC *E. coli* O157 has been discovered in river water (Liu et al., 2008), confirming its presence in the environment. This research applies that premise directly to the food industry, showing that VBNC pathogens do exist in that environment and therefore their detection by industry is necessary for the improvement of food safety. In detecting VBNC *E. coli* O157 in nature, further questions are raised about the prevalence of VBNC pathogens in the environment. This is particularly true for agricultural land, where numerous aforementioned factors suggest that it is especially prone to contamination. The methods outlined in the first paper can be employed with the experimental evidence presented in the second, so that future research can attain a comprehensive

understanding of the extent of VBNC contamination of agricultural land, and the potential impact it has on human health.

The paper was prepared for publication in the journal Environmental Microbiology. Author Callum Highmore carried out all experiments, determined the experimental direction, analysed the data, and prepared the paper. Author Professor Bill Keevil managed the research and reviewed the submitted manuscript.

Finally, the third paper 'VBNC *Listeria monocytogenes* induced by chlorine stress remain infective in *Caenorhabditis elegans*' addresses the role of the agricultural industry in creating VBNC pathogens that would then go undetected by culture techniques, where the previous papers investigate the propensity for pathogens to reach food processing factories already in the VBNC state. The focus was shifted to foodborne pathogens *L. monocytogenes* and *Salmonella enterica* serovar Thompson, as examples of Gram-positive and Gram-negative produce-associated foodborne pathogens that characteristically result in relatively severe and mild infections respectively. The role of chlorine in VBNC induction of these pathogens was assessed, both independent of and adhered to the spinach leaf phylloplane, using chlorine concentrations similar to those used during washing of fresh produce in industry. The infectivity of both pathogens while in the VBNC state was then assessed using the animal model *C. elegans*. In this way, the practices of the agricultural industry were simulated from contamination of plant material to ingestion by consumer.

Previous studies have researched VBNC induction on the phylloplane of ready-to-eat foods (Dinu and Bach, 2011), but not in the context of the stress caused by chlorination. This study offers a direct assessment of industrial practices regarding its formation of VBNC pathogens, and the subsequent danger of their causing outbreak. The assessment of the pathogenicity of VBNC *L. monocytogenes* and *Salmonella* Thompson is necessary, in addition to determining the inefficacy of chlorine in increasing food safety, it provides an application for the previously published detection method for VBNC foodborne pathogens; the assay can be applied to fresh produce as well as growth media. The

C. elegans model used was a novel approach for the investigation of the infectivity of VBNC cells, where pathogens were ingested rather than inoculated intravenously as with other models. Additionally, the use of *C. elegans* allowed for the visualisation of the pathogens in the digestive tract.

The paper was prepared for publication in the journal PLOS Pathogens. Author Callum Highmore carried out all experiments, determined the experimental direction, analysed the data, and prepared the paper. Author Dr Jennifer Warner gathered preliminary data for the sensitivity of *Salmonella* Thompson to chlorine, and the pathogenicity of VBNC of *Salmonella* Thompson in *C. elegans*. Author Dr Steve Rothwell provided an industrial perspective for the applications of the work. Author Dr Sandra Wilks contributed experimental conception and design, and reviewed the submitted manuscript. Author Professor Bill Keevil managed the research and reviewed the submitted manuscript.

Together, these papers provide a cohesive exploration of the incidence of VBNC foodborne pathogens and their potential to cause disease. The research carried out utilises novel approaches to detect VBNC cells in complex environmental matrices and to measure the pathogenicity of VBNC cells *in vivo*. With particular focus on the agricultural industry, they expand upon existing research and can be directly applied for the enhancement of food safety.

Chapter 2

Improved Sample Preparation for Direct Quantitative Detection of *Escherichia coli* O157 in Soil Using qPCR Without Pre-Enrichment

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2.1 Summary

The prominence of fresh produce as a vehicle for foodborne pathogens such as enterohaemorrhagic *Escherichia coli* (EHEC) O157 is rising, where disease cases can cause hospitalisation and in some cases death. This rise emphasises the necessity for accurate and sensitive methods for detection of pathogens in soil, potential sources of contamination of fresh produce. The complexity of the soil matrix has previously proven prohibitive to pathogen detection via molecular methods without the use of a culture enrichment step, thereby excluding the detection of viable but nonculturable cells. Here, a sample preparation procedure to facilitate a direct qPCR assay is developed for the detection of *E. coli* O157 in soil, bypassing culture steps in favour of sample separation through pulsification release and filtration. In sand and peat-based compost, the method is sensitive to 10 CFU/g soil. When testing soils from agricultural sites, it was found that several were qPCR positive for *E. coli* O157 while being culture-negative, with peat-based compost possessing a concentration of 200 *tir* gene copies per gram. This procedure offers a rapid, quantitative assessment of the potential presence of *E. coli* O157 in soils which can act as a pre-screen of their suitability to grow fresh produce safely.

2.2 Introduction

The increase in popularity of fresh produce has caused a rise in prominence of fresh produce-associated outbreaks of foodborne disease. Between 2010 and 2013, two outbreaks of *Escherichia coli* O157 in the UK caused 251 and 19 disease cases, respectively (Pennington, 2014). In both outbreaks the source was considered to be contaminated growing media, an essential component of food production which poses severe limitations to the detection of bacterial pathogens due to its complex physicochemical matrix (Wilks, 2013).

Soil and growing media such as peat-based compost and coir compost can become contaminated by foodborne pathogens such as *E. coli* O157:H7 through the application of animal faeces to agricultural land, both accidentally by roaming animals and overflying birds and deliberately as fertiliser. It has been found that animals such as rats are able to shed the pathogen for up to 11 days, whereas infected pigeons shed for 29 days (Cizek et al., 2000). Enterohaemorrhagic *E. coli* (EHEC) has been detected in a range of birds, where the *stx2* toxin gene was detected by qPCR in 23% of 412 *E. coli* isolates from 8 of 15 avian species. The species in which the gene was most prevalent were raven, turkey and pigeon, where isolates also originated in mallard ducks and pheasants (Chandran and Mazumder, 2014).

Manure and slurries are a major source of fertiliser for agricultural land. In Scotland they comprise 96% of organic waste spread on land (Anon., 1997b). Several studies have assessed the prevalence of EHEC in cattle, one using traditional culture techniques to examine the presence of *E. coli* O157:H7 in 3570 dairy cattle across 60 herds. It was found that 10 cattle were positive for the bacterium (0.28%), across 5 herds (Hancock et al., 1994). Another study compared the prevalence of the pathogen across herds associated with *E. coli* O157:H7 outbreaks in humans. Within herds, prevalence ranged from 1.3% to 9.5%, whereas in herds of cattle not associated with any *E. coli* O157:H7 cases in humans, it was found that 0% to 6.1% of cattle were infected with the pathogen (Anon., 1997a). In addition to contaminating agricultural land, there is evidence that the application

of biosolids can increase the population of EHEC in the soil; one study notes an increase of 2.62 orders of magnitude of indicator *E. coli* (Unc et al., 2006).

Currently, detection of pathogens in growing media is achieved through traditional culture methods (Islam et al., 2004a; Avery et al., 2005; Wadamori et al., 2016), where even assays using molecular techniques such as qPCR commonly depend on an enrichment culture step to amplify a signal and dilute out potential inhibitors (Nam et al., 2005). However, enrichment culture methods exclude the presence of dead and viable but nonculturable (VBNC) cells (Colwell and Grimes, 2000; Li et al., 2014), both of which are important for determining the safety of agricultural growing media. Dead cells indicate previous contamination of a sample and VBNC cells present in a sample pose a direct threat to the safety of a growing medium. Dinu and Bach have shown that a VBNC state can be induced in *E. coli* O157:H7 through exposure to conditions present on the phylloplane of lettuce (Dinu and Bach, 2011), highlighting the potential population of produce-associated pathogens that could go undetected by currently used culture methods. The pathogenicity of VBNC *E. coli* O157:H7 cells has been established, where expression of *stx* toxin genes has been documented despite the stressed state (Liu et al., 2010). Resuscitation of VBNC cells is also possible; allowing the establishment of infection provided they could reach an appropriate host. Other pathogens, *Listeria monocytogenes* (Cappelier et al., 2007) and *Vibrio alginolyticus* (Du et al., 2007a), have retained their pathogenicity following resuscitation.

While culture methods may produce false-negative results in pathogen detection, testing large volumes of growing media through molecular methods is also restricted by the complexity of the matrix. Separation of the bacterial sample is essential due to the presence of PCR inhibitors such as humic acids (Tebbe and Vahjen, 1993). Attachment of bacterial cells to soil particles must be reduced as much as possible prior to sample concentration to effectively utilise molecular detection methods for large samples of soil. The necessity of sample concentration is emphasised by the limitations of most commercial DNA extraction kits, recommending only up to 0.25 g of soil per sample.

To best overcome these problems and understand the risk of *E. coli* O157 in soil and growing media, this study employs a cost effective, qPCR assay that removes the need for culture-based pre-enrichment. The Pulsifier is used to facilitate bacterial sample separation from the soil matrix through high frequency oscillation, degrading the matrix less than a paddle-based homogeniser (Wu et al., 2003). Vacuum filtration is then used to concentrate a large soil sample (25 g) to a volume suitable for DNA extraction by an effective commercial kit (Mo Bio, USA) (Mahmoudi et al., 2011) and the *E. coli* O157 population is quantified using qPCR. The assay targets the *E. coli* O157 *tir* (translocated intimin receptor) gene, as it is specific to serotype O157 strains. The primer is also available in a commercial kit (PrimerDesign, UK), which allows the procedure to be more easily applied in industrial laboratories. Using this diagnostic assay, it was revealed that several soils of agricultural origin were positive for *E. coli* O157 although there was no corresponding culture recovery.

2.3 Results

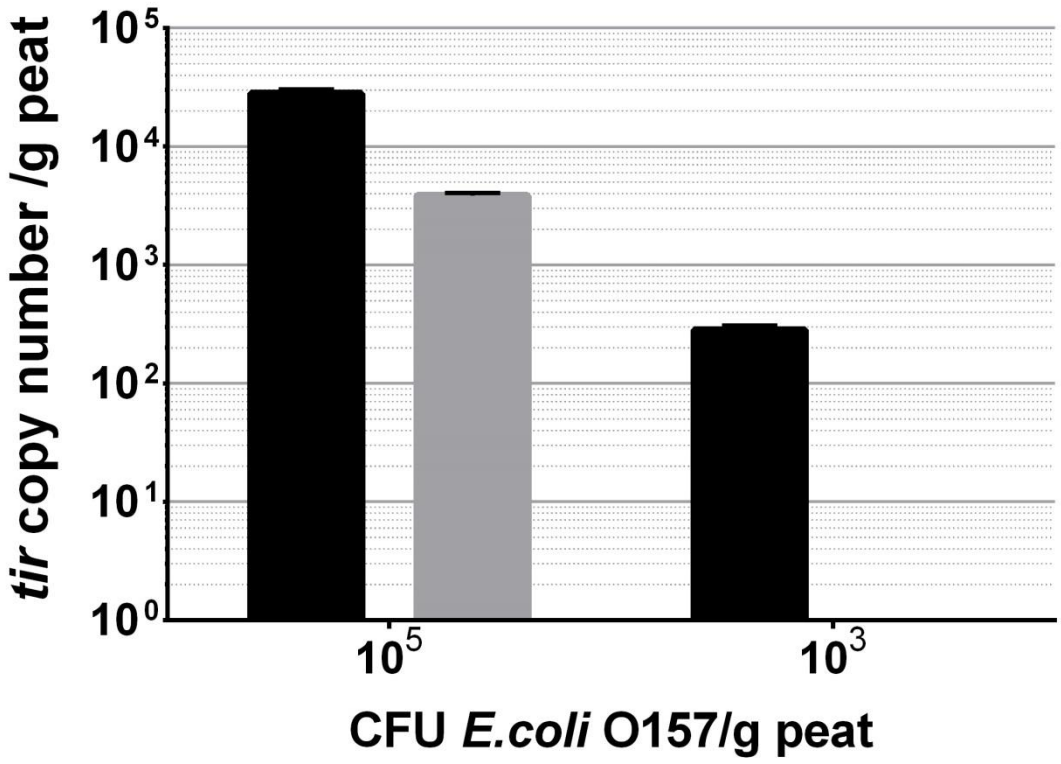

Figure 4. *tir* gene copy number detected in peat-based compost using the Pulsifier (black) and the Stomacher (grey). Error bars represent SEM. P<0.005.

To optimise separation of bacterial suspension from the complex matrix of soil, the Pulsifier was tested against the better established Stomacher. This sample separation step was followed by filtration, DNA isolation, and qPCR, to determine which process is more appropriate for the pathogen detection assay. In peat-based compost, a greater recovery of the bacterial sample was attained when using the Pulsifier (Figure 4). In samples inoculated with 10^5 CFU *E. coli* O157/g, the Stomacher causes a log reduction of detection when compared with the Pulsifier. At an inoculum concentration of 10^3 CFU *E. coli* O157/g, the Stomacher prevents the detection of any *tir* genes by the qPCR assay.

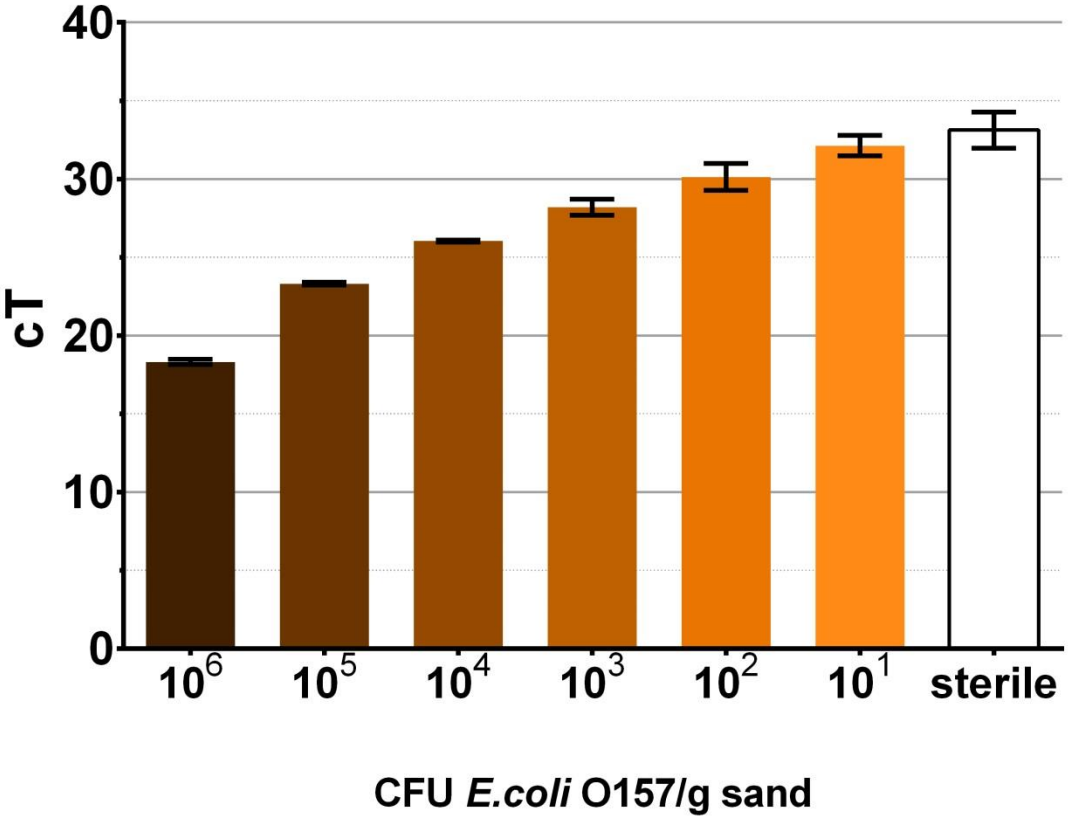

Figure 5. cT values of sand inoculated with different concentrations of *E. coli* O157. Sterile indicates sterilised sand not inoculated with bacteria. cT refers to cycle threshold. Error bars indicate SEM. R^2=0.9756.

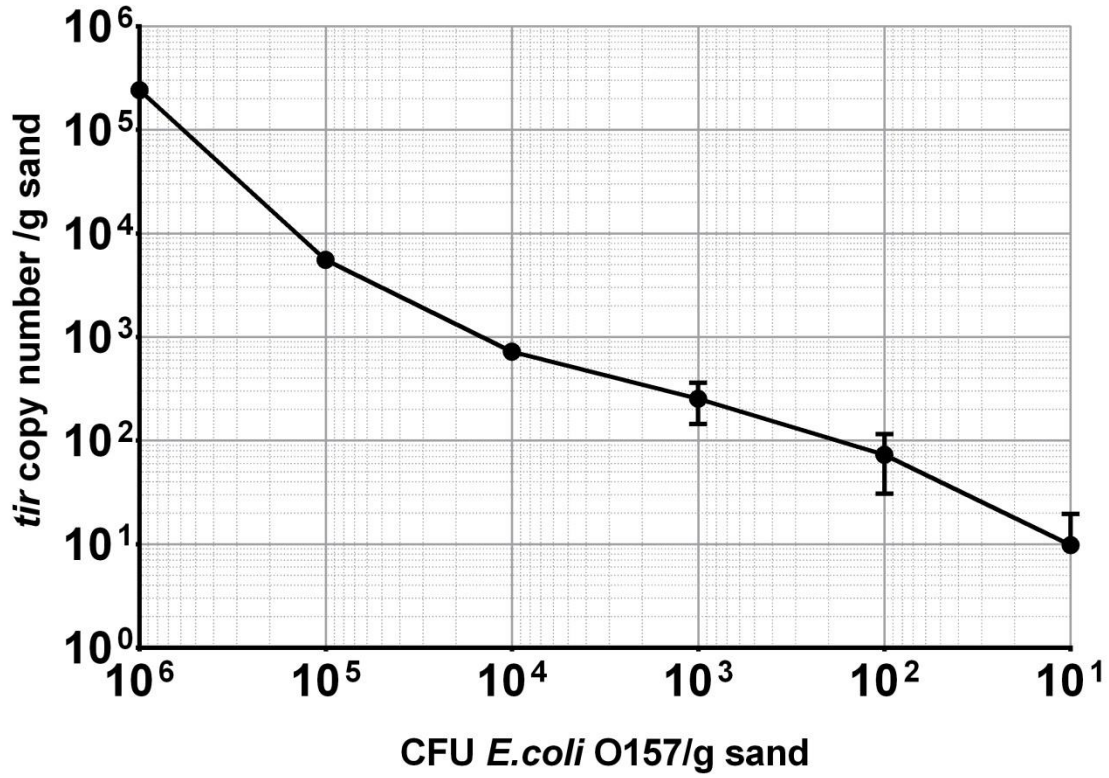

Figure 6. *tir* copy number detected in sand containing a range of concentrations of *E. coli* O157. Error bars indicate SEM.

The assay is capable of detecting *E. coli* O157 in sterilised sand to a sensitivity of 10 CFU/g (Figure 5). The control sample, containing sterile, uninoculated soil, showed gene amplification passing the cycle threshold with an average cT of 33. Following inoculation with *E. coli* O157, the sample containing 10^6 CFU/g soil passed the cycle threshold at cycle 18 and each successive log dilution of inoculum had a correspondingly greater cT value. Despite this, a log increase of inoculation concentration does not correspond to a log increase in *tir* copies detected (Figure 6). Using a standard curve, the cT values are converted to *tir* gene copy numbers to quantify bacterial numbers within the soil (Figure 6, Figure 8). The detected gene copies closely correspond to low inoculum concentrations, 73 *tir* copies were detected per gram of soil at an inoculum of 100 CFU *E. coli* O157,

and 10 copies were detected when 10 CFU were inoculated (Figure 6). At higher inoculum concentrations, fewer *tir* gene copies are detected per inoculated CFU (Figure 6). Despite heat sterilisation, an average of 2 gene copies was found in sterile sand.

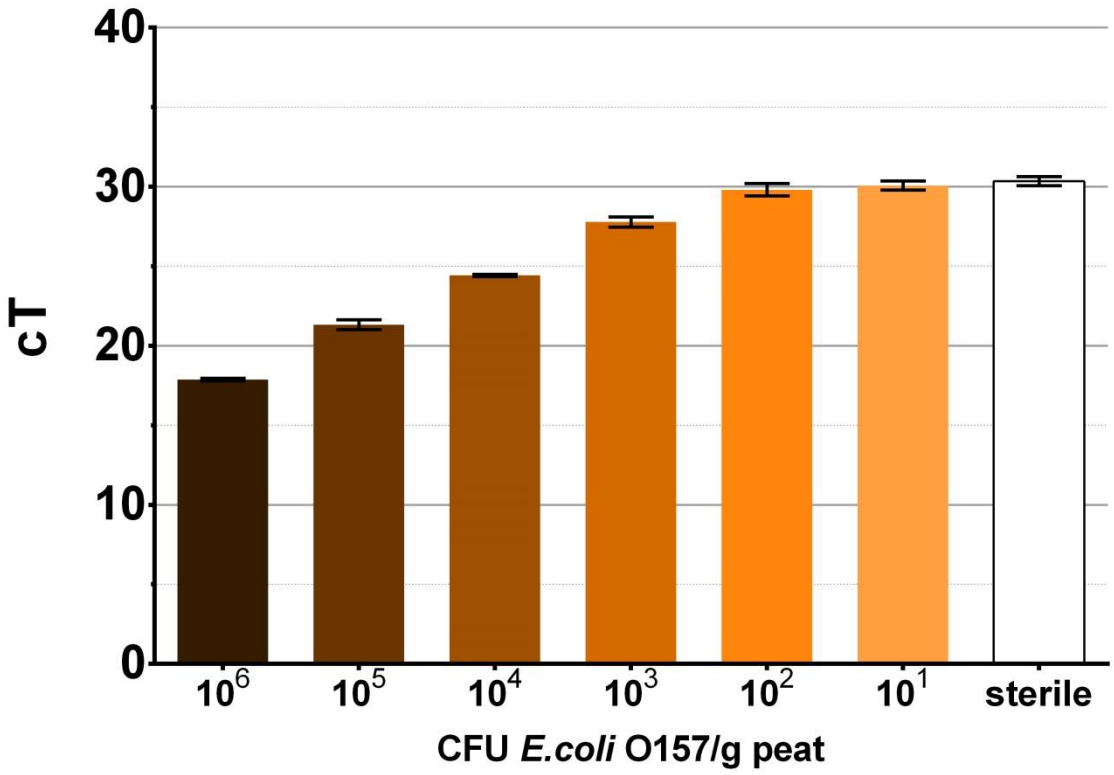

Figure 7. cT values of peat-based compost inoculated with different concentrations of *E. coli* O157. Sterile indicates soil not inoculated with bacteria, heat treated and DNase I treated. cT refers to cycle threshold. Error bars indicate SEM. R^2=0.9878.

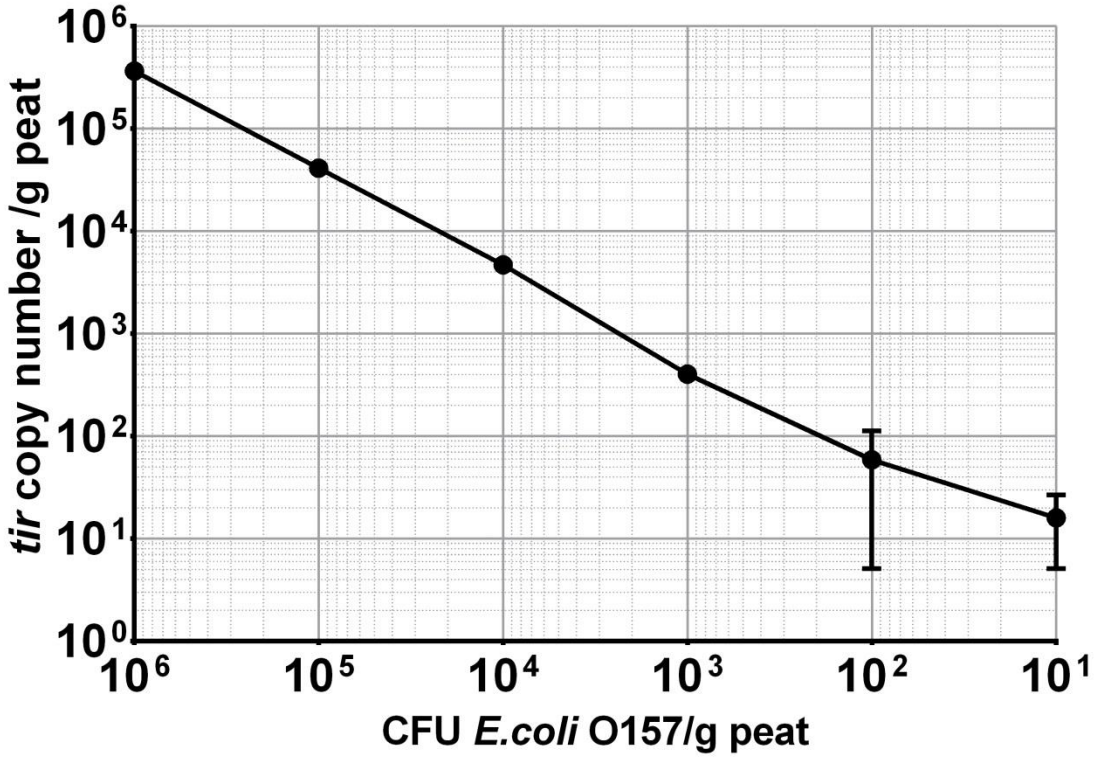

Figure 8. *tir* copy number detected in sand containing a range of concentrations of *E. coli* O157. The average copy number generated by the sterile sample was subtracted from the other columns to remove the *tir* gene background. Error bars indicate SEM.

In peat-based compost, the assay can reach a similar sensitivity of detection. To reduce the quantity of *tir* genes detected in sterilised soil, peat samples were treated with DNase I (Sigma-Aldrich) before inoculation, however no further reduction in *tir* gene detection was achieved. The peat sample inoculated with 10^6 CFU/g soil *E. coli* O157 passed the threshold at cycle 17, and similarly to sand and untreated peat, each reduction in inoculum concentration caused an increase in average cT value (Figure 7). 59 gene copies per gram of peat based compost were detected in the DNAse I treated sample. This average was subtracted from the total detected *tir* genes as a background level of the gene in the compost, so remaining *tir* gene detection could be attributed to the *E. coli* O157

inocula. In the absence of the background *tir* gene copies, the sensitivity of the assay in peat-based compost is consistent with that in sand. At an inoculum concentration of 10 CFU/g, an average of 16 gene copies was detected. At 10^6 CFU/g, an average of 3.7 x 10^5 was detected, indicating reduced detection at higher inoculum concentrations (Figure 8).

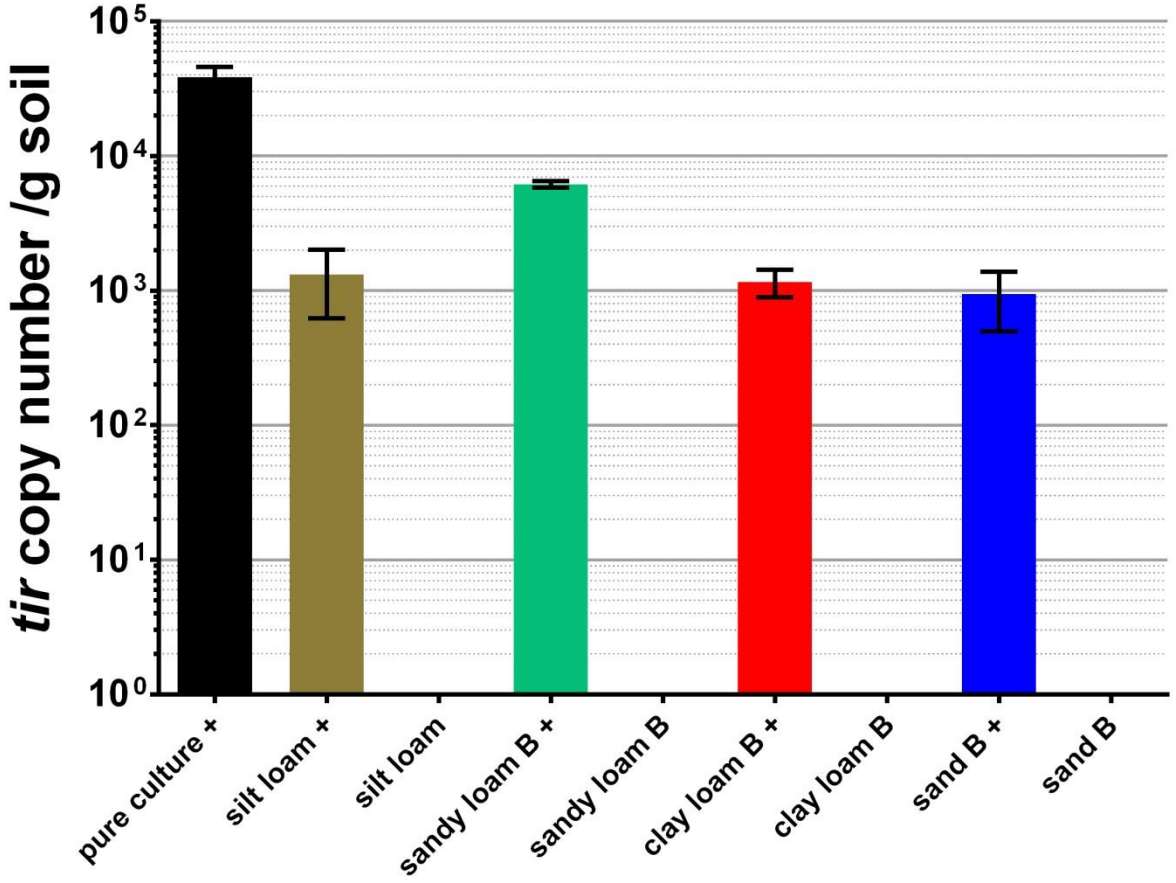

Figure 9. Pristine soil samples in which no *tir* gene copies were detected. + indicates samples inoculated with 10^5 CFU *E. coli* O157/g. Pure culture indicates inoculated water without any soil, as a positive control. Each condition was tested in duplicate. Error bars indicate SEM.

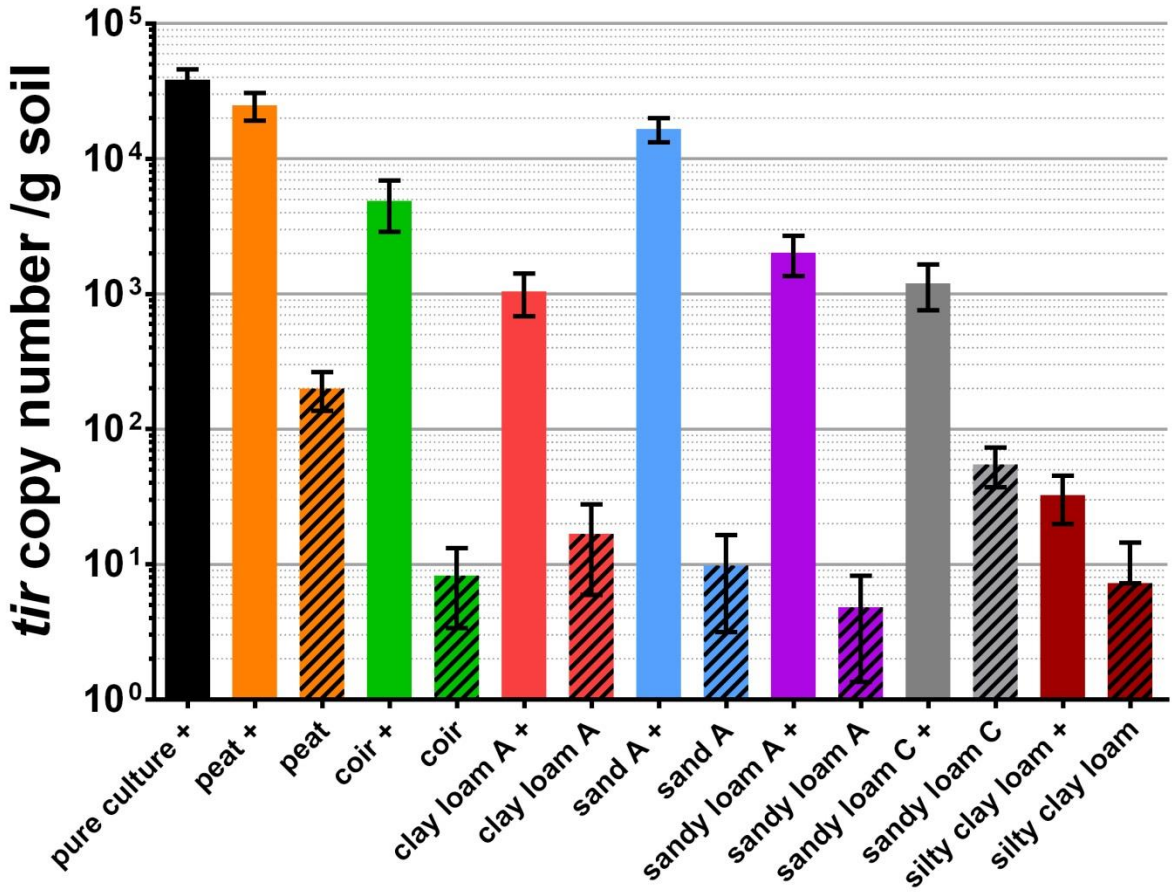

Figure 10. *tir* copy numbers detected in a range of pristine soil and growing media samples, compared with samples inoculated with 10^5 CFU *E. coli* O157/g. + indicates samples inoculated with the bacteria. Pure culture indicates inoculated water without any soil, as a positive control. Each condition was tested in duplicate. Error bars indicate SEM.

The assay was tested on 9 soil samples of agricultural origin, in addition to peat and coir composts. Each sample was tested with and without an inoculation of 10^5 CFU *E. coli* O157/g soil. A positive control was generated by inoculating the same concentration of bacteria into sterile water and a negative control was produced by using pure sterile water. For each sample, inoculating with bacteria increased detection of *tir* copy number, however each inoculated sample was found to be

statistically lower than the positive control (Figure 9, 10). Uninoculated peat-based compost contained 200 *tir* copies per gram, while sandy loam C contained 55 copies per gram and clay loam A contained 17 copies per gram. The negative control contained no *tir* gene copies.

Using the Pulsifier and filtration to concentrate large volumes of soil allows for the assessment of larger soil samples than previously possible, bypassing the need for a culture-based pre-enrichment step while allowing for the detection of VBNC pathogens, and reducing sample preparation time to less than two hours.

2.4 Discussion

2.4.1 Sample separation by pulsification

The Pulsifier proved to be far more effective than the Stomacher at facilitating the detection of *E. coli* O157 by qPCR (Figure 4). Previous studies have shown that in food samples, there is not a significant difference in bacterial recovery between the two processors (Sharpe et al., 2000; Kang et al., 2001). However, both reports corroborate in describing the reduced debris produced by the Pulsifier. In this study, the structural integrity of the assessed soil matrices coupled with the reliance on filtration for sample separation and concentration affirm the need for minimal debris. It is likely that the reduction in *tir* gene detection observed from using the Stomacher is caused by excessive soil particulate forming a physical barrier over the filter membranes, preventing bacterial elution. Therefore we recommend that the Pulsifier is used over the Stomacher in the assessment of complex environmental matrices.

2.4.2 Sensitivity of detection

The method developed is capable of detecting *E. coli* O157 in peat-based compost and in sand, sensitive to 10 CFU/g. It can also detect the pathogen in a range of other growing media and soil of different compositions. The difference in sensitivity across soil types could be due to the different rates at which they block the 5 µm pore filter membrane, the step at which most sample loss occurs. This had been explored during the method development and to optimise sample elution for different soil types, the use of filter membranes with greater pore sizes could be implemented. Replacing the filter membranes more frequently increases the quantity of sample retrieved following the filtration steps, but at the cost of more rapidly using resources.

There is evidence for loss of bacterial sample to filter membranes described in Figure 9 and 10; in inoculated soils, fewer *tir* gene copies were detected than in the positive control, which contained no soil. This reduction in *tir* genes could have been caused by the build-up of soil on the filter membrane during the filtration steps, preventing the elution of the complete bacterial sample.

In this way, the importance of protocol optimisation is emphasised. The peat sample allowed for the detection of *tir* genes closest to the concentration of the positive control, at 64.6%. As the method was developed and optimised using peat-based compost, it could be expected that a higher percentage of the gene would be detected in peat samples than in other soil types. The method developed would have to undergo further optimisation to increase its sensitivity for soil of different physicochemistry. While there is the potential for this assay to be attuned to other soil types, it could also be adjusted to different pathogens. The specificity of the method is dependent entirely on the primer used in the qPCR assay; therefore any pathogen present in soil could theoretically be detected by using this assay.

The reduction in sensitivity of *tir* gene detection at high inoculum concentrations could be due to saturation of DNA molecules during the DNA isolation process, or in the qPCR reaction wells. This is not necessarily detrimental to the sensitivity of the assay, one North American study ranging across 50 cattle herds found that the upper limit of *E. coli* O157 shedding was at a concentration of 10^5 CFU/g faeces (Zhao et al., 1995). Contamination of this magnitude can easily be detected by the assay, although greater accuracy may be achieved with lower concentrations of the pathogen.

2.4.3 tir *gene detection in pristine soil*

The reason for the discrepancy in the correlation between *tir* copy number and CFU inoculated into the soil sample could be that there is a background level of *tir* genes in bacteria present in soil. There is evidence for this in that agricultural soil samples tested show copy numbers up to 200/g in peat (Figure 10), despite having no bacteria inoculated into the sample. Using media selective for *E. coli* O157, traditional culture methods found the pristine peat compost negative for the pathogen. This limits the origin of the detected *tir* genes to either dead cells or those in a VBNC state.

Different quantities of the gene were also detected across pristine samples of different soil types, for example none were detected in silt loam, and 8 copies per gram were found in coir compost (Figure 10). It is also worth mentioning that as the silty clay loam positive control samples detected only a fraction of the *tir* genes inoculated into them (Figure 10), the quantity detected in their corresponding pristine samples may also be a fraction of the true copy number of *tir* genes they harbour.

The number of *tir* gene copies detected in pristine soils differed across soil samples of similar compositions but from different locations, for example sandy loam A and C (Figure 10). This suggests that contamination of agricultural soils by *E. coli* O157 may depend more on circumstances specific to each site, than an endemic contamination of soils by the pathogen favouring certain soil compositions. Optimisation of the assay for different soil types will help to provide a more accurate reflection of the presence of endemic pathogenic genes across a range of sites.

Considering the sources of contamination, the existence of *tir*-positive *E. coli* O157 in soils across disparate agricultural sites is plausible. Birds are known to shed the pathogen (Wallace et al., 1997; Chandran and Mazumder, 2014), one study mentioning a potentially large population of infected gulls roosting near to farmland (Wallace et al., 1997). There is also the possibility of the land being fertilised with contaminated manure or slurry, where cattle have been known to shed $>10^4$ CFU *E. coli* O157:H7/g faeces (Matthews et al., 2006). This study has detected the *tir* gene in seven of the

eleven soil samples tested, however a range of factors may affect their detection by the described qPCR assay. PCR inhibitors or continued adhesion to soil particles of certain types could possibly impede the detection of the gene.

Preliminary work carried out in this study determined that the sterilisation procedure of autoclaving soil for 30 minutes at 123^{o}C could reduce gene copy number by 5 orders of magnitude, however this procedure was unable to destroy the DNA entirely. In addition to heat sterilisation, soil samples were also sonicated and treated with deoxyribonuclease I (Sigma-Aldrich), although this failed to further reduce the *tir* gene signal. This could be due to protection of DNA molecules from degradation by adsorption to soil particles. This protection has been observed in clay particles, where DNase I treatment failed to completely degrade DNA molecules in the presence of clay minerals (Demaneche et al., 2001).

We advise that if used to screen soil samples for contamination, this assay should use sterile soil as a negative control. As neither the culture data nor the qPCR assay eliminate the possibility that the *tir* gene copies detected in pristine peat-based compost originate in VBNC *E. coli* O157 cells, any positive result in agricultural soil should be investigated further as a potential threat to food safety. By screening a greater number of soil samples and subsequent investigation, we will have a better understanding of the food safety implications of a positive result generated by this assay, and the cause of their apparent ubiquity.

With minor alterations, this method could be utilised to detect and monitor a range of pathogens contaminating a range of soil types. Future research could work to optimise the protocol for different soil compositions or even plant matter, to provide a post-harvest screen for contamination of fresh produce. At present, the procedure offers a rapid, quantitative risk assessment of the presence of *E. coli* O157 in soils and growing media, with applications in the diagnostics of occurring outbreaks for source identification and the prevention of those that may otherwise go undetected.

2.5 Experimental procedures

2.5.1 Bacteria and soil preparation

The strain used was *E. coli* O157:H7 NCTC 12900. Peat-based compost was used in the initial method development, purchased from a commercial supplier (Wickes, UK). Coir compost was supplied by Vitacress Salads ltd (Dr Graham Clarkson); agricultural soil samples from sites around the UK were kindly supplied by ADAS (Dr Lizzie Sagoo).

Soil and growing media were sterilised prior to inoculation with bacteria by thinly spreading each sample inside a loosely closed bag and autoclaving in a Priorclave benchtop autoclave at 123^{o}C for 30 minutes. The testing of agricultural samples was carried out without sterilisation.

2.5.2 Bacterial growth and soil inoculation

E. coli O157 was grown in brain heart infusion broth (Oxoid) overnight at 37^{o}C to stationary phase. Bacterial suspensions of different concentrations were produced by diluting in PBS (Oxoid).

Soil samples (25 g) were added to separate BagFilter P Stomacher bags (Interscience). One millilitre of each suspension was added to each soil sample and left to adhere to the sample for 5 minutes; 225 ml ddH_2O was then added to each bag.

To determine the presence of viable *E. coli* O157 in soil samples, pristine soil samples were prepared as described above and 100 µl of pulsified sample was added to Petri dishes containing selective medium CHROMagar O157 (Invitrogen). The agar plates were incubated at 37^{o}C overnight.

2.5.3 DNase treatment of soil

Soil samples (200 g) were autoclaved at 123^{o}C for 30 minutes, then added to 400 ml water with 5 mM Ca^{2+}, and 10 µg/ml DNase I (Sigma-Aldrich). Samples were incubated for 1 hour at 37^{o}C with gentle agitation. Soil samples were drained of water and autoclaved again under the same conditions as before, prior to inoculation.

2.5.4 Sample separation from soil matrix

The bagged sample was pulsified in a Pulsifier™ (Microgen Bioproducts ltd., Camberley, UK) for 30 seconds, then the suspension was aspirated through the BagFilter P bag 250 µm pore filter, so that larger soil particles were excluded from downstream processes. To test the Stomacher, bagged samples were homogenised for 2 minutes using a Stomacher 400 (Tekmar, UK). Aliquots of the suspension (25 ml) were added to a filtration unit fitted with a 47 mm diameter PVDF filter membrane with a pore size of 5 µm (Millipore). Using vacuum filtration, the suspension was filtered through into a receiver beneath. After the completion of filtration of the first 25 ml, another 25 ml was added. The membrane was renewed for each 50 ml that had been filtered; 200 ml of the suspension in total for each sample was filtered through to the receiver.

The filtrate underwent a second round of vacuum filtration, using a 47 mm diameter mixed cellulose-ester membrane with a pore size of 0.22 µm (Millipore). Once the entirety of the sample had filtered through, the filter membrane was removed and added to a 15 ml tube containing 1 ml PBS. The tube was shaken using a vortex mixer for 2 minutes, in the manner previously described by Wolffs et al. (Wolffs et al., 2006); then the membrane was removed.

2.5.5 DNA extraction and qPCR assay

The 1 ml suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 10000 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl in PBS for DNA

extraction using the Powersoil DNA Isolation kit (Mo Bio, USA) according to the manufacturer's instructions. DNA samples were stored at -20^{o}C until use.

qPCR was carried out according to the Genesig *E. coli* O157:H7 kit instructions, targeting the *tir* gene specific to *E. coli* O157. The primer was labelled with a FAM dye and quencher. The qPCR method consisted of a 10 minute warming stage at 95^{o}C, then 50 cycles of a 10 second step at 95^{o}C, followed by a 60 second step at 60^{o}C (Primer Design, Southampton, UK). Amplification was carried out using a Bio-Rad iQ5 cycler. Each sample was measured with at least three replicate wells. The quantity of the target gene was quantified by measuring the cycle threshold (cT) of the sample, the number of amplification cycles required for the fluorescence generated by the target gene amplification to exceed the background fluorescence.

2.5.6 Data analyses

Statistical difference between qPCR data sets was calculated using one-way analysis of variance and Fisher's least significant difference test.

To calculate *tir* gene copy number per gram of soil, each qPCR plate contained a standard curve containing known concentrations of the gene. The cT values for test samples were compared against the standard curve, giving *tir* copy number per microlitre of DNA sample. These values were multiplied to correspond to the quantity of DNA sample per gram of soil.

2.6 Acknowledgements

We thank Dr Graham Clarkson and Dr Lizzie Sagoo for the gift of the soil and coir compost samples.

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Chapter 3

Survival of viable but nonculturable *Escherichia coli* O157 in horticultural growing media

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3.1 Summary

Produce-associated outbreaks of the foodborne pathogen *Escherichia coli* O157 are responsible for a number of disease cases, hospitalisations, and deaths. In many cases, the source of contamination can be linked to the growing media of the food vehicle. In this study, qPCR was used to detect 310 copies of the translocated intimin receptor gene, specific to *E. coli* O157, in horticultural growing media purchased from a commercial supplier. The pathogen could not be cultured on selective media but was visualised using peptide nucleic acid fluorescence *in situ* hybridisation and cell elongation, confirming the VBNC state. Enumeration of elongated *E. coli* O157 determined that there were 205 cells per gram soil. The pathogen was resuscitated and separated from the soil sample with selective enrichment and immunomagnetic separation. The detection of VBNC foodborne pathogens in environmental samples challenges current understanding of the nature of foodborne pathogen contamination, and requires further investigation to determine the extent of VBNC *E. coli* O157 in the environment and the influence it has on food safety.

3.2 Introduction

Fresh produce is being increasingly recognised as a vehicle by which foodborne pathogens infect humans, causing more disease cases than any other food commodity, and accounting for 38% of hospitalisations (Painter et al., 2013). The prominence of produce-associated outbreaks of the foodborne pathogen *Escherichia coli* O157 has also increased (Lynch et al., 2009; Gould et al., 2013), with recent outbreaks across the UK in 2010 and 2013 causing 251 and 19 cases of illness, respectively (Pennington, 2014). In February of 2016 an outbreak of the pathogen associated with alfalfa sprouts caused 11 cases of illness with 2 hospitalisations (Anon., 2016b).

E. coli O157 is a pathogenic serogroup of the Gram-negative bacterium, where ingestion can result in infection, commonly causing abdominal cramps and bloody diarrhoea, with approximately 5% of patients developing HUS (haemolytic uremic syndrome) as a result of the production of Shiga-like toxins (Chart, 2000). It has been found that the pathogen can enter a viable but nonculturable (VBNC) state when inoculated onto the lettuce phylloplane at low temperatures (Dinu and Bach, 2011), excluding it from commonly used culture-dependent detection methods (Colwell and Grimes, 2000; Li et al., 2014). Furthermore, there is evidence that *E. coli* O157 can retain its pathogenicity while in this state, one study identifying expression of the Shiga-like toxin gene 19 months after VBNC induction (Liu et al., 2010).

In both previously mentioned UK outbreaks, soil was considered to be the source of the pathogen (Lynch et al., 2009; Gould et al., 2013). The pathogen is able to contaminate vegetables through its contact with the soil in a number of ways, from being splashed onto the phylloplane by rain or irrigation (Heaton and Jones, 2008; Patel et al., 2010; Oliveira et al., 2012), to uptake by the rhizosphere permitting transmission to the leaf tissues (Solomon et al., 2002) and even by insect vectors (Sela et al., 2005). As a pathogen of the gastrointestinal tract, *E. coli* O157 can be disseminated across agricultural land through both the manure of roaming animals and birds, and manure deliberately applied to land as fertiliser. One study utilised qPCR to detect the *stx2* gene in 23% of 412 *E. coli* isolates collected across 15 avian species, where samples collected from ravens,

pigeons and turkeys had the highest incidence of the gene (Chandran and Mazumder, 2014). Pigeons infected with 10^9 CFU of *E. coli* O157 were able to shed the bacterium for 29 days following inoculation, while rats shed the pathogen for up to 11 days (Cizek et al., 2000). A further study in Denmark isolated non-O157 VTEC (vero cytotoxin-producing *E. coli*) from a starling and a brown rat, identical to isolates found in infected cattle in separate farms (serogroups O2 and O136, respectively). This demonstrates the capacity for farm animals to transmit foodborne pathogens to wild animals, which could further contaminate soil on agricultural land (Nielsen et al., 2004).

The persistence of *E. coli* O157 in soil has been well studied, with inocula surviving in soil from the Salinas Valley for 31 days (Ma et al., 2012), for an average of 80 days across 36 different Danish soils (Franz et al., 2008) and for 217 days in manure-amended compost (Islam et al., 2004a). While these results will depend considerably on the varying environmental factors during incubation of the pathogen, each of these studies was only able to detect culturable cells. There is the possibility that VBNC *E. coli* O157 could exist in the soil long after it loses its culturability. Previous studies have analysed water samples for the pathogen, where the existence of VBNC cells have been indicated in Japanese river water (Kogure and Ikemoto, 1997), and in South African river water (Ndlovu et al., 2015). Analyses of more complex environmental samples have detected the *E. coli* O157 specific *tir* gene in peat-based compost at a concentration of 200 copies per gram (Highmore et al., 2017).

This study seeks to further that work, utilising peptide nucleic acid fluorescence *in situ* hybridisation (PNA-FISH) in conjunction with cell elongation to visualise VBNC cells in growing media samples. These cells are then resuscitated from the soil using immunomagnetic separation and selective media. In this way, contribution of VBNC cells to the populations and distribution of *E. coli* O157 in horticultural growing media can be determined; we become closer to understanding the extent of the presence of the pathogen in the environment, and the threat VBNC cells pose to food safety.

3.3 Results

3.3.1 qPCR detection of* E. coli *O157 genes in soil

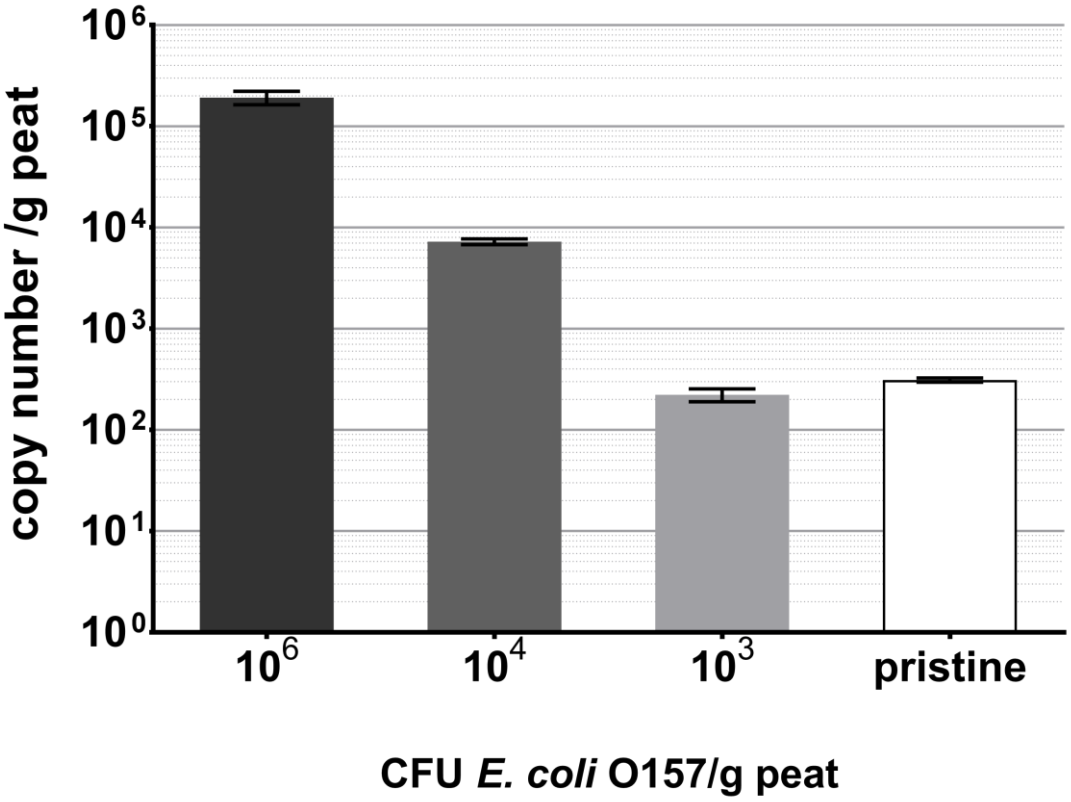

Figure 11. *tir* gene copy numbers of *E. coli* O157 inoculated into peat-based compost and detected in pristine peat-based compost. Samples inoculated with known concentrations of bacteria were sterilised prior to inoculation, pristine samples were unsterilised with no bacterial inoculation. Error bars indicate SEM.

In sterilised soil samples inoculated with 10^6 CFU *E. coli* O157 per gram, 1.9 x 10^5 *tir* gene copies were detected per gram of soil, 7261 were detected in soil samples inoculated with 10^5 CFU/g, and 222 were detected in samples inoculated with 1000 CFU/g. A one-way ANOVA determined that

there was a statistical difference between the detected copy number ($p<0.0001$). In pristine soil samples, 310 *tir* gene copies were detected per gram of soil. A t-test determined that the number of *tir* genes in soil samples inoculated with 1000 CFU/g were not statistically significant compared to uninoculated, unsterilised pristine soil samples (Figure 11). A background of 31 *tir* gene copies detected in sterilised, uninoculated soil samples was subtracted from the total *tir* gene copy number of all samples.

3.3.2 Detection of E. coli *O157 using PNA-FISH*

Figure 12. Representative epifluorescence micrograph of sterilised compost. Scale bar represents 10 µm.

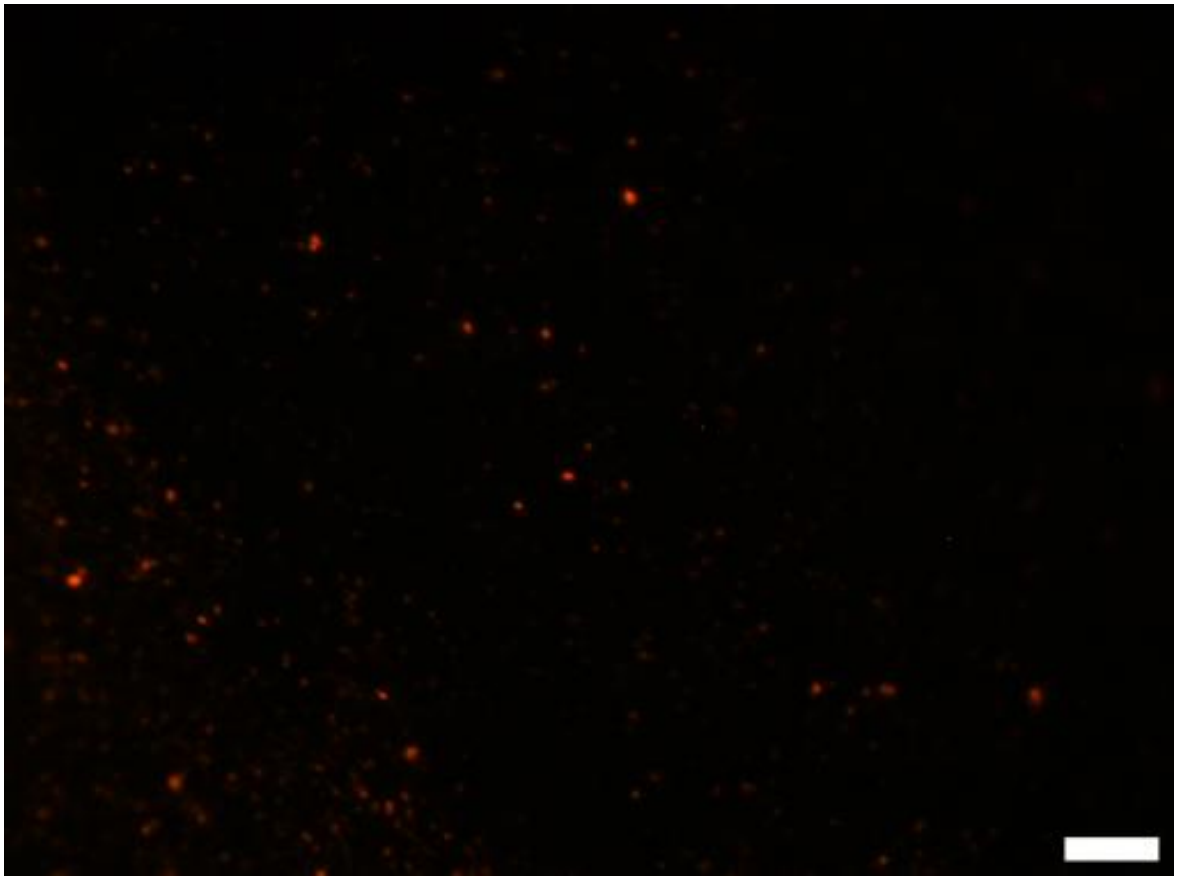

Figure 13. Representative epifluorescence micrograph of sterilised compost inoculated with 10^6 CFU *E. coli* O157/g. Scale bar represents 10 µm.

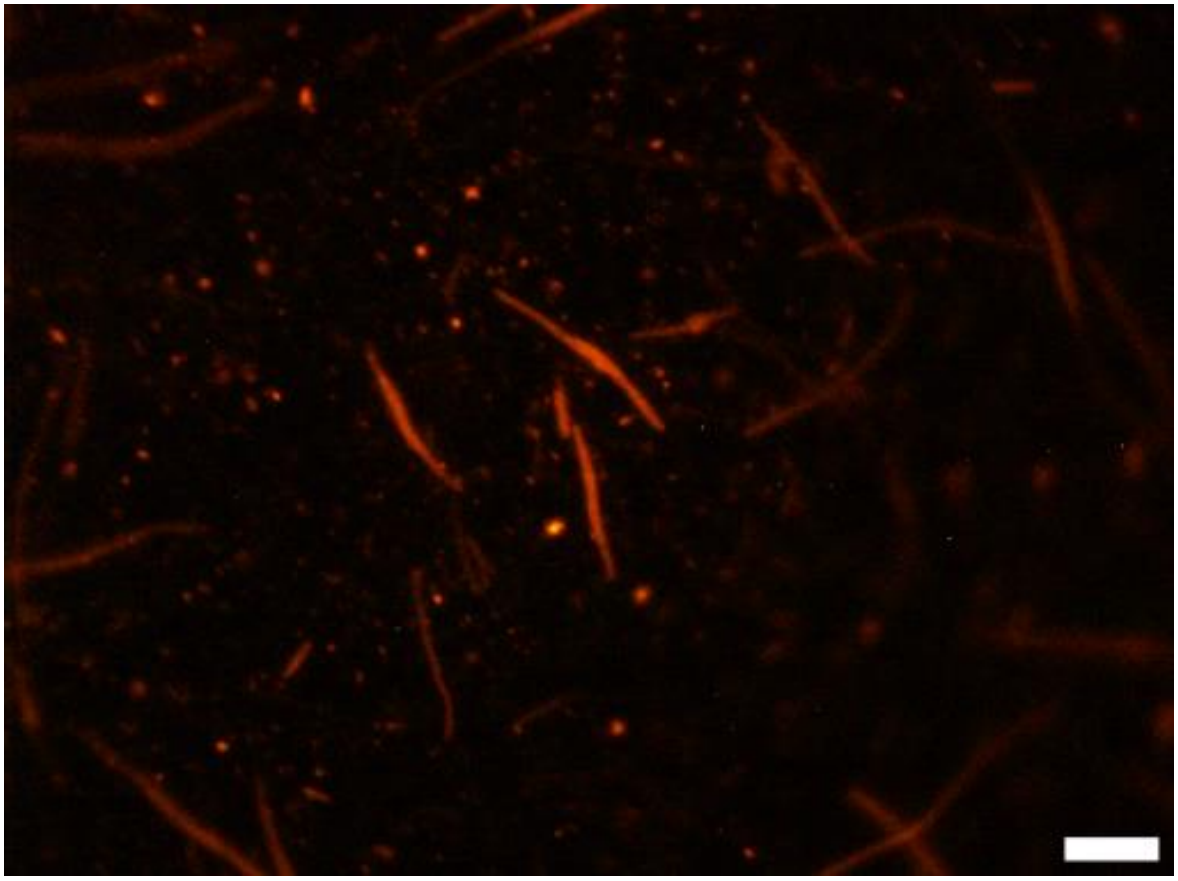

Figure 14. Representative epifluorescence micrograph of sterilised compost inoculated with 10^6 CFU *E. coli* O157/g and incubated to induce cell elongation. Scale bar represents 10 µm.

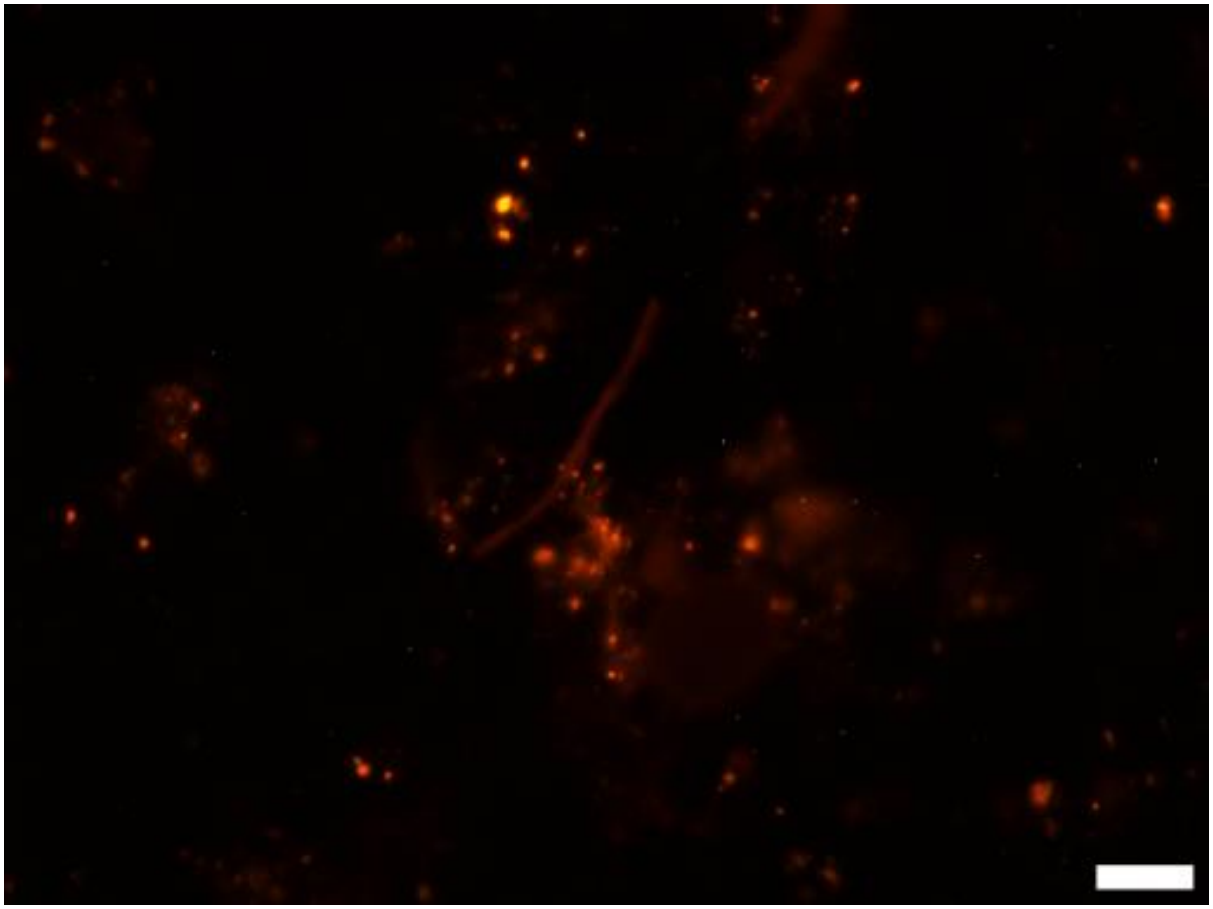

Figure 15. Representative epifluorescence micrograph of pristine compost incubated to induce cell elongation. Scale bar represents 10 µm.

Sterilised peat samples were assessed using the *E. coli* O157 specific PNA probe (Figure 12), showing no living cells, with dim auto-fluorescence of some soil particles. When the peat was spiked with 1.8 x 10^6 *E.coli* O157/g, brightly fluorescing cells were visible throughout the sample (Figure 13). Additionally, some cells appear to fluoresce less brightly than others.

Inoculated peat samples that had undergone cell elongation treatment displayed highly elongated cells, in some cases to lengths greater than 30 µm. Despite this, some cells remained at their original length, with varying degrees of fluorescence (Figure 14). Exposing pristine peat to the cell

elongation treatment permitted the visualisation of fluorescing, elongated cells (Figure 15). Six elongated cells were counted across 100 fields of view in pristine peat samples, correlating to 205 cells per gram soil sample.

3.3.3 Resuscitation of E. coli *O157*

Pristine soil samples were homogenised in PBS before culturing on CHROMagar O157, producing no mauve colonies. When incubated at 37^{o}C for 24 h in BHIB prior to culturing on CHROMagar O157, pristine soil samples produced blue confluent lawns indicating non-O157 *E. coli* or faecal indicator bacteria, with similar results when immunomagnetic separation targeting *E. coli* O157 was used following incubation. Pristine soil samples incubated in mTSB at 37^{o}C for 24 h, which then underwent immunomagnetic separation before culturing on CHROMagar O157 produced >1000 blue colonies per plate. However, across 8 plates, 10 mauve colonies were counted, indicating the presence of *E. coli* O157 in the pristine soil sample. From this, it can be extrapolated that 9 *E. coli* O157 cells were successfully recovered per gram of soil sample, when taking into consideration the loss of bacterial sample caused by retention on the filter membrane during filtration.

3.4 Discussion

The pristine peat-based compost tested positive for the *E. coli* O157 *tir* gene, at 310 gene copies per gram (Figure 11), but was culture-negative on selective media. The discrepancy between the number of cells inoculated into the sample and the *tir* gene copy number retrieved from the qPCR assay is caused by the loss of bacterial sample to the filter membrane during vacuum filtration (Highmore et al., 2017). The known concentrations of bacteria inoculated into sterilised peat-based compost can act as a standard curve to estimate the true *tir* gene copy number in pristine soil, and therefore the total number of *E. coli* O157 cells. This brings the estimated copy number of the gene to 1113 copies per gram of pristine soil.

The use of PNA-FISH and cell elongation on pristine peat samples confirms the survival of *E. coli* O157 in horticultural growing media (Figure 15), while the inability to culture the pathogen indicates its survival in a VBNC state. It has been previously confirmed that the VBNC state can be induced in *E. coli* O157 (Dinu and Bach, 2011), as has its capacity to continue expression of virulence factors such as the Shiga-like toxin gene *stx1* while in the VBNC state (Liu et al., 2010). *E. coli* O157, along with other foodborne pathogens including *Listeria monocytogenes* have been resuscitated from the VBNC state, and even implicated in outbreaks of foodborne disease (Makino et al., 2000; Cappelier et al., 2007). Combined, these factors present a risk to public health by VBNC foodborne pathogens and pose a challenge to the agricultural industry where microbial safety is dependent on culturability of pathogens for detection. These data show that those risks can be realised in nature.

Viability of bacterial cells is often measured using the *Bac*Light™ LIVE/DEAD stains, however it was decided unsuitable for this analysis. The stains, using SYTO9 and propidium iodide to indicate live and dead cells respectively, depend on cell membrane permeability to assess viability, and are frequently used without proper validation. This can lead to the use of incorrect quantities of stain, causing binding to eDNA and the enumeration of artefacts in place of VBNC cells. Additionally, the non-specific nature of the stains and the heterogeneity of the soil sample analysed would result in false-positive results by non-specific binding, both to other bacterial cells and soil particles. The use

of PNA-FISH and cell elongation is specific to the target bacteria, and confirms viability beyond an undamaged membrane.

Using PNA-FISH, it was calculated that pristine soil contained 205 VBNC *E. coli* O157 cells per gram, prior to correction for bacterial sample lost to the filter membranes. The true number of *E. coli* O157 cells in pristine soil is estimated at 978 cells per gram. The *tir* gene data from the qPCR assay corroborates with the estimation generated using the direct viable count facilitated by PNA-FISH, where the discrepancy can be attributed to the low number of cells in the sample and the assumption that there is an even distribution across of cells across the soil sample.

In sterilised soil, sterilisation by autoclave could not completely destroy all DNA, leaving 31 *tir* gene copies per gram. This remaining *tir* gene copy number was removed from each sample while analysing the data, to provide a more accurate estimation of true *E. coli* O157 population. As the quantity of VBNC *E. coli* O157 cells exceeds this background level, these genes can be attributed to incomplete destruction of DNA of dead *E. coli* O157 cells in pristine soil. However, quantitation of dead cells would be difficult using PNA-FISH due to the fluorescence caused by soil particles.

Some of the cell elongation exhibited in the pristine soil sample (Figure 15) was to a lesser degree than in soil spiked with *E. coli* O157 (Figure 14). This could be due to the stressed VBNC cells having a lower metabolism than the recently cultured cells in the control sample (Li et al., 2014). However, some cells in the pristine sample did elongate to sizes of approximately 30 µm (Figure 15), showing viability and the potential for resuscitation. Reduced elongation compared with culturable cells also corroborates with *E. coli* cells becoming smaller when entering a VBNC state (Signoretto et al., 2002).

Confirmation of the presence of VBNC *E. coli* O157 in peat-based compost was determined by a peptide nucleic acid probe specific to the *E. coli* O157 23S ribosomal subunit and detection of the *E. coli* O157 specific translocated intimin receptor gene in uninoculated soil samples, while it could not be cultured on selective media. Resuscitation was achieved through prolonged incubation in two *E. coli* O157 selective media, as it has been concluded in previous studies that the removal of the

inducing factor can resuscitate VBNC cells (Whitesides and Oliver, 1997). This is a controversial approach, other research has found that starved *E. coli* O157 cells require the presence of H_2O_2 degrading compounds for true VBNC resuscitation (Ravel et al., 1995; Mizunoe et al., 1999), however that treatment was not necessary in this particular environmental sample.

While these selection procedures confirm that *E. coli* O157 has been recovered from horticultural growing media, the pathogenicity of the recovered cultures have not yet been confirmed. Although rare, there have been *E. coli* O157 disease cases thought to have been caused by contaminated growing media in the garden; one outbreak led to four cases of the disease, following consumption of vegetables grown in manured compost containing the pathogen (Cieslak et al., 1993). *E. coli* O157 has also been found on the surface of lettuce and cabbage plants and internalised in their tissues after the plants had been grown in media contaminated with the pathogen (Mootian et al., 2009; Ongeng et al., 2011), emphasising the importance of growing media contamination by *E. coli* O157.

These factors do not necessarily indicate that the peat-based compost analysed in this study could cause an *E. coli* O157 outbreak. The exploration of environmental samples such as soil has previously been inhibited by its complex matrix, relying on traditional culture-based methods of detection that will exclude VBNC cells. With further exploration of environmental samples, particularly those used for the growth of fresh produce, the prevalence of VBNC foodborne pathogens in the environment will be better understood and the threat they pose to human health fully assessed.

3.5 Experimental procedures

3.5.1 Bacteria and growth conditions

Bacteria used for controls were non-pathogenic *E. coli* O157 NCTC 12900. Before inoculation they were grown in brain heart infusion broth (BHIB) (Oxoid, UK) for 18 h at 37^{o}C. Samples were diluted in PBS (Oxoid, UK).

Cell elongation was carried out using a modification of the method by Juhna et al. by adding 1 ml sample to 5 ml R2 broth (0.1% w/v peptone, 0.05% w/v yeast extract, 0.05% w/v glucose, 0.05% w/v starch, 0.03% w/v potassium dihydrogen phosphate, 0.03% w/v sodium pyruvate and 0.0024% w/v magnesium sulphate), 4 ml ddH_2O and 100 µl pipemidic acid at a concentration of 1 mg/ml. The sample is incubated for 22 hours at 22^{o}C in darkness, and concentrated by centrifuging 10 ml sample for 15 minutes at 4000 rpm using a Heraeus Megafuge 1.0 and resuspending in 1 ml PBS prior to PNA-FISH (Juhna et al., 2007).

3.5.2 Growing media preparation

Growing medium analysed was peat-based compost from a commercial supplier (Wickes, UK). Samples of 25 g were added to BagFilter P stomacher bags (Interscience, France), 225 ml ddH_2O was added and the sample was pulsified using a Pulsifier™ (Microgen Bioproducts ltd., UK) for 30 seconds. Appropriate soil samples were sterilised by spreading in a thin layer and autoclaving using a Priorclave benchtop autoclave at 123^{o}C for 30 minutes. Prior to pulsification, positive control samples were inoculated with 10^6 CFU *E. coli* O157 per gram growing medium.

3.5.3 Filtration

Growing medium samples were aspirated through a 250 µm pore filter in the BagFilter P stomacher bag and filtered as previously described (Highmore et al., 2017). Following filtration, samples were removed from the membrane by vortexing for 2 minutes in 1 ml PBS. Samples then underwent cell elongation, were prepared for PNA-FISH or prepared for qPCR.

3.5.4 PNA-FISH procedure

Soil samples of 50 µl were spread across Cyclopore 0.2 µm pore membranes (Whatman, UK), dried and fixed with 90% (v/v) ethanol. Cells were hybridised using 50 µl hybridisation buffer (10% w/v dextran sulphate, 10 mM NaCl, 30% v/v formamide, 0.1% w/v sodium pyrophosphate, 0.2% w/v polyvinylpyrrolidone, 0.1% v/v Triton X-100, 0.2% w/v Ficoll, 5 mM disodium EDTA, 50 mM TrisHCl) with 200 nM PNA probe. Membranes were incubated in darkness for 90 minutes at 59^{o}C, and then washed by soaking in wash buffer (5 mM NaCl, 1% v/v Triton X-100) in darkness for 30 minutes at 59^{o}C. Probe used was EcoPNA1169, specific for the *E. coli* serogroup O157 23S ribosome with the sequence 5'-CAA CAC ACA GTG TC-3' (Almeida et al., 2013), with the fluorescent marker Alexa Fluor 546, with absorption maximum of 556 nm and emission maximum of 573 nm. The probe tested negative for *Enterobacter cloacae* ATCC 13047, *Acinetobacter baumannii* strain W1 and *Streptococcus pneumoniae* strain D39. Slides were viewed at 1000 times magnification using oil immersion and episcopic differential interference contrast/epifluorescence microscopy.

3.5.5 Enumeration and image analysis

Enumeration of elongated cells in pristine soil was carried out by counting the total number across 100 fields of view, and multiplying the average number of cells by the average area of a soil suspension droplet (7.01 mm^2) to find the number of cells per soil suspension sample. This was used to calculate the number of cells per gram of soil sample. Calculation of droplet area was carried out using ImageJ 1.46r.

3.5.6 DNA extraction and qPCR assay

Filtered soil samples were centrifuged at 10000 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl in PBS for DNA extraction using the Powersoil DNA Isolation kit (Mo Bio, USA) according to the manufacturer's instructions.

qPCR was carried out according to the Genesig *E. coli* O157:H7 kit instructions, targeting the *tir* gene specific to *E. coli* O157 labelled with a FAM dye. The qPCR method consisted of a 10 minute warming stage at 95^{o}C, then 50 cycles of a 10 second step at 95^{o}C, followed by a 60 second step at 60^{o}C (Primer Design, UK). Amplification was carried out using a Bio-Rad iQ5 cycler, and copy numbers were calculated using a standard curve containing known *tir* gene copy numbers. The quantity of the target gene was quantified by measuring the sample cT, the number of amplification cycles required for the fluorescence generated by the target gene amplification to exceed the background fluorescence. Statistical analysis of qPCR data was carried out on GraphPad Prism 7 using one-way ANOVA with Tukey's multiple comparisons test.

3.5.7 Resuscitation of VBNC cells

Samples of pristine soil weighing 5 g were incubated in 50 ml modified tryptone soya broth (mTSB) (Oxoid, UK) supplemented with a selective supplement containing vancomycin, cefixime, and cefsoludin (Sigma-Aldrich, USA) for 24 hours at 37^{o}C. Samples then underwent filtration as described above and were resuspended in 8 ml PBS, providing 8 immunomagnetic separation samples using Dynabeads anti-*E. coli* O157 (Thermo Fisher, USA) according to the manufacturer's instructions. The resulting 100 µl suspension was plated onto CHROMagar O157 in its entirety (CHROMagar, France) and incubated for 24 hours at 37^{o}C. Mauve colonies were considered *E. coli* O157 isolates.

3.6 Acknowledgements

This study is funded by the BBSRC CASE Studentship BB/K012797/1 in conjunction with Vitacress Salads Ltd.

Chapter 4

VBNC *Listeria monocytogenes* induced by chlorine stress remain infective in *Caenorhabditis elegans*

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4.1 Abstract

The microbiological safety of fresh produce is monitored almost exclusively by culture-based detection methods. However, bacterial foodborne pathogens are known to enter a viable but nonculturable (VBNC) state in response to environmental stresses. Chlorine is currently used in the agricultural industry to sanitise fresh produce, and has been known to induce the VBNC state in a range of pathogens. Here, complete VBNC induction of green fluorescent protein (GFP) tagged *Listeria monocytogenes* was achieved by exposure to 12 ppm chlorine for 2 minutes in ddH_2O. The same was achieved in GFP-tagged *Salmonella enterica* serovar Thompson at 3 ppm. For phylloplane studies, the pathogens were incubated on spinach leaves for 24 hours which were then subjected to chlorine washes. Culture data revealed that total viable *L. monocytogenes* and *Salmonella* Thompson populations became VBNC by 50 and 100 ppm chlorine, respectively, while enumeration by direct viable count revealed a loss of total bacteria of less than 1 log. The pathogenicity of chlorine-induced VBNC *L. monocytogenes* and *Salmonella* Thompson was assessed using the animal model, *Caenorhabditis elegans*. Ingestion of VBNC *L. monocytogenes* by *C. elegans* resulted in a significant reduction of lifespan (p=0.0064), but no significant difference in lifespan reduction was observed between VBNC and culturable *L. monocytogenes* treatments. Using episcopic differential interference contrast microscopy and epifluorescence microscopy, GFP-tagged *L. monocytogenes* was visualised inside the nematode beyond the intestinal lumen, indicating resuscitation and cell invasion. Ingestion of *Salmonella* Thompson was not found to reduce *C. elegans* lifespan. These data emphasise the inability of chlorine to decontaminate fresh produce of foodborne pathogens, and the risk that VBNC foodborne pathogens could pose to the public should they continue to go undetected.

4.2 Introduction

Foodborne disease presents a consistent but frequently preventable threat to public health, and is responsible for an estimated 2.2 million deaths worldwide annually. In the UK, it is estimated that each year one million people suffer a foodborne illness, resulting in 500 deaths. In 2010, the bacterial foodborne pathogens *Listeria monocytogenes* and *Salmonella* spp. were responsible for more than half of these deaths (Anon., 2011b). Another UK study spanning 17 years determined that of foodborne outbreaks, *Salmonella* spp. were responsible for the highest number of disease cases, and the greatest proportion of deaths was caused by *L. monocytogenes* (Gormley et al., 2011).

Fresh produce such as lettuce and spinach provides an effective vehicle for these pathogens, as they are often sold as ready-to-eat foods. As consumer habits are tending towards healthier eating with more fresh produce, the risk of disease outbreaks increases (Lynch et al., 2009). In 2016, an outbreak of *L. monocytogenes* associated with packaged salads caused 19 cases each resulting in hospitalisation across 9 states in the USA (Anon., 2016a). In the UK, an outbreak was caused by *L. monocytogenes* contaminating sandwiches sold at a hospital, affecting 5 pregnant women (Dawson et al., 2006). Although *Salmonella* spp. outbreaks are proportionally less severe, they are more far-reaching. One produce associated outbreak of *Salmonella enterica* serovar Saintpaul resulted in 1500 disease cases across 43 USA states, which hospitalised 21% of those affected and may have caused 2 deaths (Barton Behravesh et al., 2011).

To detect contamination by foodborne pathogens, the agricultural industry relies almost exclusively on the use of culture recovery techniques. However, these techniques exclude the detection of viable but nonculturable (VBNC) cells. The VBNC state is a survival state entered into by bacteria, including produce-associated foodborne pathogens, in response to a range of stresses including starvation, low temperature, antibiotic pressure and oxidative stress (Dinu and Bach, 2011; Pasquaroli et al., 2013; Lin et al., 2017). Despite their nonculturability, VBNC foodborne pathogens still pose a risk to consumers. While there is conflicting data on the pathogenicity of VBNC cells there is evidence for their resuscitation under more favourable conditions, potentially allowing pathogens

to cause disease prior to or even following ingestion by humans. Research carried out on *L. monocytogenes* has found that VBNC cells induced by starvation were avirulent when exposed to human adenocarcinoma cells, but were resuscitated when inoculated into embryonated chicken eggs and regained virulence (Cappelier et al., 2005; Cappelier et al., 2007). Similar results have been observed for *Salmonella enterica* serovar Typhimurium, where VBNC cells induced by ultraviolet irradiation were unable to cause infection in a mouse model (Smith et al., 2000), however another study using *Salmonella* Oranienburg induced into the VBNC by osmotic stress found that resuscitation could be achieved following injection into a mouse model (Asakura et al., 2002). Other pathogens have been shown to retain aspects of their virulence while VBNC; the toxin genes of *Shigella dysenteriae* and *Escherichia coli* O157 have been detected while nonculturable (Rahman et al., 1996; Liu et al., 2010).

The parameters of the VBNC state and the infectivity of VBNC pathogens have been explored with a focus on VBNC induction via harsh conditions that bacteria are likely to encounter in a natural environment, but food production provides alternate stressors for foodborne pathogens. Chlorine is widely used to decontaminate fresh produce of both foodborne pathogens and spoilage bacteria. Previously, the efficacy of chlorine against *L. monocytogenes* has been measured using culture techniques, reporting that there were no viable cells recovered after using 50 ppm chlorine (Brackett, 1987). The presence of VBNC cells was not measured. Chlorine has been shown to induce the VBNC state in *Salmonella* Typhimurium biofilms (Leriche and Carpentier, 1995). Further work concentrating on chlorinated drinking water and wastewater found that chlorine induces the VBNC state in a range of pathogens including *E. coli*, *Salmonella* Typhimurium and *Helicobacter pylori* (Oliver et al., 2005; Gião et al., 2010).

The mechanisms responsible for the antimicrobial activity of chlorine are not fully understood, though studies indicate that reactive chlorine species attack the bacterial inner membrane, where the dose of HOCl required for cell killing is similar to the dose required for ATP loss, loss of DNA

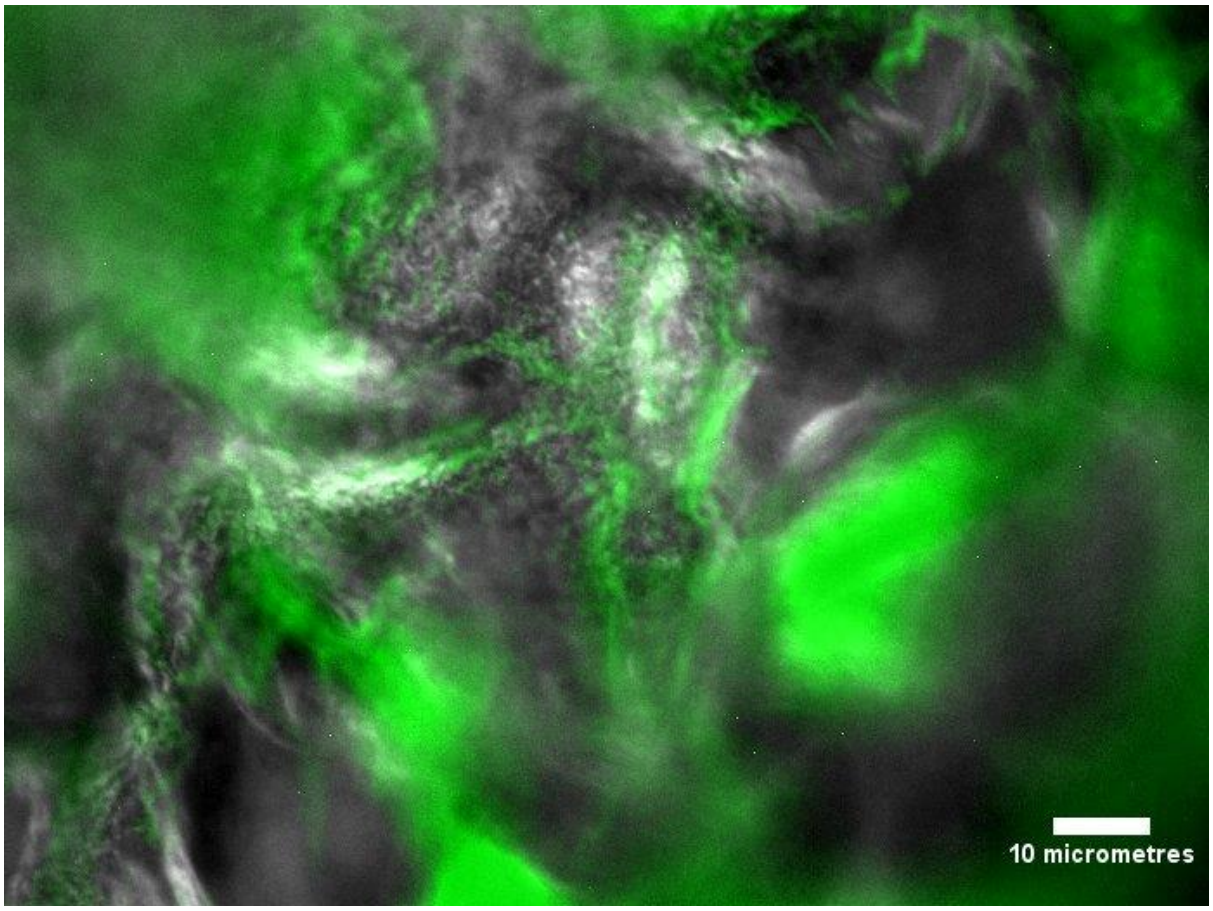
replication and prevention of protein transport across the inner membrane (Rosen et al., 1990; Gray et al., 2013).

This study will simulate the passage of spinach contaminated with *L. monocytogenes* and *Salmonella* Thompson from farm to ingestion. In this way, VBNC induction of the pathogens by chlorine will be assessed *in situ* on the spinach leaf phylloplane, comparing culture techniques to direct viable counts. The potential for infection by VBNC pathogens will then be determined by using the animal model *C. elegans*.

4.3 Results

4.3.1 Visualisation of pathogen adherence to spinach phylloplane

A.



B.

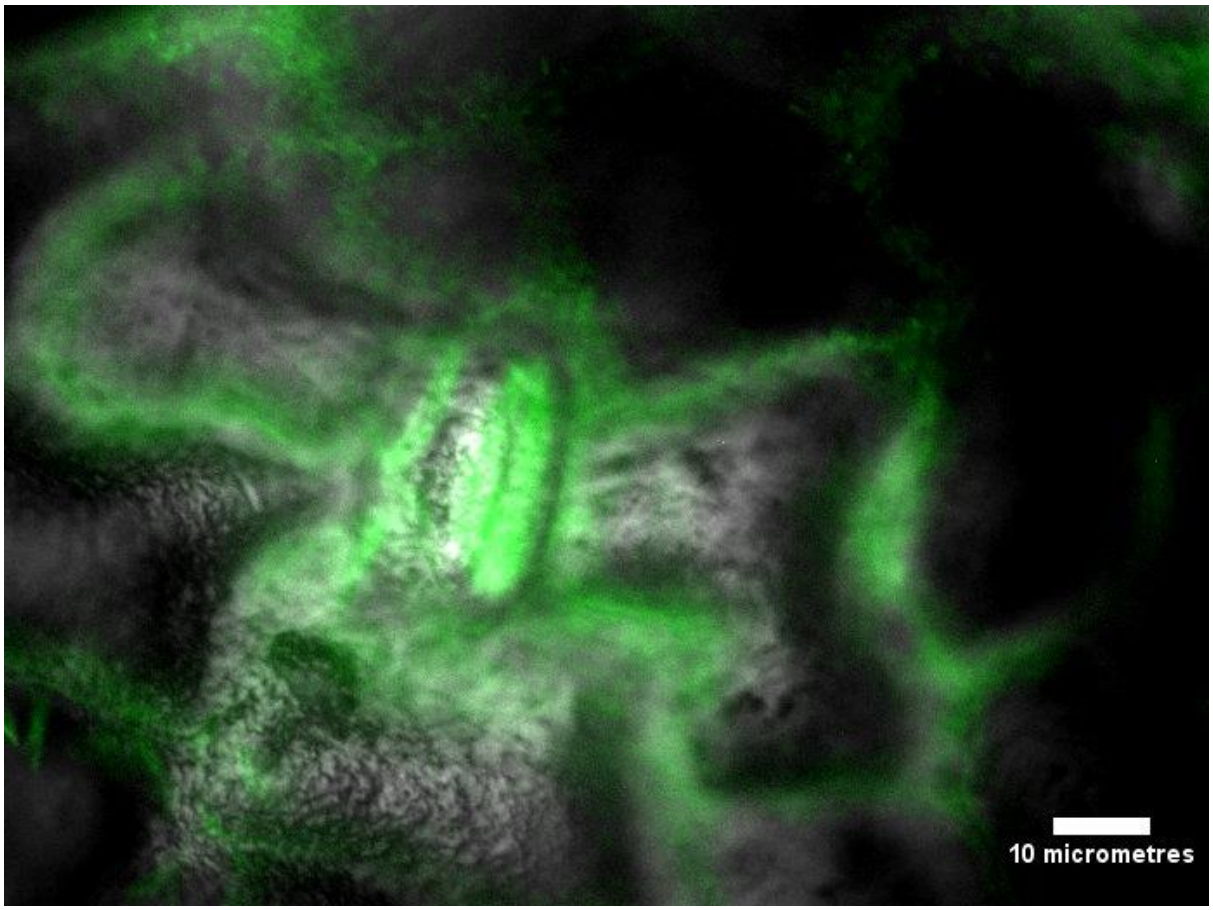


Figure 16. A. EDIC/EF micrograph of fluorescent *L. monocytogenes* adhered to the spinach phylloplane after 24 hours incubation. Scale indicates 10 µm. B. EDIC/EF micrograph of fluorescent *Salmonella* Thompson adhered to the spinach phylloplane after 24 hours incubation. Scale indicates 10 µm.

L. monocytogenes and *Salmonella* Thompson were visualised under EDIC/EF microscopy following 24 hour incubation on the spinach phylloplane. Green fluorescence indicates that the pathogens are primarily localised inside the spinach stomata, and at cell junctions. Both spinach samples possess a rough, uneven surface indicative of biofilm growth (Figure 16).

4.3.2 VBNC induction of L. monocytogenes and Salmonella Thompson in chlorinated water

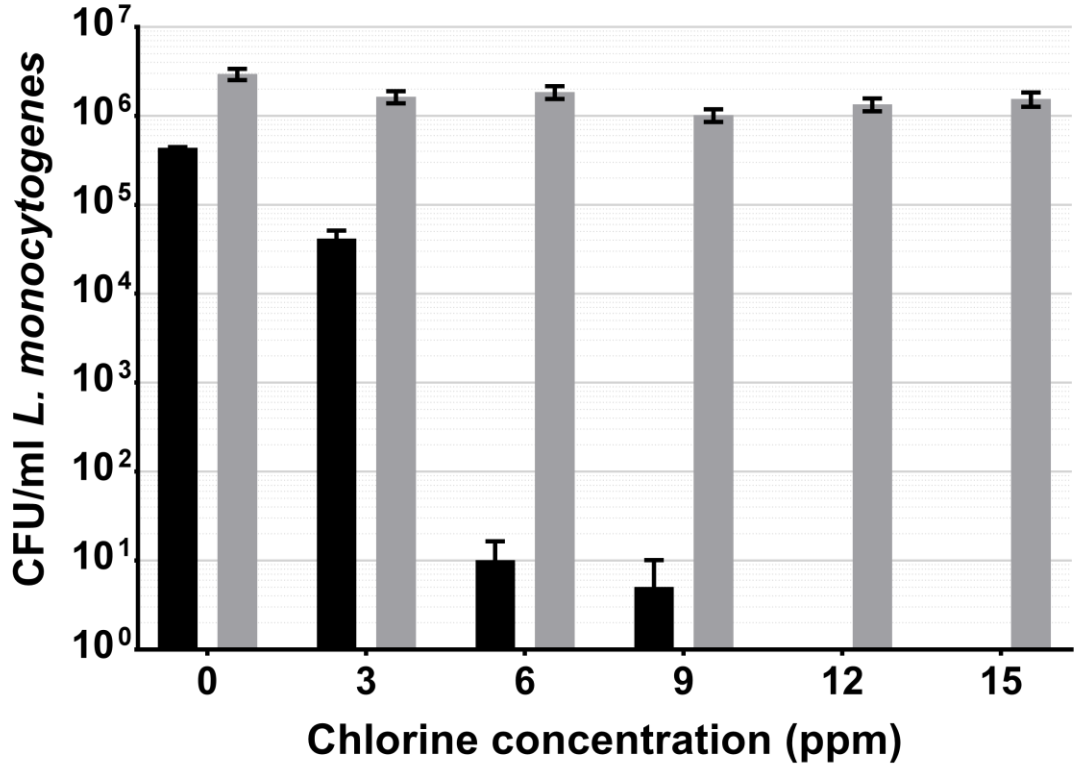


Figure 17. *L. monocytogenes* exposed to chlorinated water cultured on selective media (black) and quantified using DVC (grey). Error bars indicate SEM for 2 replicates.

L. monocytogenes became fully VBNC after 2 minutes of exposure to 12 ppm chlorine, with just under a 1-log reduction of culturability at 3 ppm ($p<0.0001$), and above a 4-log reduction by 6 ppm (Figure 17). Between 0 and 15 ppm, 47.64% of viable cells counted by DVC are lost ($p=0.0075$).

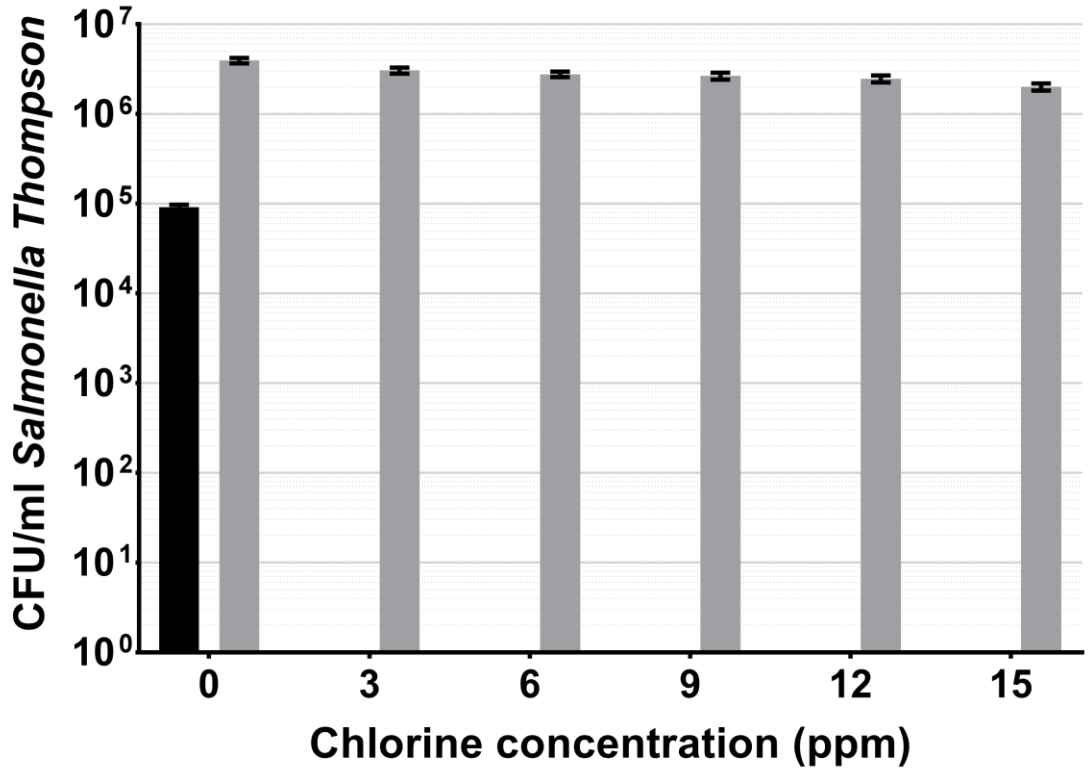


Figure 18. *Salmonella* Thompson exposed to chlorinated water cultured on selective media (black) and quantified using DVC (grey). Error bars indicate SEM for 2 replicates.

Salmonella Thompson became fully VBNC after 2 minutes of exposure to 3 ppm chlorine ($p<0.0001$). Each increase in chlorine concentration was met with a loss of *Salmonella* Thompson cells, with a 49% reduction between 0 ppm and 15 ppm chlorine ($p<0.0001$). There is also a 1.4 log difference between culturable cells and those enumerated by DVC ($p<0.0001$) at 0 ppm chlorine (Figure 18).

4.3.3 VBNC induction of L. monocytogenes and Salmonella Thompson adhered to the spinach phylloplane

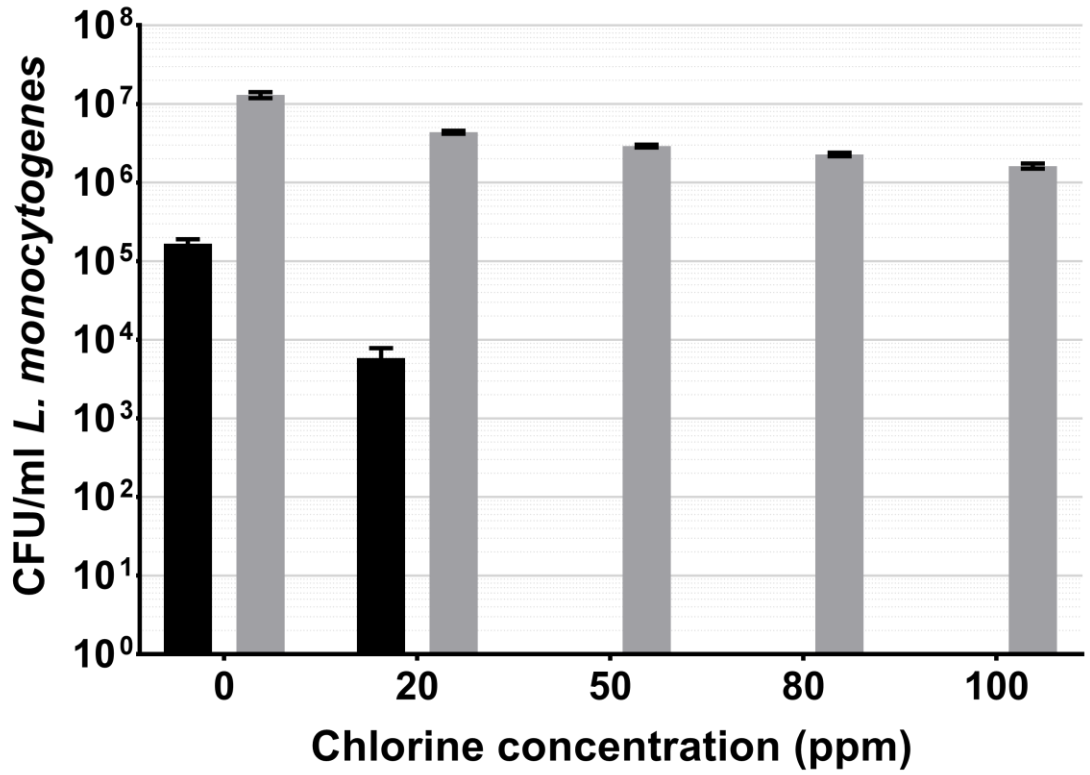


Figure 19. *L. monocytogenes* adhered to spinach leaves washed in chlorinated water, cultured on selective media (black) and quantified using DVC (grey). Error bars indicate SEM for 4 replicates.

Spinach adhered *L. monocytogenes* became fully VBNC after 2 minutes of exposure to 50 ppm chlorine, with a culturability reduction of 96.5% at 20 ppm. Direct viable counts declined with each increase in chlorine concentration, where only the decrease between 20 and 50 ppm was not statistically significant. Despite this, there was less than one log reduction between 0 and 100 ppm. There is also a discrepancy of 1.7 log between culture data and DVC data at 0 ppm (Figure 19).

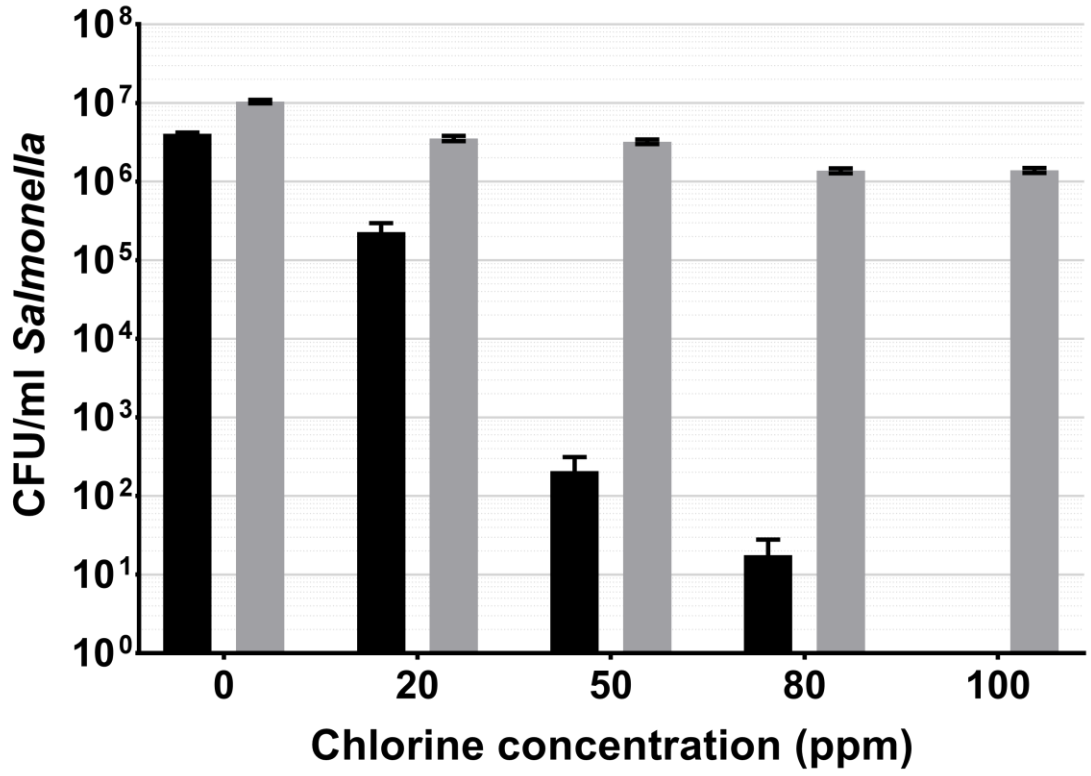


Figure 20. *Salmonella* Thompson adhered to spinach leaves washed in chlorinated water, cultured on selective media (black) and quantified using DVC (grey). Error bars indicate SEM for 4 replicates.

Salmonella Thompson adhered to spinach leaves became fully VBNC after a 2 minute exposure to 100 ppm chlorine, with a mean CFU/ml of 207 at 50 ppm and 18 CFU/ml at 80 ppm (Figure 20). Consistent with *L. monocytogenes*, a reduction in DVC is observed with each increase in chlorine concentration, until a plateau is reached at 100 ppm. Again, there is less than a 1-log reduction in DVC between 0 ppm and 100 ppm (Figure 20).

4.3.4 Virulence of VBNC L. monocytogenes and Salmonella Thompson ingested by C. elegans

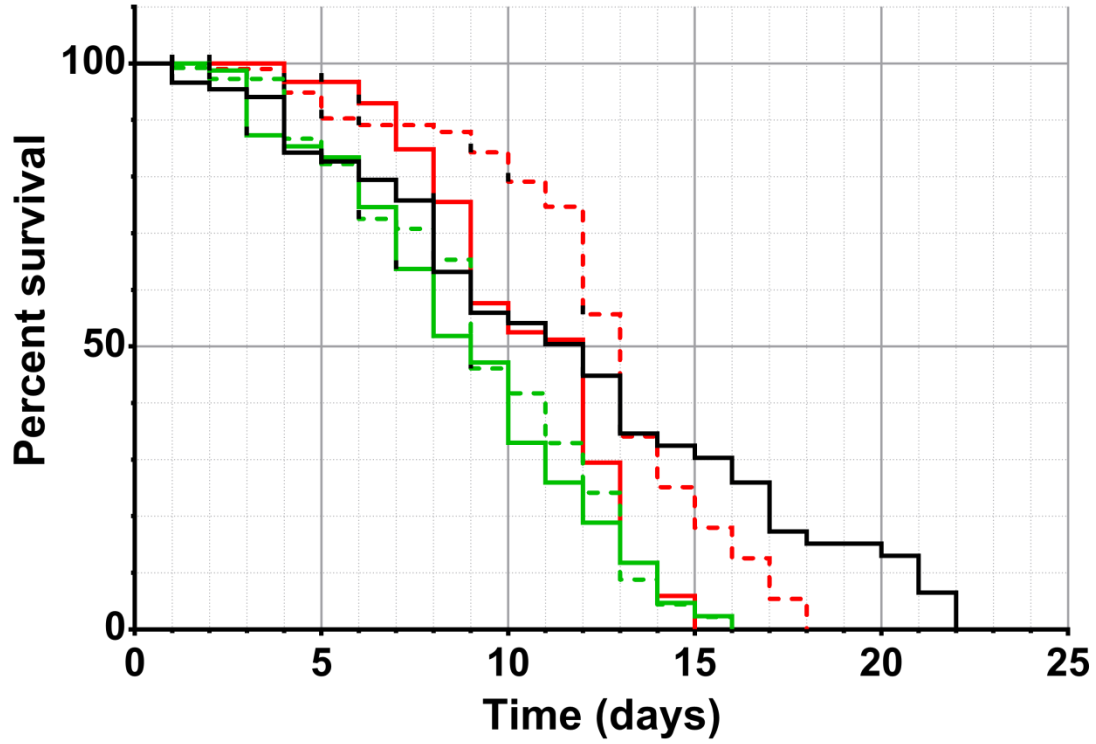
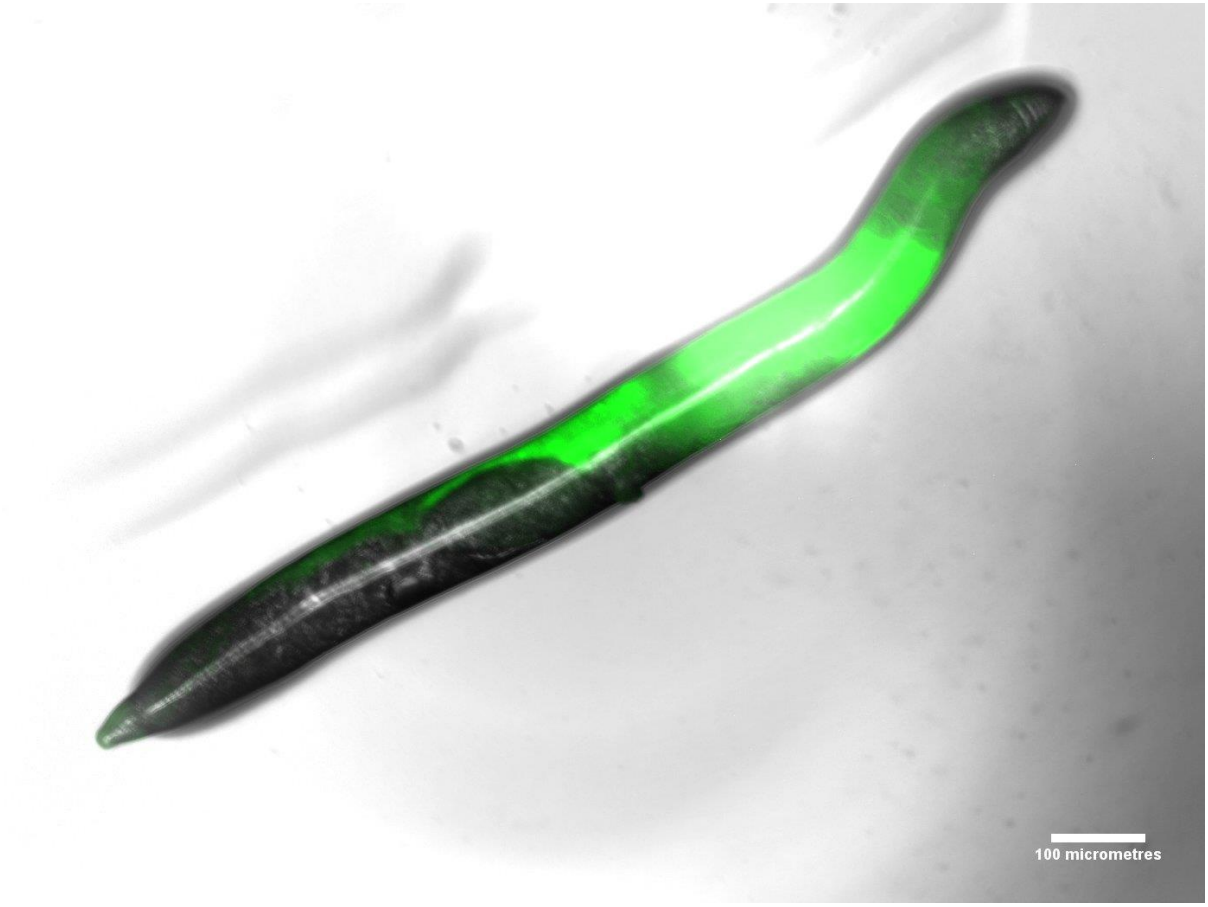
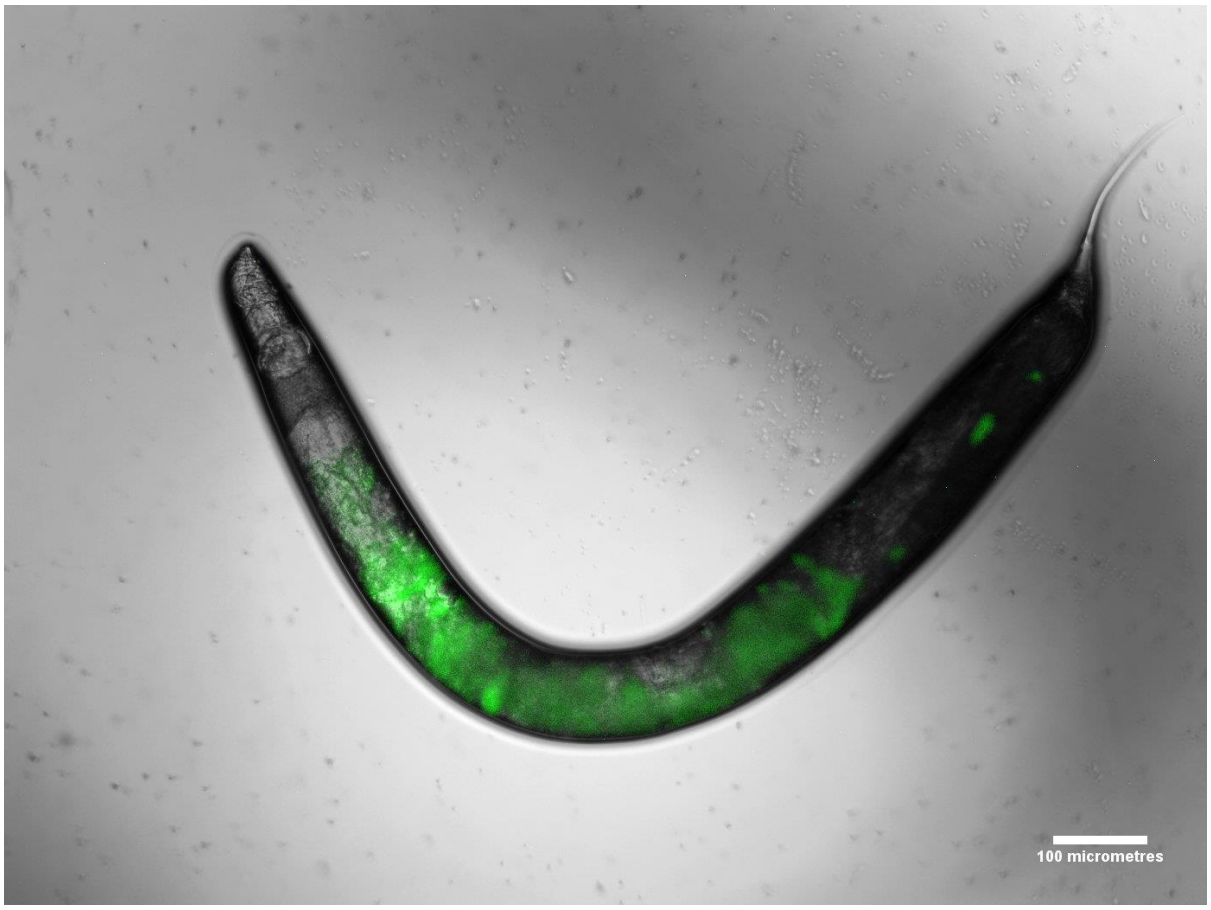


Figure 21. Survival of *C. elegans* exposed to culturable (solid line) and VBNC (broken line) *L. monocytogenes* (green) and *Salmonella* Thompson (red). *E. coli* OP50 (black) is used as a non-pathogenic control.

A.



B.



C.

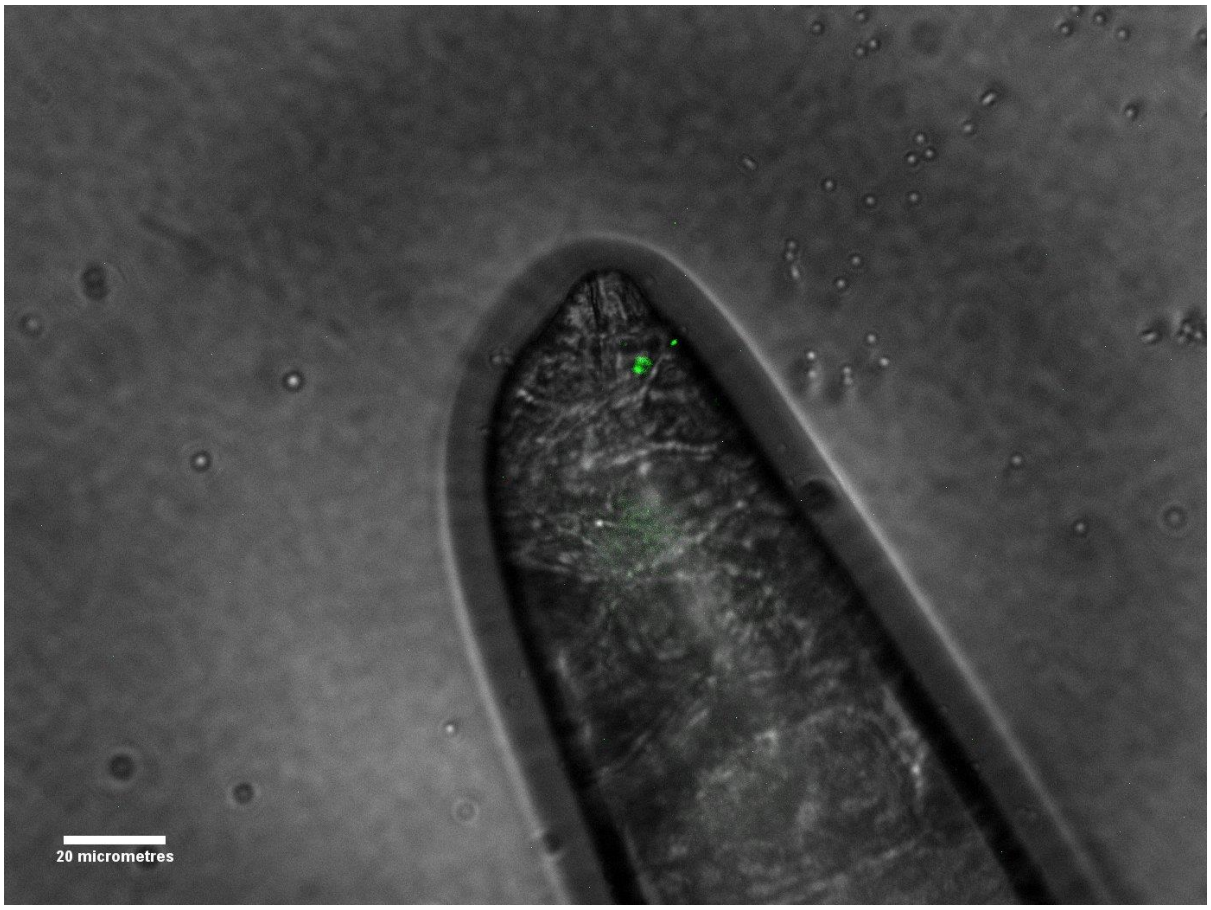


Figure 22. A. EDIC/EF micrograph of fluorescent VBNC *L. monocytogenes* ingested by *C. elegans*. Scale indicates 100 µm. B. EDIC/EF micrograph of fluorescent VBNC *Salmonella* Thompson ingested by *C. elegans*. Scale indicates 100 µm. C. EDIC/EF micrograph of fluorescent VBNC *Salmonella* Thompson ingested by *C. elegans* at the head of the nematode. Scale indicates 20 µm.

C. elegans that had only ingested *E. coli* OP50 survived for a maximum time of 22 days. All those exposed to culturable and VBNC *L. monocytogenes* died by day 16, no statistical difference between the two conditions. *C. elegans* exposed to culturable *Salmonella* Thompson died by day 15, and those exposed to VBNC *Salmonella* Thompson by day 18. Despite this, a statistical difference was not found between *E. coli* OP50 and either of the *Salmonella* Thompson treatments, but a difference was found between culturable and VBNC *Salmonella* Thompson ($p<0.0001$). Significant reductions in nematode lifespan were found between *E. coli* OP50 and culturable *L. monocytogenes* ($p=0.0012$)

and between *E. coli* OP50 and VBNC *L. monocytogenes* (p=0.0064), where the median lifespan of *C. elegans* feeding on *E. coli* OP50 was 12 days, and only 9 days for both *L. monocytogenes* treatments (Figure 21).

GFP fluorescence from each pathogen assessed was observed filling the intestinal lumen of *C. elegans* (Figure 22), and in the case of *L. monocytogenes*, permeating into the surrounding tissues (Figure 22A). Pathogen cells were still visible when nematodes were returned to *E. coli* OP50 plates.

4.4 Discussion

As chlorine is commonly used in the agricultural industry to decontaminate fresh produce, foodborne pathogens will be exposed to the sanitiser during food production, both adhered to the phylloplane and detached in suspension. Here we show that in both cases, exposure to chlorine can induce the VBNC state in *L. monocytogenes* and *Salmonella* Thompson (Figure 17-20). In water, *L. monocytogenes* becomes fully VBNC when exposed to 12 ppm chlorine, although 50 ppm is required following incubation on the spinach phylloplane (Figure 17, 19). Similarly *Salmonella* Thompson becomes fully VBNC following exposure to 100 ppm on the phylloplane, but only 3 ppm is required in chlorinated water (Figure 18, 20). This could largely be explained by the colonisation of the bacteria on the phylloplane. Both are primarily localised in and around stomata, and at cell junctions, potentially providing physical protection from the sanitiser.

A further benefit to phylloplane adherence is the facilitation of biofilm formation, where the production of an extracellular polysaccharide matrix presents a barrier for chlorine molecules. Previous studies have shown chlorine and hypochlorite to have limited penetrative ability in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* biofilms (De Beer et al., 1994; Stewart et al., 2001), as well as in *Salmonella* biofilms (Lapidot et al., 2006). This effect could be supplemented by the autochthonous bacterial species present on the phylloplane. Non-fluorescent bacterial growth observed on the spinach cell surface indicates biofilm formation by indigenous species (Figure 16), where an agonistic interaction with the inoculated foodborne pathogen may serve to reduce chlorine efficacy. These interactions could account for the relative decrease in sensitivity to chlorine observed in *Salmonella* Thompson on the phylloplane, where in ddH_2O the pathogen lost culturability more easily than *L. monocytogenes* (Figure 17-20). It was postulated in one study that when attached to the spinach phylloplane, the biofilm forming capability of foodborne pathogen *E. coli* O157 may be augmented by the presence of indigenous epiphytic bacteria (Carter et al., 2012). Despite the protective effect of biofilm, 5.5 ppm chlorine exposure has previously been shown to induce the VBNC state in *Salmonella* biofilm (Leriche and Carpentier, 1995).

This corroborates with the data in this study. The total population of *L. monocytogenes* and *Salmonella* Thompson lost culturability following exposure to 100 ppm chlorine (Figure 19-20), where the approximate 1 log reduction in bacteria counted by DVC can be attributed to cell death by chlorine exposure. Here, that reduction resulted in 1.6 x 10^6 CFU/ml VBNC *L. monocytogenes* and 1.4 x 10^6 CFU/ml VBNC *Salmonella* Thompson. Typically in the agricultural industry, 90 ppm chlorine is used to wash fresh produce. While these data show that an increase of chlorine concentration does result in a loss of viable bacteria, the chlorine use of industry is limited by the damage it causes to the food product, particularly leafy vegetables. Decontamination of food products by chlorination may be ubiquitous across food production; however a wealth of research has shown chlorine to be ineffective at killing foodborne pathogens, including *L. monocytogenes* and *E. coli* O157 inoculated on lettuce (Beuchat and Brackett, 1990; Niemira, 2008).

In water, the relatively greater sensitivity to chlorine observed in *Salmonella* Thompson (Figure 17-18) could be due to the nature of damage of reactive chlorine species in bacteria. Chlorine is thought to cause bacterial cell death by impeding the functions of the inner membrane (Gray et al., 2013). As *Salmonella* Thompson is Gram-negative, whereas *L. monocytogenes* is Gram-positive, and the Gram-positive thick peptidoglycan layer could influence susceptibility to chlorine stress. Previously, it has been shown that inactivation by exposure to singlet oxygen is affected by the presence of the peptidoglycan layer (Dahl et al., 1989). Preliminary data corroborates with this as the Gram-negative pathogen *E. coli* O157 shows a similar sensitivity to *Salmonella* Thompson in response to chlorinated water (data not shown).

The data obtained shows a pronounced difference between untreated cells quantified by culture and by DVC, particularly in Figure 17. In this case, it could be that the osmotic stress placed upon *L. monocytogenes* in ddH_2O resulted in some loss of culturability without having been exposed to chlorine. It is also possible that the discrepancy is a consequence of the assumption that cells are evenly distributed across each microscope slide.

The data obtained in this study suggest that the reduction observed in previous research can in part be attributed to VBNC induction by chlorine. In the food industry, the use of chlorine to decontaminate minimally processed food results in the inability of 'gold standard' culture techniques to detect foodborne pathogens, which may then go on to cause disease outbreaks.

The nematode killing assay revealed that there is no difference between the virulence of *L. monocytogenes* in culturable and VBNC states, and that both cause a reduction in *C. elegans* lifespan (Figure 21). Previous work on *L. monocytogenes* has provided evidence that in the VBNC state, the pathogen is avirulent (Cappelier et al., 2005). The results in this study could contradict this for several reasons; this study focuses on VBNC induction by chlorine exposure, where Cappelier *et al.* generated VBNC cells via starvation. Using human cell lines as a model, virulence was previously measured by assessing the invasive properties of *L. monocytogenes*, and was injected into the bloodstream of a mouse model. In this study, infection is modelled in *C. elegans* by ingestion. It has been shown that VBNC *E. coli* O157 maintains expression of its Shiga-like toxin genes once VBNC (Liu et al., 2010), so while there is limited research on *L. monocytogenes*, it is possible that toxin expression causes disease in the digestive tract, while cell invasion in the VBNC state is impaired.

The suggestion that there are differences in the VBNC states of the same pathogen dependent on method of VBNC induction has not been explored, but could present further challenges for the food industry. Prior to harvest, the phylloplane is a harsh environment for bacteria, with exposure to ultraviolet radiation and limited moisture, providing conditions that could induce the VBNC survival state in foodborne pathogens before exposure to chlorination. There is evidence for this as VBNC induction has been shown to occur in *E. coli* O157 on the lettuce phylloplane in response to low temperatures (Dinu and Bach, 2011). While these data show that VBNC *L. monocytogenes* induced by chlorine can cause disease, VBNC pathogens induced by physical stimuli on the phylloplane may require a separate assessment, comparing VBNC expression profiles where the fundamental mechanisms of the state have yet to be fully understood.

Compared with *E. coli* OP50, *Salmonella* Thompson did not significantly reduce the lifespan of *C. elegans* (Figure 21). This is because the median survival time of culturable *Salmonella* Thompson was 12 days and VBNC *Salmonella* Thompson at 13 days, compared to the control median at 12 days. Despite a better survival rate in the first two weeks of the experiment, the total population of nematodes fed on *Salmonella* Thompson died 7 and 4 days before those fed on *E. coli* OP50. Research carried out on the cell invasion ability of VBNC *Salmonella* Typhimurium has indicated that VBNC cells have an impaired ability to invade epithelia (Passerat et al., 2009), and those induced by antibiotic pressure are unable to cause disease in mice (Smith et al., 2002). Conversely, immunocompromised mice that ingested VBNC *Salmonella* Oranienburg were affected by the pathogen, suggesting that there is still risk of infection by VBNC *Salmonella* under certain conditions (Asakura et al., 2002). The relative success of *L. monocytogenes* in reducing *C. elegans* lifespan could be due to the ability of the pathogen to grow at lower temperatures (Walker et al., 1990), whereas VBNC *Salmonella* Thompson may require a higher temperature to resuscitate and establish infection.

Both pathogens in the VBNC state could be seen fluorescing inside the intestinal lumen of *C. elegans* (Figure 22). *L. monocytogenes* completely fills the intestinal tract and has invaded the surrounding tissues, with the ovary of the nematode masking the terminal end of the tract (Figure 22A). The high level of fluorescence observed, even when nematodes are removed from the pathogen, provides evidence that they have colonised the gut which may suggest resuscitation once inside a host. This is supported by the fluorescence extending beyond the intestine, which is consistent with the cell invasion that occurs with *L. monocytogenes* infection (Cossart et al., 2003). A similar phenomenon has been observed before in *L. monocytogenes*, where resuscitation occurred following introduction to embryonated eggs but not in non-embryonated eggs (Cappelier et al., 2007). Resuscitation inside the host could explain the data in this study, where *Salmonella* Thompson was not found to reduce *C. elegans* lifespan despite contradicting evidence in previous studies (Labrousse et al., 2000). *C. elegans* that fed on *Salmonella* Thompson died rapidly from day 12, which could be a result of colonisation and in the case of VBNC cells, resuscitation.

As such, these data support the use of the *C. elegans* invertebrate model for the study of VBNC foodborne pathogens: it is more cost and space efficient than the use of vertebrate models, and free from ethical constrains. In addition, the presence of a well-defined nervous system and digestive tract, with a mouth, pharynx that pumps the food into the intestines, a digestive system that enables them to process the food, and an excretory system, make this animal model more applicable to higher organisms than others such as the unicellular amoebal or wax moth larvae infectivity models.

The data obtained in this study does not discern whether VBNC *L. monocytogenes* causes disease by resuscitation stimulated by ingestion into a host, or by continued expression of virulence factors while in the VBNC state. However, it does provide evidence that the use of chlorine to decontaminate fresh produce is not only ineffective, but permits virulent *L. monocytogenes* to reach the public undetected by standard methods. Outbreaks of foodborne disease where no food vehicle can be identified do occur (McFarland et al., 2017), and it is possible that the VBNC state plays an important role. Consequently, new methods are required to rapidly detect VBNC pathogens which are still capable of causing disease, despite accepted sanitisation procedures, to protect the public health. Indeed, it may be better to not sanitise foodstuffs and rely instead on rapid pathogen detection methods and positive release of those foodstuffs deemed safe for human consumption.

4.5 Materials and methods

4.5.1 Bacterial strains

Bacteria used were *Listeria monocytogenes* Scott A, expressing green fluorescent protein (GFP) on plasmid pPL3-GFP, and *Salmonella enterica* serovar Thompson RM2311, expressing GFP on plasmid pWM1007 which also contains a kanamycin resistance gene (Miller et al., 2000; Dell'Era et al., 2009). Both were cultured for 18 hours at 37^{o}C in brain heart infusion broth (Oxoid, UK). *L. monocytogenes* was cultured on agar using the selective medium PALCAM (Oxoid, UK) with *Listeria* selective supplement (Sigma-Aldrich, USA), and *S. enterica* was cultured on agar using CHROMagar Salmonella Plus with its cognate supplement (CHROMagar, France). *Escherichia coli* OP50 were used as a non-pathogenic control for the nematode killing assay. Cultures were grown in Luria-Bertani broth (Formedium, UK) for 18 hours at 37^{o}C prior to use.

4.5.2 Leaf samples

Leaf samples used were raw material unwashed spinach leaves supplied by Vitacress Salads Ltd, UK. Leaves were inoculated within 48 hours of delivery: 25 g leaf sample were placed in a Stomacher bag (Interscience, France) and inoculated with 1 ml bacteria in Brain heart infusion broth (BHIB) at a concentration of 5 x 10^{7} CFU/ml. Inoculated samples were shaken vigorously and incubated at 22^{o}C for 24 hours prior to washing with chlorine.

4.5.3 Chlorine washing

4.5.3.1 Water

A stock solution of 2500 ppm free chlorine was produced by dissolving one Haz-Tab (Guest Medical, UK) in 1 litre of ddH_2O, which was further diluted in ddH_2O to generate working solutions. Bacterial suspensions of 10^8 CFU in phosphate buffered saline (PBS) (Oxoid, UK) were inoculated into 50 ml ddH_2O in a Stomacher bag, to which 50 ml of the appropriate chlorine dilution was added. The sample was shaken vigorously for 2 minutes and then filtered through a 0.22 µm pore, mixed cellulose ester membrane (Millipore, USA) using vacuum filtration. Bacteria were removed from the membrane by placing in another Stomacher bag with 100 ml PBS and shaken using the Pulsifier for 30 seconds, producing a final concentration of 10^6 CFU/ml. Samples were then taken for culture and direct viable counts (DVC).

4.5.3.2 Spinach

Following 24 hour incubation, 225 ml ddH_2O containing the appropriate volume of chlorine solution was added to inoculated spinach samples. Samples were vigorously shaken for 2 minutes and the liquid was discarded, retaining the leaf samples; 225 ml PBS was then added and the bag was shaken in the Pulsifier for 30 seconds. Samples of the resulting bacterial suspension were then taken for culture and DVC.

4.5.4 DVC and visualisation of samples

Samples taken for DVC were concentrated by centrifuging 10 ml sample for 15 minutes at 4000 rpm using a Heraeus Megafuge 1.0. The sample was then resuspended in 1 ml PBS. To aid visualisation, samples were subjected to cell elongation, carried out using a modification of the method by Juhna *et al* (Juhna et al., 2007). The 1 ml sample was added to 4 ml ddH_2O and 5 ml R2 broth (0.1% w/v peptone, 0.05% w/v yeast extract, 0.05% w/v glucose, 0.05% w/v starch, 0.03% w/v potassium dihydrogen phosphate, 0.03% w/v sodium pyruvate and 0.0024% w/v magnesium sulphate), with 10 µl pipemidic acid at a concentration of 10 µg/ml. The suspension was incubated for 18 hours at 22^{o}C in darkness. The suspension was concentrated prior to DVC in the same manner as before.

All samples were imaged using episcopic differential interference contrast (EDIC) and epifluorescent (EF) microscopy and a QImaging Retiga EXi camera. Bacteria were quantified by counting visible cells across at least 30 fields of view per sample. Images were merged using ImageJ.

4.5.5 **Caenorhabditis elegans** ***killing assay***

C. elegans were maintained on 5 cm nematode growth medium (NGM) agar plates, prepared according to standard methods (Brenner, 1974) with a lawn of *E. coli* OP50. To prepare experimental plates, 50 µl of *E. coli* OP50, *L. monocytogenes*, or *Salmonella* Thompson cultures were added to the centre of the plates and incubated at 22^{o}C for 24 hours. To produce VBNC cells, cultures of *L. monocytogenes* and *Salmonella* Thompson were pelleted by centrifugation and resuspended in 10 ml 200 ppm chlorine solution for 30 minutes. Chlorinated water was removed by vacuum filtration as described above, and bacteria were removed from the membrane by vortexing in 1 ml PBS for 2 minutes (Highmore et al., 2017), concentrating the sample to compensate for the growth of their culturable counterparts on the NGM plate. Plates were then inoculated with 50 µl VBNC cells and incubated at 22^{o}C for 24 hours. VBNC cells were plated on selective media to verify the VBNC state.

C. elegans were transferred to experimental plates at the L4 stage; 20 animals were used per plate and each condition was tested with at least 4 replicates. Nematodes were counted daily and transferred to fresh plates every other day. Nematodes that did not respond to being prodded with a pick were considered dead.

4.5.6 Statistical analyses

Culture data and DVC were separately analysed using a one-way ANOVA with Tukey's multiple comparisons test. Comparisons between culture data and DVC data were carried out using multiple t-tests. Nematode killing assay was analysed using the survival curve comparison Mantel-Cox test. All statistical analyses were carried out using GraphPad Prism 7.

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Chapter 5

General Discussion

5.1 The state-of-the-art of the VBNC state

In previous studies, VBNC induction has been shown to occur due to conditions found in growing media, on the phylloplane of fresh produce, and due to chlorine treatment (Leriche and Carpentier, 1995; Liu et al., 2008; Dinu and Bach, 2011). The papers presented in this study link previous research to the challenges VBNC foodborne pathogens present the agricultural industry, and further understanding of their prevalence and significance to food safety.

The results presented here also provide insight into the philosophical question of what the term 'viable but nonculturable' can refer to. In Chapter 2, the research goal is to produce an assay that facilitates the detection of VBNC foodborne pathogens in the complex environmental matrix of soil. Here, the term VBNC is defined wholly by its functionality; regardless of whether there is a biochemical imperative underpinning the VBNC state of the bacterial targets, the assay aims to include the detection of any potentially infective pathogen that would circumvent detection by routine culture-based techniques that the agricultural industry employs.

This extends to the broadest definition of the term employed across previous studies (McDougald et al., 1998; Nystrom, 2003), where VBNC cells are non-replicative, moribund cells transitioning to cell death. Through the use of qPCR, even the DNA of dead cells will be detected by the assay described. This is valuable to the agricultural industry due to a limitation of this method of pathogen detection, while the assay allows for a larger volume of 25 g sample to be tested, it is still comparatively miniscule against the total volume of soil in any agricultural plot. Therefore, the detection of dead cells or those in a moribund VBNC state may be indicative of pathogenic cells elsewhere in the land, and so would reveal a risk of foodborne disease to the public.

The research question addressed by this chapter is an example of the response of research to the current understanding of the VBNC state; exhaustive investigation of culture conditions for bacteria *in situ* is not feasible, so alternative detection methods were developed to include any nonculturable bacterial target.

Chapter 3 further explores the definition of VBNC, and directly faces the controversy of resuscitation. After being unable to culture *E. coli* O157 on selective media, viable cells were detected using a PNA-FISH probe specific to the pathogen (Figure 15). To ensure viability, pipemidic acid was used to inhibit DNA replication but cause the elongation of cells via continued protein synthesis. This invites the paradox of identifying VBNC cells by their replicative potential. Previous work has used a similar compound, nalidixic acid, to determine potential culturability in *V. parahaemolyticus* that was not detectable on agar (Jiang and Chai, 1996). In this case, the technique was used to conclude that the perceived return of the *V. parahaemolyticus* population to culturability was caused by the regrowth of a few surviving cells. However, the study is consistent with the results presented here; in both cases elongation was observed in bacterial samples that were deemed nonculturable on growth media.

The hypothesis that the elongated *E. coli* O157 cells visualised using PNA-FISH correspond to culturable cells among a larger population of irreversibly nonculturable cells conflicts with the data, as enumeration under the microscope is consistent with data obtained via qPCR (Figure 11). The concentration of VBNC *E. coli* O157 is estimated at 1113 and 978 cells per gram of soil by these two methods, which should not be below the limit of detection using culture-based techniques. Previous research investigating this issue (Whitesides and Oliver, 1997) verifies resuscitation by first inducing cell cultures into the VBNC state and subjecting samples to different treatments. This methodology cannot be carried out in the present study as resuscitation of the pathogen is concomitant with its isolation from an environmental sample, with a heterogeneous microbial population. While confirmation of the VBNC state and subsequent resuscitation is valuable for stimulating future research into the state, the reality of culturing *E. coli* O157 from a commercially available soil sample raises further questions into the prevalence of the pathogen in the environment, and the possibility that it is currently underrepresented.

The functional definition of the VBNC state does not acknowledge the intricacies of microbial survival in nature, and this incongruity may unpick the Chapter 3 paradox. The observation of cell

elongation coupled with the inability of the same bacterial population to produce colonies on culture media could be a result of a gradient of culturability responding to a huge number of stressors. The complexity of detecting and isolating confirmed VBNC cells from environmental samples has led to little research conducted on the topic, which in turn impedes the characterisation of the nonculturable state or states that allow bacteria to persist in the environment. In this study, the need for this characterisation is emphasised, as a nonculturable isolate of an important human pathogen has been detected in the environment.

The research presented further suggests diversity within a supposedly singular VBNC state regarding the pathogenicity of nonculturable pathogens. Chapter 4 focuses on assessing the pathogenicity of *L. monocytogenes* and *S. enterica* following chlorine mediated VBNC induction. It was found that while in the state, ingestion of the bacteria by *C. elegans* causes a significant reduction of lifespan (Figure 21). This conflicts with previous research, which has shown that VBNC *L. monocytogenes* could neither cause disease in mice, nor infect a human cell line (Cappelier et al., 2005). *S. enterica* was also found to be unable to cause infection in mice while in the VBNC state (Smith et al., 2000). While further research in the area shows that both pathogens are capable of resuscitation when inoculated into a host (Asakura et al., 2002; Cappelier et al., 2007), in this study there is no definitive differentiation between VBNC and resuscitated pathogens increasing mortality in *C. elegans*.

The aforementioned studies on VBNC *L. monocytogenes* in mice are a more direct contradiction to the results presented here, as resuscitation was conducted by inoculation onto embryonated eggs, rather than via passage in the mouse model (Cappelier et al., 2007). This could be explained by several experimental differences: the pathogen strain used, or the fundamental differences in the nature of the animal model, but it could also be that mode of VBNC induction and environmental conditions while in the state elicit different responses in nonculturable bacteria. In literature there has been little focus on this subject, but other VBNC studies exploring induction and resuscitation provide a valuable precedent. By examining factors that influence resuscitation of *V. vulnificus*, Pinto et al (Pinto et al., 2011) observed that no resuscitation of VBNC suspensions was achieved when the

state was induced at room temperature, but each suspension of VBNC cells induced at 4^{o}C could be resuscitated by at least one of four conditions. The cytotoxic activity of VBNC *E. coli* O157 was also found to be dependent on induction conditions (Liu et al., 2009; Liu et al., 2010). Following 19 month incubation in river water, VBNC *E. coli* O157 was found to produce more Stx toxin corresponding to more Vero cell death than VBNC cells incubated in PBS for the same length of time. The authors note that VBNC cells induced in river water could pose a greater threat to the public than those produce under different conditions, but no further distinction between cell populations is identified.

As early as 1998, it has been asserted that the term 'viable but nonculturable' is unsuitable (Kell et al., 1998), considering the limitations of the functional nature of the definition. The authors provide the alternative possible term 'active but nonculturable' (ABNC), to reflect contradicting notions of viability. As the focus of research has shifted from the existence of the state towards resuscitation and infectivity of VBNC pathogens, use of the term ABNC does not fully describe the state or the relevance to public health regarding nonculturable pathogens. Toxin-producing nonculturable (TPNC) cells might be a more appropriate description, and through its connotations could promote further research into the topic. Another distinction could be made between nonculturable cells and non-replicative cells, where the latter do not elongate in response to pipemidic acid treatment. However, these proposals still rely on the bacterial response to laboratory techniques. There is still a lack of knowledge into fundamental aspects of the VBNC state, and future work may bring the transcriptomic and proteomic profiles necessary to bring the concept of the VBNC state into the confines of a biological definition that remains relevant to nature.

5.2 The detection of VBNC foodborne pathogens in soil by qPCR

The analysis of complex environmental matrices has been facilitated with the techniques employed in this study, so that in peat-based compost and sand, foodborne pathogens can be detected to a sensitivity of 10 CFU/g (Figure 6, 8). The Stomacher is still widely used in laboratories focusing on

food microbiology, yet evidence provided in this study shows that the homogenisation of soil samples reduces the efficacy of isolation of bacterial populations (Figure 4). The introduction of the Pulsifier and the use of simple, low cost sample preparation via vacuum filtration can help to bypass the need for culture techniques in pathogen detection in agricultural soil. The qPCR method of detection was selected for this assay in place of newer, high throughput technology such as Next-Generation DNA sequencing or RNA-Seq to keep the assay relatively cost-effective and using the equipment likely to be available in an industrial laboratory. The assay could be adapted to support multiplex qPCR, permitting the detection of multiple foodborne pathogens in one assay.

The assay was used to analyse nine soil samples of agricultural origin, in addition to two commercially available compost types, detecting the *E. coli* O157 *tir* gene in seven of the samples. As the assay does not discriminate between DNA from living and dead cells, further analysis would need to verify any potential contamination. It is arguable that any positive result generated by the qPCR assay could be considered a threat to public health; genes detected from dead cells still indicate that contamination by the target pathogen of the soil has taken place, and internalisation of the pathogen into the plant could have already occurred (Solomon et al., 2002).

Positive results obtained from the assay were explored during the project, using the compost sample for which the assay was optimised. The optimisation of the assay for this soil type may be responsible for the highest concentration of *tir* genes detected in this sample; gene detection levels in other samples may have been underestimated due to loss of the bacterial sample during sample preparation. Deeper analyses of the other soil samples could not take place due to time constraints, but further investigation of soil samples without a predisposition to contamination by foodborne pathogens found in agricultural soils could improve understanding of the extent of VBNC foodborne pathogen dissemination. Previously, *L. monocytogenes* was found to be more prevalent in the soil of

uncultivated fields than in cultivated field soil samples, although this study was only carried out using culture techniques (Dowe et al., 1997).

The sample was analysed for the presence of viable *E. coli* O157 cells using PNA-FISH and DVC, in conjunction with culture data to determine VBNC status of the pathogen. In this case, the analysis was already primed by a lack of culture data to indicate VBNC presence, however in an industrial setting, testing may be carried out using further molecular methods such as RT-qPCR, where detection of pathogen gene expression will confirm viability and it requires little more equipment than would already be present for conducting the qPCR assay.

In this study, determining that the contaminating pathogen was in the VBNC state was necessary, as little is currently known about the prevalence of VBNC populations in the environment due to the difficulties in their isolation. Should the assay described be used in the agricultural industry, the distinction may carry less significance due to the research also carried out in this study, assessing the pathogenicity of VBNC *L. monocytogenes* and *Salmonella* Thompson. Using a *C. elegans* killing assay, it was determined that ingestion of VBNC *L. monocytogenes* induced by chlorine significantly reduced *C. elegans* lifespan, indistinguishable from the reduction caused by culturable *L. monocytogenes* (Figure 21). By measuring the virulence of different pathogens in the VBNC state, the agricultural industry will be able to effectively discriminate between positive results when testing agricultural soil for foodborne pathogens using the methods outlined in this study.

5.3 Chlorine VBNC induction and detection

This research has also examined the role of food production practices in the generation of VBNC foodborne pathogens through the use of chlorine as a sanitiser of fresh produce. It was found that in water, populations of *L. monocytogenes* and *Salmonella* Thompson became fully VBNC following

exposure to 12 ppm and 3 ppm chlorine respectively (Figure 17-18). When adhered to the spinach leaf phylloplane, the tolerance to chlorine increased, requiring 50 ppm to induce the state in *L. monocytogenes* and 100 ppm to do so in *Salmonella* Thompson (Figure 19-20). Chlorine treatments of 100 ppm resulted in less than a 1-log reduction of viable bacteria for both strains assessed and regardless of the presence of spinach leaves. This corroborates with previous research showing the inefficacy of chlorine as an antimicrobial agent (Niemira and Cooke, 2010). It also supports previous research into VBNC induction of pathogens by chlorine, but with a focus on fresh produce rather than wastewater (Oliver et al., 2005).

The data obtained across this study could be utilised to apply the VBNC pathogen detection assay to fresh produce washed in chlorine, as currently microbial safety of produce is dependent on culture techniques. In addition to the decontamination of foodborne pathogens, chlorine washing of fresh produce is used to remove spoilage bacteria for an improved shelf life (Behrsing et al., 2000). This could result in industrial producers of fresh produce being reluctant to remove chlorine treatment from food production practices. The qPCR assay used to detect foodborne pathogens in soil could easily be adapted for VBNC pathogen detection following factory processing, as samples of fresh produce in suspension would require less preparation than soil.

Alternatively, removal of chlorine treatment for fresh produce would reduce the potential for VBNC induction of foodborne pathogens during food production, and in doing so increase the strength of culture-based detection techniques. One commercial supplier of fresh produce in the UK, Vitacress Salads Ltd, has eliminated chlorine washes for fresh produce in favour of washing in spring water. However, VBNC induction of pathogens could still occur in the soil or on the phylloplane, so a direct method of detection is favourable over culture-based methods.

This study was focused on the implication of VBNC induction of foodborne pathogens by chlorine, and as such the factors affecting chlorine efficacy were not thoroughly explored. In particular, it was observed that adherence to the spinach leaf phylloplane provided *Salmonella* Thompson populations with a substantial increase in tolerance to chlorine stress, relative to *L. monocytogenes* (Figure 19-20). This may be due to its proposed adaptations to surviving on the phylloplane (Charkowski et al., 2002), but could in part be caused by interactions with autochthonous epiphytic bacteria; episcopic differential interference microscopy and epifluorescence microscopy showed the pathogen colonising the spinach leaf surface together with apparent biofilm growth from indigenous bacteria (Figure 16B). Previous research has examined the potential for using antagonistic interactions with other bacterial species synergistically with chlorine treatment to enhance decontamination of *E. coli* O157 on the spinach phylloplane (Gragg and Brashears, 2010), so conversely further research could characterise agonistic interactions with indigenous bacteria impeding sanitisation of fresh produce.

In summary, the data obtained suggest that washing fresh produce with chlorine is not only an ineffective treatment, but facilitates the generation of VBNC foodborne pathogens. Without a direct detection method that bypasses culture-based pre-enrichment techniques, these pathogens may compromise food safety unimpeded.

5.4 Infectivity of VBNC foodborne pathogens

The purpose of researching effective methods of VBNC foodborne pathogen detection is to enhance the microbial safety of food. This relies on the characterisation of the threat that the pathogens present the public while in the VBNC state. Previous studies have assessed this in response to VBNC induction by starvation and incubation at low temperatures (Cappelier et al., 2005; Liu et al., 2010). While important, VBNC induction under these circumstances is not assured in an agricultural environment. This research examines the pathogenicity of *L. monocytogenes* and *Salmonella*

Thompson induced by chlorine treatment, simulating the treatment of fresh produce in the food industry which currently acts as the primary decontamination method for ready-to-eat food.

A *C. elegans* killing assay determined that VBNC *L. monocytogenes* maintained its full infectivity while in the VBNC state, but neither culturable nor VBNC *Salmonella* Thompson significantly reduced *C. elegans* lifespan, despite the total population of nematodes that had ingested culturable *Salmonella* Thompson dying 7 days faster than the control nematodes (Figure 21). These data conflict with previous research, which has suggested that *Salmonella* Typhimurium significantly decreases the *C. elegans* lifespan (Labrousse et al., 2000). This difference could simply be caused by the strain used, *Salmonella* Thompson is a relatively rare serovar, in 2003 it was attributed to only 0.26% of *Salmonella* disease cases across Europe (Nygård et al., 2004), whereas *Salmonella* Typhimurium is a prominent foodborne pathogen, responsible for 5.3% of total illnesses caused by foodborne pathogens associated with leafy vegetables in the USA between 1998 and 2008 (Painter et al., 2013). Another potential cause of the lack of virulence is that the bacteria used contained plasmid pWM1007, containing GFP and a kanamycin resistance gene. This insertion could have sufficiently reduced fitness to reduce infectivity in the *C. elegans* worm.

The pathogenicity of *L. monocytogenes* inside a *C. elegans* host has previously been documented (Thomsen et al., 2006); the data in this study corroborate with previous research and provide new insight into its pathogenicity, infecting the nematodes with cells in the VBNC state. While this research shows no loss of virulence by the pathogen in the VBNC state, previous research has found VBNC *L. monocytogenes* to be avirulent in a mouse model (Cappelier et al., 2005). There are several potential causes for this. The VBNC cells were produced by storing *L. monocytogenes* in distilled water in darkness for 10 weeks, starving them. In this study, chlorine stress was used. There has been very little research conducted on the differences between VBNC cells inducted by different

methods, and as there is such a diverse range of VBNC inducing factors, the phenotype observed in VBNC cells induced by one stressor should not be assumed for cells induced by another.

Another potential cause of the contradiction to previous literature is the use of the *C. elegans* model. The model has been used to study several foodborne pathogens with notable advantages over other animal models. There are no ethical concerns as there would be with vertebrate models, and as *C. elegans* naturally feeds on bacteria, they can model infection by ingestion in place of intravenous inoculation. *C. elegans* also bears the benefit of being able to visualise pathogens inside the intestinal lumen of living animals. However, *C. elegans* is grown at room temperature. Where colonisation of a mammalian host would occur at 37^{o}C, this temperature is prohibitive for the use *C. elegans* (Zevian and Yanowitz, 2014). *L. monocytogenes* is known to thrive at lower temperatures (Walker et al., 1990), which could explain the infectivity observed in *C. elegans* where avirulence was determined in mice.

By visualising *L. monocytogenes* colonisation inside a living *C. elegans* worm, it was observed that the pathogens had invaded tissues beyond the intestinal lumen (Figure 22). This either shows the resuscitation of VBNC *L. monocytogenes* inside a living host restoring infectivity of the pathogen, or continued virulence while in the VBNC state. It has previously been observed that the cell invasion characteristic of *L. monocytogenes* infection is impaired by entry of the pathogen into the VBNC state (Cappelier et al., 2005). Further exploration of this subject could include homogenisation of the *C. elegans* host and attempt to culture resuscitated *L. monocytogenes*. The factors determining resuscitation would then be defined, including the effect of incubation temperature, so that the danger to humans from chlorine induced VBNC foodborne pathogens can be fully characterised.

The pathogenicity of *E. coli* O157 resuscitated from peat-based compost was not determined. With more time, a *C. elegans* killing assay would have been carried out in the same manner as the experiments with *L. monocytogenes* and *Salmonella* Thompson. Previously, starvation induced VBNC *E. coli* O157 cells have been observed as having a cytotoxic effect on Vero cells through continued expression of Shiga-toxin genes, and foodborne pathogens have been documented as regaining pathogenicity upon resuscitation (Cappelier et al., 2007; Liu et al., 2010). This research, and the results obtained in this study, supports the hypothesis that resuscitated *E. coli* O157 is able to infect hosts following ingestion, and its discovery in horticultural growing media brings that threat to public health away from theory and into the environment.

5.5 Limitations of the study

The research conducted in this study is limited by time and cost, producing limitations and raising questions that could be addressed by future research. Although the limitation of the VBNC state being poorly defined is pervasive throughout the study, this has been discussed in Chapter 5.5 and so will not be addressed here.

The pathogen detection assay described in Chapter 2 was constructed with the aim of allowing for greater sample size of soil in agricultural land. However, multiple smaller samples could have been taken from which bacterial DNA could have been isolated directly, removing the need for bacterial sample separation and concentration. This argument is supported by the loss of bacterial sample observed on the filter membranes following filtration (Figure 10). Further investigation into this approach was prevented by the sample loss being observed in an advanced stage of assay development, so it was decided that optimisation of the assay would utilise the available time more effectively.

Another specification of the assay is that it must be suitable for use by industry, taking into account the limitations of industrial laboratories. This came at the expense of sensitivity of the resulting assay, and does not factor the advancing technology that may become ubiquitous in industrial

laboratories in the near future. The BAX® system expands upon the PCR technique to detect foodborne pathogens from food samples, by automating detection with 96 samples at a time in a fluorescence based assay (Nugen and Baeumner, 2008). PCR based techniques have been further improved (Mester et al., 2017), where in place of the culture-based pre-enrichment step used for signal amplification, molecular enrichment is used to amplify the whole genome of the target bacteria. This was used in conjunction with a 'Matrix-Lysis technique which facilitates processing of large volumes of food samples and reduces DNA background from dead cells. While environmental samples were not mentioned in the study, these emerging techniques could develop foodborne pathogen detection with minimal sample preparation.

Alternative detection methods to PCR are also being used to detect foodborne pathogens. Microarray technology allows for a rapid, high-throughput detection of foodborne pathogens where identification of individual genes permits discrimination between pathogen strains (Li et al., 2017). This approach can also be used in samples of mixed bacterial populations, bypassing one of the challenges that come with testing environmental samples. A recent pilot study also assessed the use of proteomics to detect bacterial contaminants in food samples via mass spectrometry (Jabbour et al., 2017); while routine testing by this method is currently prohibitively expensive, the trend towards streamlining and automating high-throughput technologies will change the equipment commonly seen in industrial laboratories in the near future.

Originally, it was assumed that the inclusion of DNA from dead cells would be indicative of contamination of agricultural land. When applied to soils of agricultural origin, DNA from the target *E. coli* O157 was found in 7 of 11 samples (Figure 10); it has previously been noted that adsorption of DNA to clay particles can prevent its degradation for years (Demaneche et al., 2001). Future research into the presence of foodborne pathogens in soil could take this into account, and exclude DNA from dead cells using RT-qPCR to focus on gene expression of pathogens, ensuring continued detection of VBNC cells. This methodology would also be appropriate for determining the nature of the *tir* gene detection in other agricultural soils tested Figure 10).

While the results obtained in Chapter 4 by PNA-FISH were corroborated by qPCR, improvements could have been made to the methodology to improve the quality of the data. Capture antibody target fluorescence *in situ* hybridisation (CAT-FISH), as the name suggests, uses antibodies to isolate bacteria from complex samples alongside a conventional FISH probe (Stroot et al., 2012). This permits increased specificity at the protein level of the bacterial target and has been implemented in the complex matrices of spinach rinse and blood culture, making it suitable for the experiments carried out in this study.

Alone, the specificity granted by a PNA-FISH probe is subject to change as further genomes are sequenced. The probe used was developed by Almeida et al with the sequence 5'-CAA CAC ACA GTG TC-3' (Almeida et al., 2013), specific to *E. coli* O157. The probe was tested for false positives using several bacterial species, including *Salmonella* Typhimurium. While no positive result was observed using the PNA probe with this bacterium, using a BLAST nucleotide search (Altschul et al., 1997) the probe sequence was found to share 100% homology with *Salmonella* Typhimurium, several other *S. enterica* serovars, and *Vibrio natriegens*. In this study, the qPCR data set a precedent for the presence of *E. coli* O157, however the limitations of this methodology should be considered before it is applied to similar studies.

C. elegans has not previously been used to investigate the VBNC state, so while novel, some limitations have been revealed in the emerging application of this animal model. *C. elegans* is unable to survive at the mammalian core temperature of 37^{o}C (Zevian and Yanowitz, 2014), which could affect the growth and resuscitation capability of ingested VBNC pathogens. Modelling pathogen infection is also limited in *C. elegans* as it is limited to an innate immune system (Ermolaeva and Schumacher, 2014), although this limitation may have greater significance for potential subsequent studies concerning resuscitation of VBNC pathogens in the nematode intestinal lumen. Another possible factor affecting the study is that *C. elegans* maintained in the laboratory purely on *E. coli* OP50 is unlikely to accurately simulate the human gut microflora. These bacterial communities could have a role in the survival of VBNC pathogens in a host; previous research has found that factors

produced by culturable bacteria can induce resuscitation in VBNC foodborne pathogens (Reissbrodt et al., 2002). One further limitation of using *C. elegans* over a mammalian model is that the primary determinant of pathogenicity in *C. elegans* is a reduction in lifespan; symptoms of less severe foodborne infection cannot be identified. Mouse models have previously been used to determine these symptoms, such as the diarrhoea associated with *Salmonella* infections (Asakura et al., 2002).

Despite the limitations of the *C. elegans* animal model, a methodological reason VBNC foodborne pathogens have not been explored with this model is that the bacteria to be ingested by the nematode is grown on an agar plate prior to exposure. This cannot be achieved for VBNC cells. To meet this challenge, cells induced into the VBNC state were concentrated to provide the same number of culturable cells as those that grew on the agar. However, this cannot account for the regrowth of culturable cells that occurs while the assay is underway and so remains a limitation of this study.

Due to time constraints, a nematode killing assay was not performed on the resuscitated *E. coli* O157 isolate. This would have been a valuable assay that could verify the virulence of a resuscitated pathogen induced into the VBNC state *in situ*, and could clearly illustrate the risk to food safety posed by VBNC foodborne pathogens.

5.6 Conclusions

The work presented here applies the theory of VBNC pathogenicity to the agricultural industry, and demonstrates the potential for foodborne outbreak through VBNC carriage on minimally processed foods. By investigating aspects of the VBNC state, the nature of nonculturable bacteria has been questioned. The results here (Figure 21) in conjunction with literature (Smith et al., 2000; Cappelier et al., 2005) suggest that the functional parameters that define the VBNC state are insufficient to describe the intricacies of nonculturable bacterial survival in response to stress.

In the absence of a complete understanding of nonculturable bacteria, a detection method for foodborne pathogens, regardless of culturability, was developed for use on agricultural land. This assay improves sample preparation through the use of the Pulsifier and vacuum filtration, and in this case detects *E. coli* O157 using qPCR targeting the *tir* gene. In peat-based compost and sand, it was sensitive to 10 CFU/g soil (Figure 6, 8). Applying the assay to soils of agricultural origin, it was found that the *tir* gene was detected in samples of disparate composition and geographic origin (Figure 9, 10). While this indicates that the foodborne pathogen *E. coli* O157 is abundant in soils used for agriculture, further research is required to confirm the presence of living pathogen cells and characterise the factors responsible for its widespread contamination.

Further examination of this nature was carried out in *tir* gene positive peat-based compost, verifying the presence of VBNC *E. coli* O157 with PNA-FISH and cell elongation (Figure 15), and negative culture data. Enumeration of the VBNC cells determined that *tir* gene detection in the sample is nearly wholly accounted for by the VBNC population, although the estimation of total cells may be subject to some error caused by sample loss on the filter membrane. Further experimentation on this sample led to the resuscitation of VBNC *E. coli* O157, supporting the use of the detection assay on agricultural land and demonstrating the presence of VBNC foodborne pathogens in the environment and the potential for their resuscitation to a culturable state.

In addition to VBNC cells present in agricultural land, this study examined VBNC induction caused by sanitisation of leafy greens. Foodborne pathogens *L. monocytogenes* and *S. enterica* were induced into the state by the commonly used industrial sanitiser chlorine, both in water and adhered to the spinach leaf phylloplane. The total number of bacteria decreased by less than 1 log, and the remaining pathogens became VBNC in water at concentrations of 12 ppm for *L. monocytogenes* and 3 ppm for *S. enterica* (Figure 17, 18). Following incubation on the spinach phylloplane, the required concentration increased to 50 and 100 ppm chlorine respectively (Figure 19, 20). The protection granted to the pathogens by the phylloplane does not exceed the concentration of chlorine

commonly used to process leafy greens, 90 ppm, so there is a very real potential of VBNC induction due to food processing treatments.

VBNC *L. monocytogenes* induced by chlorine was found to reduce lifespan of *C. elegans*, with no difference in lifespan reduction observed between treatments of the pathogen in culturable and VBNC states (Figure 21). Compared with the non-pathogenic control, the total nematode population that ingested *L. monocytogenes* died 6 days sooner, with the longest lived at 16 days. Despite previous research claiming that *C. elegans* provides a good infection model for *Salmonella* (Labrousse et al., 2000), a statistical reduction in lifespan was not observed following either *S. enterica* treatment (Figure 21). Visualisation of the GFP-tagged pathogens inside *C. elegans* indicates that resuscitation from the VBNC state or a continuation of pathogenicity while in the state has occurred, as the pathogens are clearly visible fluorescing outside of the nematode intestinal lumen (Figure 22).

Together these data both confirm the risk of VBNC pathogen contamination to ready-to-eat foods, and provide the tools with which they can be better detected. The need to determine microbial safety of agricultural land via molecular methods of detection is evident, but the agricultural industry further requires a revision of its sanitisation procedures to account for the VBNC threat. This can be achieved through the use of alternative sanitisation methods to chlorine, which has been shown to be ineffective at killing foodborne pathogens (Figure 17-20). In lieu of emerging alternative techniques, the industry must take measures to detect contamination following food processing, including the VBNC cells that they may have inadvertently created. In fact, this challenge could be met with a qPCR assay attuned to complex environmental samples for detection of VBNC pathogens.

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