RESUSCITATION OF PUTATIVE VIABLE BUT NON-CULTURABLE (VNC) FOODBORNE BACTERIA OF SIGNIFICANCE TO NEW ZEALAND

DRAFT FINAL REPORT

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by

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FINAL REPORT

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SUMMARY

In the VNC state bacteria are reported to be unable to be detected using standard media-based culture methods, however, remain viable. Reports from the literature suggest that some VNC cells may be able to be resuscitated to a fully culturable state, although there is much controversy and debate in the literature over this. The goal of this project was to assess the ability of foodborne pathogens to enter into, and emerge from, a putative viable but non culturable (VNC) state. The following bacteria of importance to New Zealand public health were selected for the study: *Salmonella enterica* serovar Brandenburg (one strain) and serovar Typhimurium (one strain), two strains of *Listeria monocytogenes*, two strains of *Campylobacter jejuni* and one strain of Shiga-toxin producing *Escherichia coli*.

Outcomes of the project were:

1. Rapid experimental conditions for VNC establishment were developed by incubating bacterial cells in 13% w/v NaCl at the human physiological temperature of 37^{o}C. This confirmed the ability of these foodborne pathogenic bacteria to become VNC under specific controlled model conditions.

2. Experiments undertaken to establish a quantitative molecular detection method for VNC cells using either DNase or propidium monoazide to remove dead cell DNA demonstrated that these reagents were not robust enough to distinguish VNC cells from any dead cell background in a culture. This was an important result as the methods that were tested have been reported in the literature and the results from this study suggest such reports may be unreliable.

3. A number of reported and empirical methods, including heat-shock, antioxidants, siderophores and autoinducers, were tested to see if VNC cultures, established using the model system developed in this study, could be resuscitated. None of the methods were able to resuscitate VNC cells of any of the bacteria studied to a state where they could be cultured using standard methods.

4. To address the question of the potential for VNC cells to be infective, VNC cells were tested to see if they could be killed when exposed to a variety of simulated human fluid environments likely to be encountered if the bacteria were ingested. Interestingly the VNC cells from different bacterial species showed different sensitivities to the fluids tested (simulated human saliva, gastric fluid and intestinal fluid), as assessed by LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) staining. VNC cells of *C. jejuni* were the most sensitive to the simulated human fluids, being killed by all three. *L. monocytogenes* VNC cells were only sensitive to simulated gastric fluid, while *S. enterica* spp. and *E.coli* VNC cells were unaffected by any of the simulated human fluids. These results indicated that it is certainly possible to kill VNC cells and some are sensitive to simulated human fluids, which may impact on their ability to cause disease.

Outputs from project: The results reported in Section 6 of this report, entitled 'VNC cells in human fluid environments', could be prepared into a manuscript to be submitted for publication in either Applied and Environmental Microbiology or the Journal of Applied Microbiology.

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1 INTRODUCTION

'Viable but non-culturable' (VNC) bacteria were first described by Xu *et al.* (1982) as bacterial cells with detectable metabolic function that are not culturable by current standard methods (Xu *et al.* 1982; Roszak and Colwell 1987). A VNC cell state could explain such conundrums as the level of human disease attributed to *C. jejuni*, although the organism is regarded as one of the least robust human gastrointestinal pathogens, and is apparently sensitive to oxygen, desiccation, ultra violet light and any number of other environmental stresses to which it may be subjected outside the host. An ability of the bacteria to enter a VNC state could protect it from such stressors, and then the ability to resuscitate could allow it to cause disease once in a human host.

The implication of VNC cell establishment for foodborne pathogens relates to the ability of cells in this state to contribute to human disease, and the ability to detect VNC cells in the food chain. As routine water and food testing depends largely on the ability of bacteria to grow in culture, the discovery of VNC cells, especially of bacterial species of relevance to human health, has fueled studies to better understand this controversial phenomenon (Oliver 2005). Despite this, to date, the significance of VNC cells in causing human intestinal disease is unknown.

The biological significance of the VNC phenomenon is also subject to an intense ongoing debate. What is undisputed is that VNC cells are formed as a result of exposure of bacterial cells to stresses in a hostile environment. One theory espouses that VNC cells form as a result of cellular deterioration, which could either be genetically programmed (Aertsen and Michiels 2004) or a stochastic event (Bogosian and Bourneuf 2001). In essence, VNC is a stage along the pathway to cell death, which may have evolved as a strategy to enhance the survival of a population during adverse conditions by providing essential nutrients to the persisting members of the population (Cuny *et al.* 2005). An alternate theory advocates VNC is a genetically-encoded adaptive pathway, likened to spore formation, for producing dormant cells capable of surviving intense periods of environmental stress (McDougald *et al.* 1999). The important difference between these two theories is that the latter implies the ability of VNC cells to 'resuscitate', become culturable again and thereby cause disease, once the environmental stress is removed and conditions are again sufficient to support bacterial growth. In contrast, the former theory implies VNC is a non-reversible state from which bacteria can neither be resuscitated nor cause disease.

In order to prove or disprove one of these two theories, much of VNC research reported in the literature has focused on resuscitation. Yamamoto *et al.* (2000) delineated three categories of resuscitation studies: 1) removal of the environmental stress, 2) repair of the damage to the bacterial cell and 3) signaling factors that stimulate the cell to replicate (Yamamoto 2000). The first category includes adding essential nutrients and shifting bacteria to optimal growth temperatures. The second category includes heat-shock, to stimulate DNA repair, and molecules such as siderophores (iron-complexing agents) and antioxidants, to help remove toxic compounds and prevent oxidative damage to the bacterial cell during regrowth. The third category includes autoinducers produced by quorum-sensing bacteria, which are secreted and stimulate cell proliferation.

Resuscitation has been reported for many bacterial species, including several of importance to foodborne disease: *Vibrio* spp. (Nilsson *et al.* 1991; Ravel *et al.* 1995; Wai *et al.* 1996;

Whitesides and Oliver 1997; Wong *et al.* 2004), *Salmonella enterica* serovar Typhimurium (Reissbrodt *et al.* 2002; Gupte *et al.* 2003; Panutdaporn *et al.* 2006), *Campylobacter jejuni* (Cappelier *et al.* 1999; Baffone *et al.* 2006) and enterohemorrhagic *Escherichia coli* (Dukan *et al.* 1997; Reissbrodt *et al.* 2002). However, demonstrating resuscitation is a controversial endeavor as it is difficult to definitely prove that culturable cells are not present in the experimental culture prior to resuscitation. Furthermore, several groups have reported that VNC cells cannot be resuscitated (Arana *et al.* 2007; Dreux *et al.* 2007). This is clearly an area of VNC research still open to debate.

Other less controversial VNC research has focused upon the physiological changes that occur when a bacterial cell becomes VNC; these include reductions in nutrient transport, respiration rates and protein synthesis (Porter *et al.* 1995; Oliver 2005). VNC cells also produce novel stress proteins (Morton and Oliver 1994; Srinivasan et al. 1998) and alter the cell membrane and cell wall composition (Signoretto *et al.* 2000; Signoretto *et al.* 2002). These changes are thought to be instrumental to surviving varied adverse environments.

This project was initiated to investigate two important aspects of VNC research, (i) detection of VNC bacterial cells and (ii) entry into and out of the VNC state, for important foodborne enteric pathogens in New Zealand. Because surveillance of the food and water supply in New Zealand is dependent upon the ability to culture a pathogen of interest, a detection method for VNC cells should be developed in order to assess the presence and prevalence of VNC cells in the New Zealand food and water supply. Detection is the instrumental first step for determining if VNC cells could contribute to human enteric disease in New Zealand; in addition, it is paramount to develop a model system to gather information that will aid in deciding whether VNC cells formed by enteric pathogens could pose a disease risk if ingested. This information is essential to support risk management initiatives by the New Zealand Food Safety Authority to control these important enteric pathogens through the food chain.

The bacteria selected for the study were *Salmonella enterica* serovar Brandenburg (one strain) and serovar Typhimurium (one strain), two strains of *Listeria monocytogenes*, two strains of *Campylobacter jejuni* and one strain of Shiga-toxin producing *Escherichia coli*. These bacteria are all of importance to New Zealand public health (Cressey and Lake 2007) and the strains chosen were from NZ or Australian isolates.

The initial objectives of this project were:

- To examine whether New Zealand strains of foodborne pathogens can enter the VNC state.
- To reproduce the resuscitation phenomenon reported in the literature for *Salmonella* and apply the same approach to determine whether other organisms of gastrointestinal significance in New Zealand (e.g. *Salmonella* Brandenburg PFGE type 14, the "outbreak" strain of *L. monocytogenes* and NZ isolates of *Campylobacter* and STEC) can be resuscitated in the same manner.
- To determine whether the addition of autoinducer molecules from other enteric microbes or other factors will resuscitate VNC cells of significant bacteria.

The project was originally designed such that a MSc student based at Waikato University would be responsible for the bulk of the experimental work. Staff at ESR would be responsible for method development to support the student's work. Due to issues with the

student that resulted in the termination of their contract, and the indication from initial work that the system for establishment of pathogens in a VNC state required more empirical experimentation than originally presumed, staff at ESR took over all of the experimental work of the project. After discussion with the NZFSA, the work was reprioritized.

Ongoing work in the project covered three broad areas related to VNC cells of foodborne pathogens:

1. Development of a model system to generate VNC cells of New Zealand strains of foodborne pathogens important to human health.
2. Investigation of a quantitative molecular method to detect VNC cells.
3. Investigation of methods to remove cells from the VNC state - by resuscitation of cells and by killing cells.

2 METHODOLOGY

2.1 Bacterial species investigated in study

Table 1: Bacterial isolates studied

Bacteria	Species	Isolate details
Salmonella	*enterica* serovar Brandenburg	NZRM 3684 NZ isolate ER 98/4135 Strain I989007. 4,12 : 1,v : e,n,z15. Ovine, NZ South Island. Outbreak Strain. PFGE Type 14.
	enterica serovar Typhimurium	NZRM 3970 NZ isolate ER 00/3165. 4,12 : i : 1,2. Phage type 104. Antibiotic resistant strain.
Listeria	*monocytogenes*	Serotype 1/2, PFGE type 96/2. LM2000/47. "outbreak" strain"[a].
	monocytogenes	NZRM 3450 NZ isolate SB92/844. Serotype 1/2a, phage type 1967, 881. Blood, perinatal case, associated with consumption of smoked mussels.
Campylobacter	*jejuni*	P145a. Poultry. Massey University. MLST sequence type ST u48; not yet found in human cases.
	jejuni	P110b. Poultry. Massey University. MLST sequence type ST 474; most common type isolated from human cases in Manawatu.
Shiga-toxin producing *Escherichia*	*coli*	NZRM 3647 (non-toxigenic strain). Strain BTA 2850. 0157:H7. Verotoxin negative (VT-). From child, Australia.

2.2 Medium and culture conditions

Table 2: Medium and culture conditions used

Bacteria	Shape	Broth	Non-Selective Agar	Selective Agar	Growth Conditions
E. coli	rod	Tryptic Soy	Tryptic Soy	CT-SMAC	37ºC, aerobic, 24 h
Salmonella	rod	Tryptic Soy	Tryptic Soy	Brilliant green	37ºC, aerobic, 24 h
Listeria	rod	Brain Heart Infusion	Brain Heart Infusion	PALCAM	37ºC, aerobic, 24 h
Campylobacter	spiral	Nutrient broth no. 2	Columbia blood	Exeter Agar	37ºC, microaerophilic, 48 h

2.3 Method for establishing viable but non-culturable bacterial cells

Viable but non-culturable bacterial cells were prepared in microcosms, small representative systems analogous to a larger system. Conditions within these microcosms were chosen to reflect various environmental conditions likely to be encountered by the bacterial species studied, which could contribute to a bacterial cell becoming viable but non-culturable. Such conditions included nutrient limitation by incubation in sterile reverse osmosis (RO) water or 0.85% w/v NaCl, temperature stress by incubation at 4°C and osmotic stress by incubation in 13% w/v NaCl.

[a] Sim J, Hood D, Finnie L, Wilson M, Graham C et al. (2002) Series of incidents of Listeria monocytogenes non-invasive febrile gastroenteritis involving ready-to-eat meats. Lett Appl Microbiol 35(5): 409-413.

2.3.1 Method for *E. coli*, *Salmonella* and *Listeria*

Microcosms were prepared with bacterial cultures grown to mid-exponential growth phase in broth. Briefly, 2-3 colonies from a fresh agar culture plate were inoculated into 5 ml of appropriate medium (Table 2) and incubated with shaking overnight in a 37°C water bath to allow the culture to reach stationary growth phase. The next day, 20 ml appropriate medium (Table 2) was inoculated with 0.1 ml overnight culture and grown with shaking at 37°C until mid-exponential growth phase was reached, as determined by optical density $OD_{650\ nm}$. Bacterial cells were then pelleted by centrifugation for 10 minutes at room temperature at 5,000 rpm. Media was removed and the cells were washed once (for 13% w/v microcosms) and twice for (RO water and 0.85% w/v NaCl microcosms) with 20 ml 0.85% w/v NaCl. Bacteria were pelleted again and resuspended in 40 ml final VNC-generating solution to an approximate concentration of 10^8 cfu/ml. Several VNC-generating solutions were explored: RO water, 0.85% w/v NaCl and 13% w/v NaCl. Microcosms were prepared in sterile 50 ml polypropylene conical tubes (Axygen Scientific, Union City, CA, USA) or separated into four 10 ml aliquots in sterile 12 ml polystyrene test tubes (Labserv, Clayton, Victoria, AUS).

2.3.2 Method for *Campylobacter*

Microcosms were prepared with bacterial cells grown to stationary growth phase on agar culture plates. Briefly, isolates were grown on Columbia blood agar plates at 37°C in a 3% oxygen/6% hydrogen/10% CO_2/81% nitrogen MACS-VA-5100 microaerophilic workstation (Don Whitley Scientific Ltd., Shipley, W. Yorkshire, UK) for 48 hours. Bacterial cells were scraped from the plates using an inoculating loop and resuspended in 40 ml VNC-generating solution at a final concentration of $OD_{650\ nm}$ of 0.5. These microcosms were then incubated at room temperature (on the bench top) in the laboratory.

2.4 Monitoring of microcosms for viable but non-culturable bacterial cells

2.4.1 Monitoring culturable bacterial cells

Microcosms were monitored daily, every other day, weekly or monthly (depending on the rate of decline of culturable bacterial cells) for culturable cells by standard spot dilution plating (Chen *et al.* 2003). Briefly, for each microcosm sample, a 10-fold dilution series (ranging from undiluted to 10^{-6}) was prepared in 0.1% (w/v) buffered peptone water; 10 µl of each dilution was then spotted in triplicate onto appropriate agar medium (Table 2) and incubated 24-48 hours at appropriate temperature (Table 2). Spots containing 30-100 colonies were counted. The counts from triplicate spots were averaged and then multiplied by the appropriate dilution factor to quantify the colony forming units per ml (cfu/ml) in the microcosm sample. When an undiluted sample from a microcosm yielded 30-100 countable colonies by the spot plating method, the microcosm was hence-forth monitored by spreading 100 µl of undiluted sample onto the appropriate agar medium in triplicate, which were then incubated as appropriate, until no bacterial cells could be recovered by plating.

2.4.2 Monitoring viable bacterial cells

Microcosms were monitored for viable bacterial cells every other day, weekly or monthly (depending on the rate of decline of culturable bacterial cells) using the LIVE/DEAD®

*Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation, Carlsbad, CA, USA). The kit was used as per the manufacturer's instructions. Briefly, the kit's dyes, SYTO 9 and propidium iodide, were mixed together in equal volumes and stored at –20ºC covered in tin foil as the dyes are light-sensitive. A 300 µl microcosm sample was incubated with 1 µl LIVE/DEAD® *Bac*Light™ dye mixture in the dark at room temperature for 15 minutes. Bacterial cells were then immobilised onto a 0.22 µm pore size 25 mm diameter round polycarbonate filter (GE, Osmonics, Minnetonka, MN, USA), using a funnel/vacuum filtration system, taking care to gently apply vacuum as to not crush or dry out the bacterial cells. Residual dye was removed by running 2-3 ml distilled water through the filter before mounting the filter onto a glass slide with optical coverslip using the non-fluorescent mounting oil provided with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation). Bacterial cells were visualised using a Leica DM6000 B microscope (Leica Microsystems, Heerbrugg, Switzerland) with an I3 filter block at 1000x magnification. Ten 0.125 mm x 0.0944 mm pictures of randomly selected fields of view were taken with a Leica DFC500 digital camera system using the Leica imaging application suite. Viable cells, appearing fluorescent green (Figure 1), were counted in each image and the following equation was used to calculate the number of viable cells per ml of microcosm:

$$\textit{Viable bacteria per ml} = \frac{FA}{MA \times n} \times \sum \textit{viable counts} / \textit{volume sample filtered}$$

Where:

FA Area of the filter viewed (mm)[b]

MA Area viewed under the microscope at 1,000× magnification (converted to mm)[c]

n The number of fields of view under the microscope where bacteria were counted

[b] The filters were 25 mm in diameter, or 490.87 mm2 (2 d.p.), or π(12.5)2.

[c] The picture taken when viewing through the 100x objective is 0.125 mm X 0.0944 mm = 0.0118 mm2.

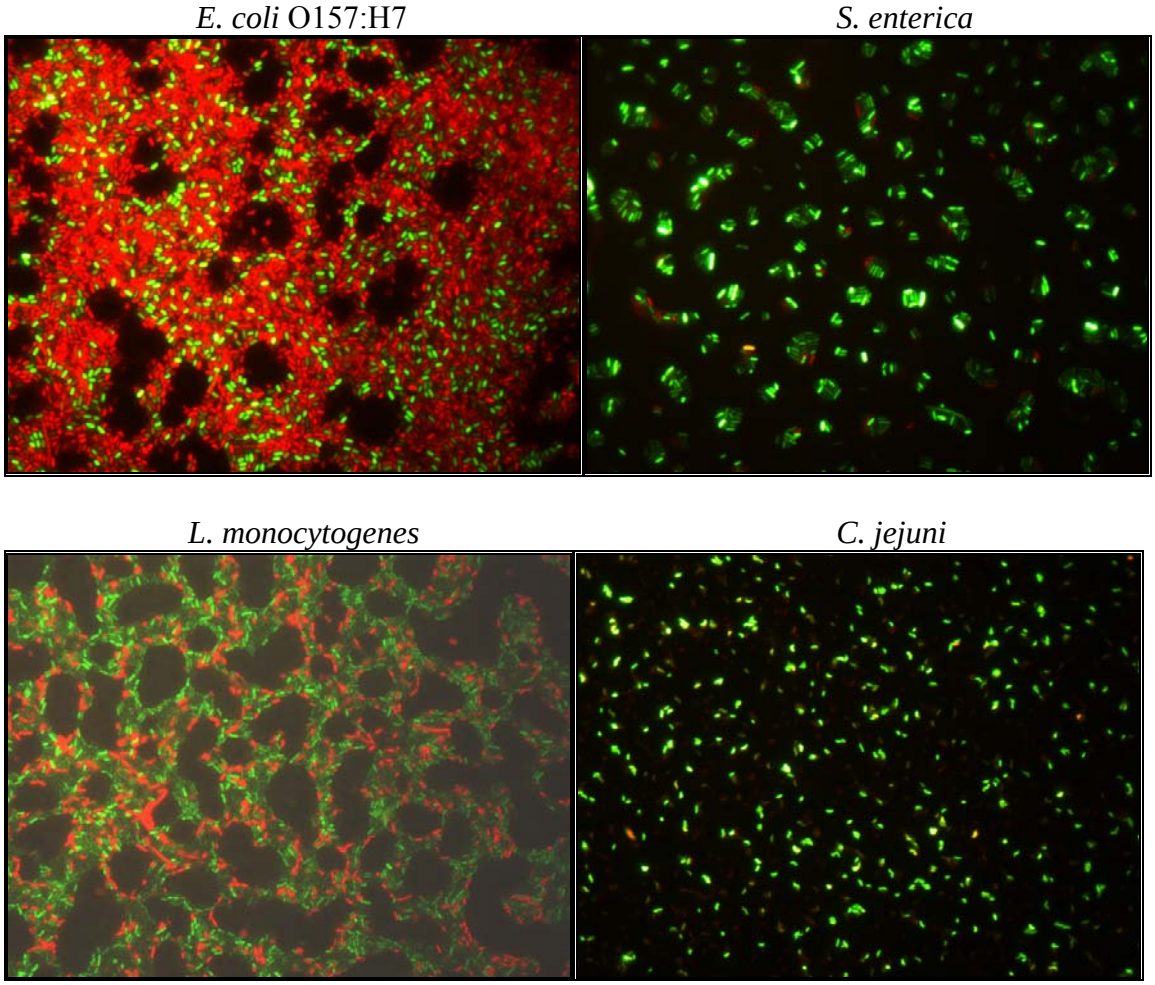

Figure 1: LIVE/DEAD® *Bac*Light™ staining of viable (green) bacterial cells

2.5 Method development: monitoring VNC cells by quantitative real-time PCR

2.5.1 Detection of bacteria by quantitative real-time PCR

Based on published methods, primer pairs (Table 3; manufactured by Invitrogen Corporation) and Taqman probe (Table 3; manufactured by Applied Biosystems, Foster City, CA, USA) were validated using genomic DNA prepared with a Qiagen DNeasy Blood & Tissue Kit (Qiagen) from mid-exponential growth phase cultures of the following bacterial species: *E. coli* O157:H7 NZRM 3647, *S.* Typhimurium NZRM 3970, *L. monocytogenes* NZRM 3450 and *C. jejuni* NZRM 2397. PCR reactions were performed in 25 µl reaction volumes using 1x Platinum qPCR supermix-UDG with ROX (Invitrogen Corporation), 500 nM forward primer, 500 nM reverse primer and 200 nM Taqman probe. Amplification and detection of fluorescent products was performed using an ABI Prism 7700 thermocycler with SDS 1.9 software package (Applied Biosystems) and the following cycling conditions: 50ºC, 2 min; 95ºC, 2 min; 50 cycles [95ºC, 15s; 60ºC 1min]. Amounts of DNA in unknown samples was determined by comparing C_t values from these samples with those generated by a standard

curve using genomic DNA from the appropriate bacterial species. Standard curve samples and controls were assayed in duplicate; unknown samples were assayed in triplicate.

In order to evaluate the ability of the real-time PCR assays to quantify numbers of bacterial cells in a microcosm for each of the bacterial species studied, genomic DNA was prepared from a 10-fold dilution series of bacterial cells and compared to the values generated by real-time PCR with colony forming units produced by standard plating methods (Table 2). The following formula was used to convert real-time PCR C_t values into bacterial cells per ml:

$$\textit{Bacterial cells per ml} = \frac{(V/v)*D*B}{n}$$

Where:

V total volume of genomic DNA extract

v volume of genomic DNA added to real-time PCR reaction

D pg amount of DNA present in real-time PCR reaction (generated by comparing C_t value in sample to a standard curve)

B Conversion factor based on the size of the bacterial genome; for *E. coli*, use 162.6; for *Salmonella*, use 183.5; for *Listeria monocytogenes*, use 312.5; for *Campylobacter jejuni*, use 531.9

n volume of microcosm from which genomic DNA was prepared (ml)

Table 3: Primers and probes for quantitative real-time PCR

Bacteria	Target gene	Primer/probe name	Oligo sequences (5' – 3') and Probe sequence (FAM-5' – 3'-TAMRA)	Reference
E. coli O157:H7	*rfb*	Ecol-rfb-F Eco1-rfb-R Ecol-rfb-PROBE	ATGCTGCCCACAAAAATAATGTAAA TTCCATAATCGGTTGGTGTGCTAA AACTGCTTTTCCTCGGTTCGTCGTGTAT	(Hsu *et al.* 2005)
Salmonella enterica spp.	*invA*	Sal-invA-F Sal-invA-R Sal-invA-PROBE	AAACGTTGAAAAACTGAGGA TCGTCATTCCATTACCTACC TCTGGTTGATTTCCTGATCGCA	(Rodríguez-Lázaro *et al.* 2003)
Listeria monocytogenes	*hly*	Lmono-hly-F Lmono-hly-R Lmono-hly-PROBE	CATGGCACCACCAGCATCT ATCCGCGTGTTTCTTTTCGA CGCCTGCAAGTCCTAAGACGCCA	(Rodríguez-Lázaro *et al.* 2004)
Campylobacter jejuni	*glyA*	Cjej-glyA-F Cjej-glyA-R Cjej-glyA-PROBE	TAATGTTCAGCCTAATTCAGGTTCTC GAAGAACTTACTTTTGCACCATGAGT AATCAAAGCCGCATAAACACCTTGATTA GC	(Jensen *et al.* 2005)

2.5.2 DNase method for removal of dead bacterial cell DNA

Lyophilized DNase I powder (Roche Diagnostics Corporation, Indianapolis, IN, USA) was reconstituted in storage buffer (20 mM sodium acetate (pH 6.5), 5 mM $CaCl_2$, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 50 % v/v glycerol) at a concentration of 100 mg/ml and then stored at -20°C in the dark. A 10 mg/ml working stock of DNase I was prepared in storage buffer.

The DNase method for removing DNA from dead bacterial cells in a microcosm was initially tested using a culture of 100% dead bacterial cells. Briefly, bacterial cells (10^8 cfu/ml) were incubated with 70% v/v isopropyl alcohol for 15 minutes at room temperature to kill the cells. Cells were pelleted by centrifugation at 5000 rpm for 15 minutes at room temperature and resuspended in an equal volume of 0.85% w/v NaCl. The method for killing the bacterial cells was verified by staining the cells with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) as outlined previously. DNase treatment was performed as described by Rueckert *et al.* (2005). Dead bacterial cells were mixed with 5-30 µl DNase I (10 mg/mL, 20 U/µl) in 1 x DNase reaction buffer (20 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 50 mM KCl in Milli-Q double-distilled water) and incubated in a 37°C water bath for 2-4 hours. Control bacterial cells were incubated with 5 µl DNase I storage buffer, using similar incubation conditions. The enzyme was then inactivated by adding 0.5 mM EDTA and incubating for 10 minutes at 70°C; control tubes were similarly treated. Genomic DNA was then prepared from these samples using the Qiagen DNeasy Blood and Tissue DNA preparation kit (Qiagen) as per the manufacturer's instructions.

2.5.3 Propidium monoazide method for removal of dead bacterial cell DNA

Lyophilised propidium monoazide (PMA) (BioScientific Pty. Ltd., Gymea, NSW, AUS) was dissolved in 20% v/v DMSO at a stock concentration of 20 mM and stored at –20°C in the dark. The PMA method for removing DNA from dead bacterial cells in a microcosm was also initially tested using a culture of 100% dead bacterial cells as described above. PMA treatment was performed as previously described (Nocker *et al.* 2006). Briefly, dead bacterial cells were mixed with 50 µM PMA in an eppendorf tube and incubated in the dark for at least 5 minutes on ice. Control bacterial cells were incubated with 20% v/v DMSO. To photo cross-link the PMA to DNA, the tubes were held on ice and exposed to a 650-W halogen light source placed 20 cm from the samples. Genomic DNA was then prepared from these samples using the Qiagen DNeasy Blood and Tissue DNA preparation kit (Qiagen) as per manufacturer's instructions.

2.6 Method development: resuscitation of VNC cells

A summary of the methods used to attempt to resuscitate VNC cells is given in Table 4.

2.6.1 Resuscitation by heat shock

Heat shock resuscitation was performed as described by Gupte *et al.* (2003). Briefly, equal volumes of microcosm and appropriate broth were mixed together in an eppendorf tube. Tubes were then incubated for 1-15 minutes at 56°C or 15 seconds at 80°C. Samples of 100

µl were plated in triplicate on appropriate agar medium (Table 2) and grown overnight at 37°C.

2.6.2 Resuscitation in minimal broth medium

Microcosms were diluted to a final volume of 10 ml M9 minimal broth medium[d] such that culturable cell numbers were <0.1 cell/ml, as determined by agar plating on appropriate medium. If microcosms did not contain any culturable cells 1 ml of microcosm was added to 9 ml M9 broth. Culture tubes were then incubated in a 37°C water bath for 24 hours to 7 days. Turbidity of the resuscitation culture was assessed daily, and 100 µl of the resuscitation culture was removed, plated and grown overnight on appropriate medium and under appropriate conditions (Table 2).

2.6.3 Resuscitation in soft agar

Overlays of 5 ml of the appropriate medium (Table 2) containing 7.5% w/v agar (soft agar) were melted by boiling and cooled to 46°C. Samples of microcosms were added to the soft agar in 1 ml volumes, mixed by vortexing and then spread across an agar plate. After solidifying, the soft agar overlay plate was placed inverted in a 37°C incubator overnight. The next day colonies were counted and compared to culturable numbers obtained by standard plating on agar plates.

2.6.4 Resuscitation in a microaerophillic environment

Microcosm samples were plated as described in the 'monitoring culturable bacterial cells' section on appropriate medium (Table 2). Plates were then grown overnight in a 37°C 3% oxygen/6% hydrogen/10% CO_2/81% nitrogen MACS-VA-5100 microaerophilic workstation. The next day colonies were counted and compared to culturable numbers obtained by standard plating on agar plates incubated at 37°C in a standard incubator.

2.6.5 Resuscitation with various growth enhancing supplements

Resuscitation with various growth-enhancing supplements was assessed in liquid culture based on the methods of Reissbrodt *et al.* (2002), where resuscitation was demonstrated by the presence of a growth factor increasing the length of time bacteria could be cultured from a microcosm in comparison to using recovery medium alone. Briefly, 150 µl of microcosm was added to 3 tubes of 5 ml sterile buffered peptone water (BPW) for *S. enterica* spp., tryptic soy broth (TSB) for *E. coli* O157:H7 or nutrient broth no. 2 for *C. jejuni*, so as to dilute the NaCl concentration to physiological range (0.85% w/v), with and without growth supplement. This was repeated daily until bacteria could no longer be cultured from the microcosm in enrichment broth with or without growth supplement.

The following growth enhancing supplements were assayed: 1% w/v 3,3' thiodipropioinic acid (TDPA) (Sigma-Aldrich Co., Milwaukee, WI, USA), added to medium before

[d] M9 minimal broth recipe: M9 salts solution. To 400ml H_2O, add 32g Na_2HPO_4-$7H_2O$, 7.5g KH_2PO_4, 1.25g NaCl, and 2.25g NH_4Cl. Stir until dissolved and then adjust volume to 500ml with distilled H_2O. Sterilize by autoclaving. To make M9 minimal broth, mix 80 mL M9 salts with 310 mL distilled H_2O. Finally, add 0.8 mL 1M MgSO4 (filter-sterile), 8 mL 20% w/v glucose (filter-sterile) and 40 µl 1M $CaCl_2$ (filter-sterile).

sterilization by autoclaving; 0.5% sodium pyruvate (Sigma-Aldrich Co.), sterilized by passing through a 0.22 µm syringe filter and added to broth after sterilization by autoclaving; 100 ng/ml ferrioxamine E (Sigma-Aldrich, Inc.), sterilized by passing through a 0.22 µm syringe filter and added to broth after sterilization by autoclaving; 20 U/ml (first experiment) and 500 U/ml (second experiment) BuGro (kindly provided by Dr. Primrose Freestone and Dr. Chris A. Jones, University of Leicester), added to broth after sterilization by autoclaving; 20 µl/1 ml Oxyrase® for Broth (Oxyrase, Inc., Mansfield, OH, USA), added to broth after sterilization by autoclaving. Due to limited quantities of Oxyrase® for Broth available, the broth recovery was performed in 1 ml final volumes.

Table 4: Resuscitation method summary

Method	Theory	Bacterial Species Tested	Reference
1) Heat shock at 56°C and 80°C	Initiate production of heat shock proteins leading to bacterial replication	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(Gupte *et al.* 2003)
2) Recovery in minimal broth media	Slower recovery may be less stressful to cells	*E. coli* O157:H7, *S.* Typhimuriu*m*, *S.* Brandenburg	(Wong *et al.* 2004)
3) Recovery on rich media (Columbia blood agar)	Higher nutrient content increases access to nutrients	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg	This study
4) Recovery in soft agar	Higher water content aids in rehydration of cells	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg	This study
5) Recovery in a microaerophillic environment	Lower oxygen environment reduces oxidative stress	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg	(Reissbrodt *et al.* 2002)
6) Recovery in broth media + sodium pyruvate	Degrades H_2O_2 reducing oxidative stress	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(McDonald *et al.* 1983)
7) Recovery in broth media + 3,3' thiodipropionic acid	Antioxidant will reduce oxidative stress	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(McDonald *et al.* 1983)
8) Recovery in broth media + Ferrioxamine E	Siderophore sequesters iron to reduce generation of oxygen radicals	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(Reissbrodt *et al.* 2000)
9) Recovery in broth media + Oxyrase®	Bacterial enzyme that acts as an O_2 reducing agent and antioxidant for the purpose of reducing oxidative stress	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(Reissbrodt *et al.* 2002)
10) Recovery in broth media + BuGro®	Autoinducer involved in quorum sensing, which can induce bacterial replication	*E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg, *C. jejuni*	(Reissbrodt *et al.* 2002)

2.7 Exposing VNC cells to human fluid environments

After VNC cells were generated in 13% w/v NaCl microcosms, 1 ml of microcosm was pelleted by centrifugation at 5,000 rpm for 1 minute at room temperature. The supernatant was removed and bacterial cells were resuspended in 0.5 ml of one of three human simulated fluids: saliva (Table 5) (Davis *et al.* 1971), gastric fluid (Table 6) (USP30NF25S2 2007-2008) or intestinal fluid (Table 7) (USP30NF25S2 2007-2008). Control bacterial cells were resuspended in 13% w/v NaCl. Bacterial cells were then incubated at 37°C for 5 minutes in simulated saliva or 1 hour in either simulated gastric or simulated intestinal fluid. These incubation periods were chosen as a representative time of exposure for a bacterial cell in this fluid environment had the cell been ingested by a person (Table 8). After this incubation period, bacterial cells were pelleted by centrifugation at 5,000 rpm for 1 minute at room temperature. Cells were then washed once with 0.5 ml 0.85% NaCl and resuspended in 0.3 ml 0.85% w/v NaCl, using gentle vortexing. Viable bacterial cell numbers were then determined as previously described using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation).

VNC cells were also exposed to human saliva collected from a volunteer. Briefly, 30 minutes after eating lunch, a human volunteer spat into a 15 ml polypropylene collection tube over a 10 minute period until 3-4 mls of saliva were collected. The pH of the saliva was determined to be pH 7.05. The saliva was sterilized by filtration through a 0.22 µm syringe filter and used immediately. VNC cells were incubated in 0.5 ml of saliva, while control VNC cells were incubated in 0.5 ml 13% w/v NaCl, at 37°C for 5 minutes (the same conditions used to expose the VNC cells to simulated human saliva).

Table 5: Simulated human saliva (pH = 7.2)

Compound	Concentration	Manufacturer
Mucin, gastric	1.0 g/L	Sigma-Aldrich Co.
α-Amylase	2.0 g/L	Sigma-Aldrich Co.
NaCl	0.117 g/L	LabServ
KCl	0.149 g/L	LabServ
$NaHCO_3$	2.1 g/L	BDH Ltd., Poole, England, UK

Table 6: Simulated human gastric fluid (pH = 1.2)

Compound	Concentration	Manufacturer
NaCl	2.0g/L	LabServ
Pepsin (porcine stomach mucosa)	3.2 g/L (800-2500 U/mg)	Sigma-Aldrich Co.
HCl	7.0 ml/L	Sigma-Aldrich Co.

Table 7: Simulated intestinal fluid (pH = 6.8 ± 1[e])

Compound	Concentration	Manufacturer
KH_2PO_4	6.8 g/L	Sigma-Aldrich Co.
NaOH	0.015 M	Sigma-Aldrich Co.
Pancreatin	10.0 g/L	Sigma-Aldrich Co.

Table 8: Time period of food transit in human fluid environments

Host environment	Time period	Reference
Stomach	3-4 hours	(Gotthard *et al.* 1986)
Small intestine	~5 hours	(Shils and Young 1988)
Colon	~15 hours	(Glober *et al.* 1977)
Total Transit Time	20-23 hours	(Glober *et al.* 1977)

[e] The US Pharmacopoeia suggests this formula to simulate intestinal fluid, in general.

3 RESULTS: GENERATING VNC CELLS

VNC cells are, by definition, unable to be cultured on standard microbiological medium. It is therefore necessary to utilize an alternative method to detect VNC cells. In this study a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) was used to detect VNC cells. This method relies on the integrity of the membrane of 'live' bacterial cells and its ability to exclude a red fluorescent dye. Live cells are detected by staining with a green fluorescent dye. Dead cells, with compromised membranes, are able to take up the red fluorescent dye and stain red. All VNC results in this study therefore rely on the premise that the VNC state reflects the continued ability of the bacterial membrane to exclude the red fluorescent dye.

3.1 Water and 0.85% w/v NaCl microcosms at 4°C

The first question addressed by this study was to explore whether New Zealand strains of foodborne pathogens *E. coli* O157:H7, *S. enterica* serovar Typhimurium, *S. enterica* serovar Brandenburg, *L. monocytogenes* and *C. jejuni* (Table 1) could form VNC cells when challenged with a simulated environmental stress. Previous studies have demonstrated that cold stress (incubation of bacterial cells at 4°C) and nutrient deprivation (incubation in either 0.85% w/v NaCl or water) can trigger bacteria to form VNC cells (Oliver *et al.* 1995; Warner and Oliver 1998; Reissbrodt *et al.* 2002; Arana *et al.* 2007). Therefore, selected bacterial cells were prepared in microcosms (small model systems of larger, complex systems) using these conditions and culturable cells were monitored for up to 2.5 months (Figure 2). These conditions did reduce culturable cell numbers, but significant reductions took months to occur and none of the microcosms reached zero culturable cfu/ml in the time period examined.

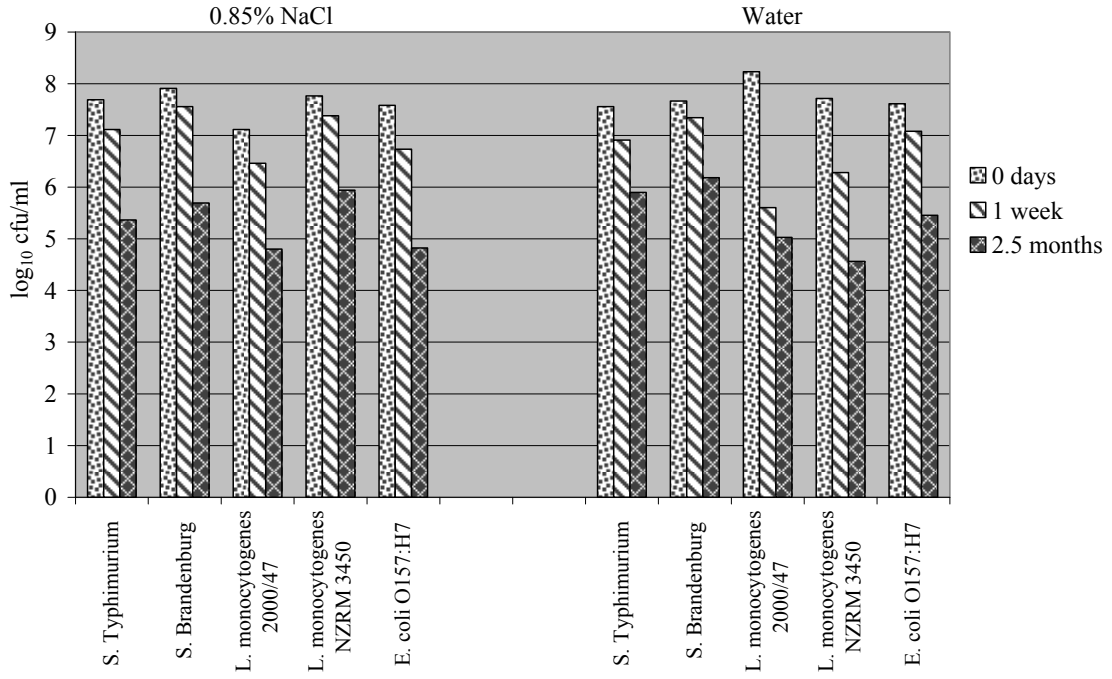

Figure 2: Decline in culturable cell numbers in 0.85% w/v NaCl or water at 4°C

When viable cells were quantified for *E. coli* O157:H7 in water and 0.85% w/v NaCl microcosms incubated at 4°C over a month-long period, the viable cell numbers declined at the same rate as the culturable cell numbers and therefore no VNC cells were formed under these conditions (Figure 3). Based on this result viable cells were not quantified for the other bacterial species of interest. Instead, the focus shifted to finding a microcosm condition that would cause each of the four bacterial species chosen for this study to form VNC bacterial cells.

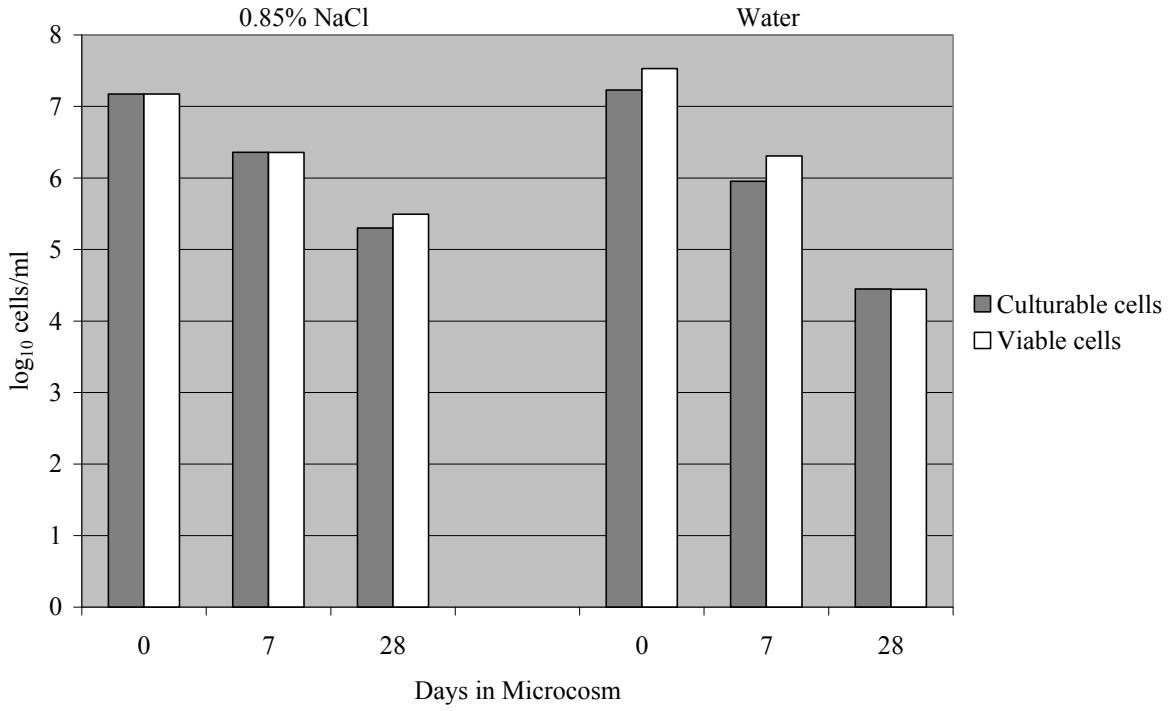

Figure 3: Culturable vs. viable *E. coli* O157:H7 in 0.85% w/v NaCl or water at 4°C

3.2 VNC cells form in 13% w/v NaCl microcosms at 37°C

Makino and co-workers demonstrated that enterohemorrhagic *E. coli* O157 incubated in high salt concentrations (7% w/v and 13% w/v NaCl) at 37°C form VNC cells in a matter of days as a response to osmotic stress (Makino *et al.* 2000). In an effort to find a model system for generating VNC cells that would mimic stress encountered by pathogens during food storage, 13% w/v NaCl (high salt that mimics desiccation conditions) microcosms for *E. coli* O157, *Salmonella* and *Listeria* were prepared, which were then incubated at 4°C to imitate storage in a domestic refrigerator. Culturable cells were monitored for one month by standard plating methods in order to determine if incubation at 4°C in 13% w/v NaCl would sufficiently reduce culturable cell numbers in a timely manner (Figure 4).

After one week in 13% w/v NaCl at 4°C, culturable cells declined little in the *Salmonella* and *Listeria* microcosms, whereas culturable cells declined by nearly 2 $\log_{10}$ cfu/ml in the *E. coli* O157:H7 microcosm. After one month culturable cells only declined 0.5-1.0 $\log_{10}$ cfu/ml in

the *Salmonella* and *Listeria* microcosms, while the *E. coli* O157:H7 microcosm dropped by almost 3 $\log_{10}$ cfu/ml. Therefore the *E. coli* O157:H7 isolate used in this study was more sensitive to high salt conditions when stored at 4°C than either of the two *Salmonella* or *Listeria* isolates tested. Given these conditions did not dramatically decrease culturable cell numbers in *Salmonella* or *Listeria* microcosms, alternate microcosm conditions were explored for these bacteria.

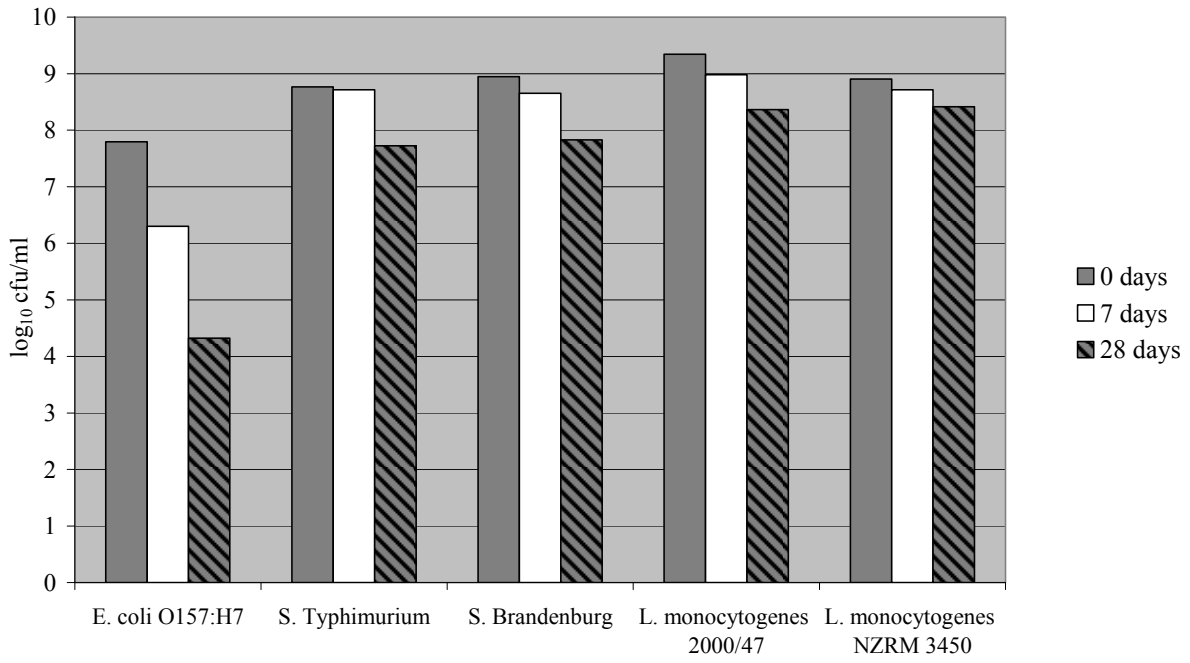

Figure 4: Decline of culturable cell numbers in 13% w/v NaCl at 4°C

As it was important to develop a rapid method for establishing model VNC cells that was effective for all bacterial species of interest in this study, microcosms in 13% w/v NaCl were prepared, which were then incubated at 37°C according to the methods of Makino *et al.* (2006). Culturable and viable cells were monitored to determine if VNC cells formed under these conditions; a representative experiment is shown in Figure 5.

All seven isolates tested (1x *E. coli* O157:H7, 1x *S.* Typhimurium, 1x *S.* Brandenburg, 2x *L. monocytogenes* and 2x *C. jejuni*) formed viable but non-culturable cells within 1-2 days in the 13% w/v NaCl microcosm incubated at 37°C. Culturable cells could not be isolated from *E. coli* O157:H7, *S.* Typhimurium, *S.* Brandenburg and *C. jejuni* within 1-4 days. In contrast, cells could be cultured from *L. monocytogenes* microcosms throughout the experiment, with culturable cell numbers stabilizing after declining approximately 4 $\log_{10}$ cfu/ml in the first two days. Viable cell numbers remained relatively constant throughout the experiment for all isolates and bacterial species examined; therefore, the isolates chosen for this study are capable of producing VNC cells under these conditions. Based on these results, we generated VNC cells in 13% w/v NaCl microcosms incubated at 37°C for all subsequent experiments.

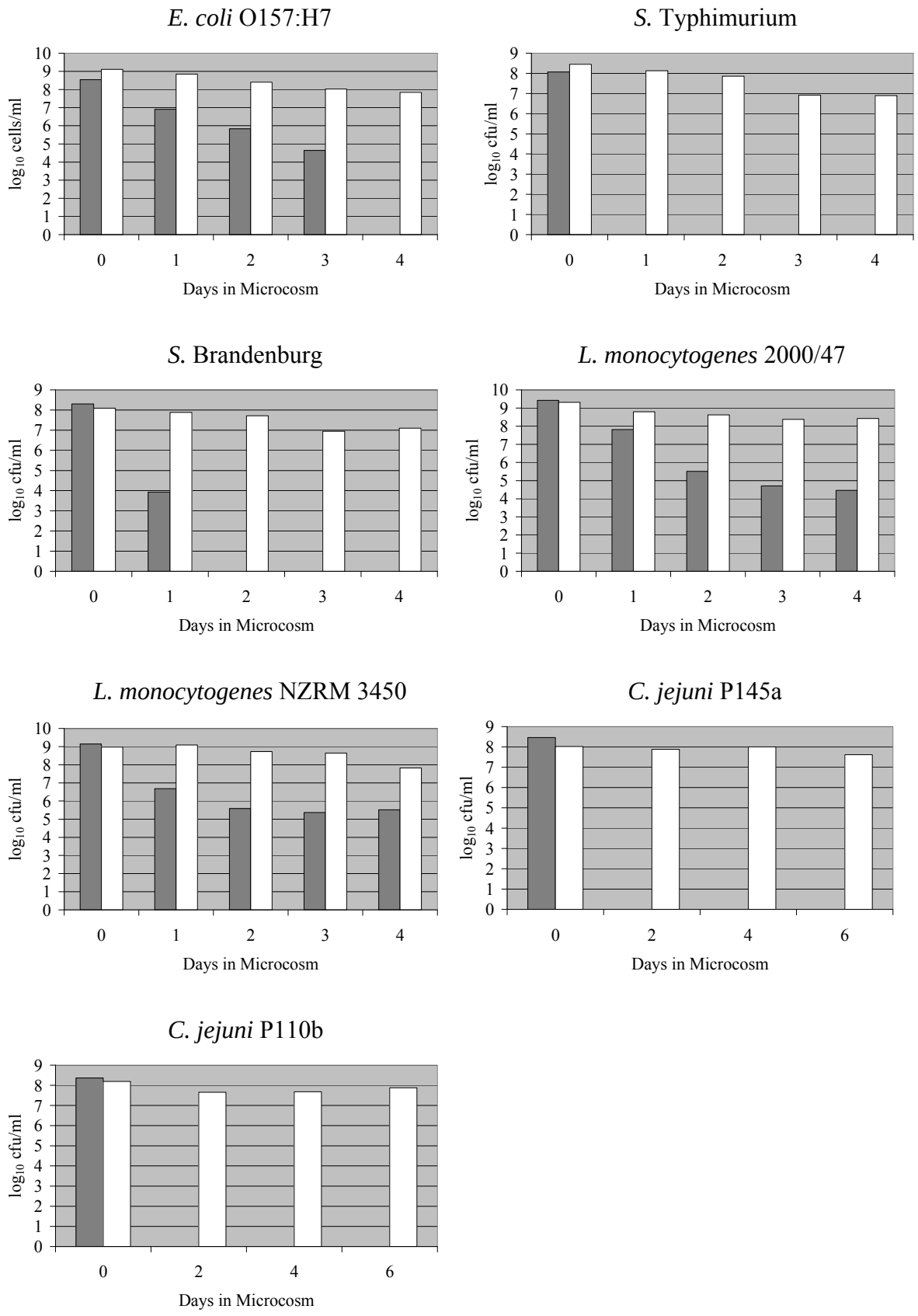

Figure 5: Culturable vs. viable bacterial cells in 13% w/v NaCl; dark bars = culturable cells, white bars = viable cells

3.3 Morphological changes to bacterial cells in a VNC state

Several groups have reported that *C. jejuni* entering the viable but non-culturable state alter their characteristic spiral shape and become round (Beumer *et al.* 1992), as do dead *C. jejuni*. The significance of this morphological alteration is unknown. This characteristic morphological change was also observed for both New Zealand isolates of *C. jejuni* in this study when bacterial cells were placed in 13% w/v NaCl microcosms (Figure 6). In general, bacterial cells of all four species used in this study appeared smaller over time in 13% w/v NaCl microcosms (data not shown). This is a common characteristic of VNC cells (Oliver 2005). Over time, VNC cells also appeared more faintly green after staining with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation), which may suggest that cell membrane integrity of these cells is slightly altered, but not in the same manner as dead cells, as the VNC cells still exclude the red propidium iodide dye.

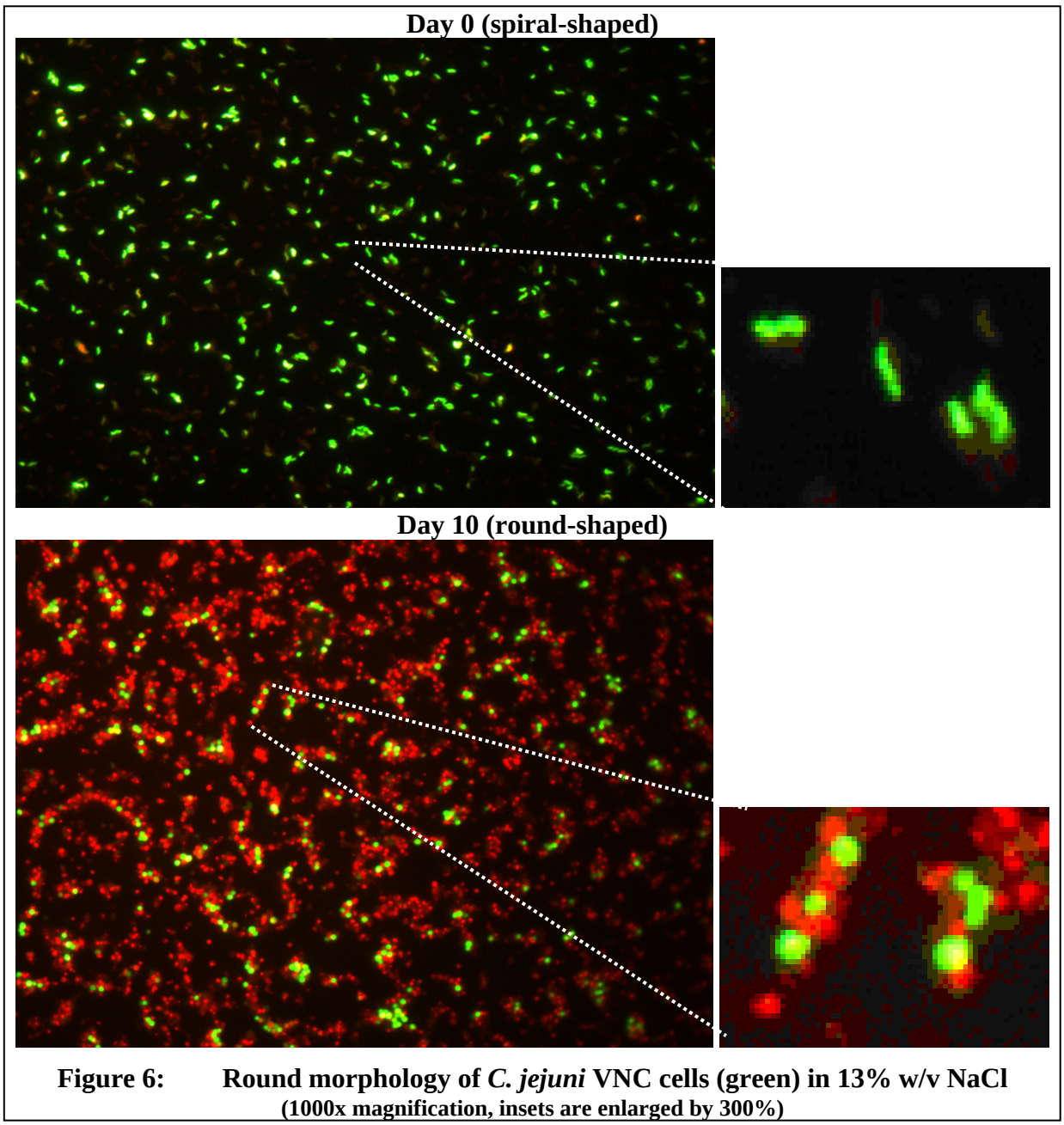

Figure 6: Round morphology of *C. jejuni* VNC cells (green) in 13% w/v NaCl
(1000x magnification, insets are enlarged by 300%)

4 RESULTS: DETECTING VNC BY QUANTITATIVE REAL-TIME PCR

4.1 Validating real-time PCR method for quantifying bacterial cell numbers

To validate a real-time PCR method for quantifying bacterial cell numbers, initial experiments were performed to compare bacterial cell numbers obtained by quantitative real-time PCR (Q-PCR) with number of colony forming units obtained by standard agar culturing methods. For this comparison, genomic DNA was isolated from a 10-fold dilution series, 10^4-10^7 cells/ml, of mid-exponential growth phase bacteria and quantitative real-time PCR was performed to determine the total amount of DNA in each sample of the dilution series. Based on the total size of a bacterial species' genome, the number of bacterial cells in a sample could be derived from the amount of DNA in the sample. Concomitantly, cfu from each dilution series sample were determined by standard plating methods. The number of cells/ml quantified by real-time PCR and by standard plating methods for *E. coli* O157, *C. jejuni*, *L. monocytogenes* and *S. Typhimurium* were compared (Figure 7).

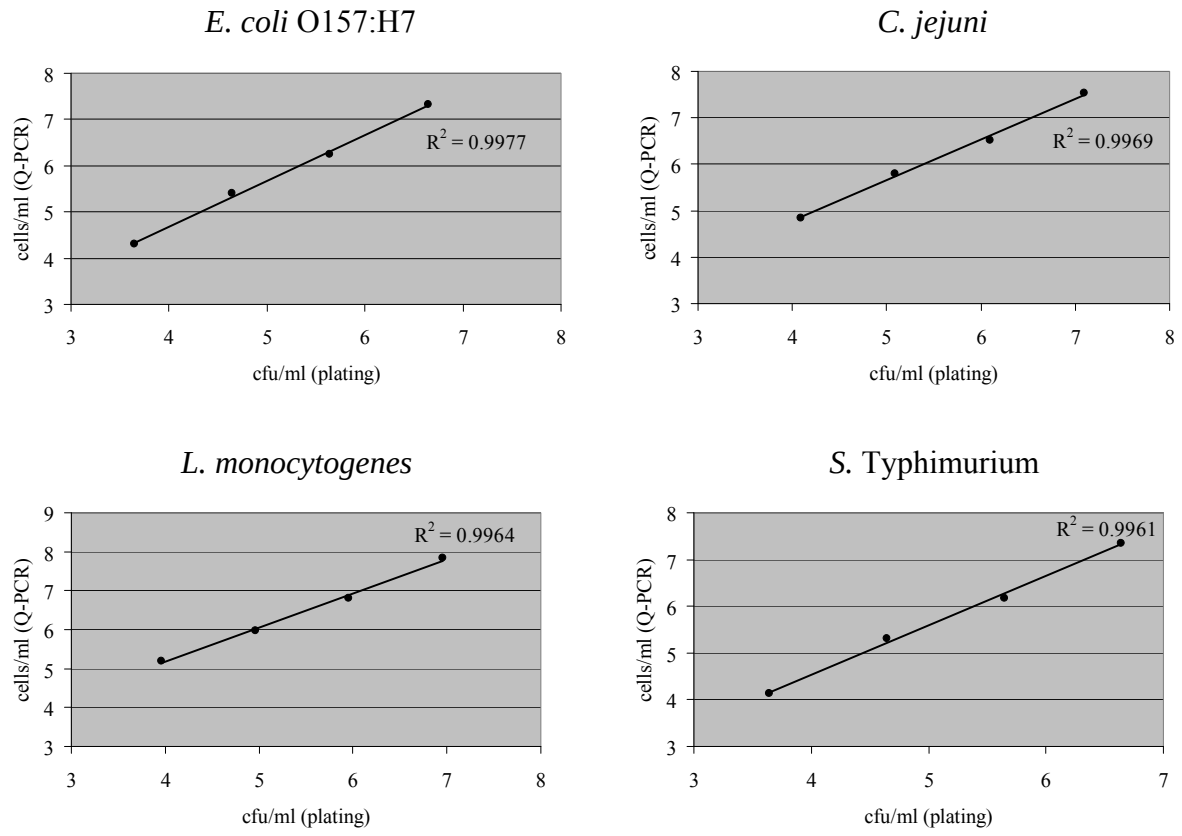

Figure 7: Comparison of cells/ml measured using real-time Q-PCR vs. plating

In these preliminary experiments, cells/ml quantified by real-time PCR and cfu/ml correlated well. All quantitative real-time PCR values for a particular dilution sample were within less than 1 $\log_{10}$ of the corresponding cfu/ml, and most were within 0.5 $\log_{10}$. Interestingly, within

a dilution series, the fold difference between cells/ml (Q-PCR) and cfu/ml (plating) was consistent across the samples, with *E. coli* O157 and *C. jejuni* consistently 4-fold different, *L. monocytogenes* 7-fold different, and *S. enterica* Typhimurium 2-fold different. In general, cells/ml values (Q-PCR) were greater than cfu/ml values (plating). This might be explained by the presence of a dead cell population within the sample contributing to the total genomic DNA isolated and could explain the consistent fold-difference between cells/ml (Q-PCR) and cfu/ml (plating) within a dilution series.

These results highlight that in order for real-time PCR to be used to quantify viable cell numbers in a microcosm, a method must be used to remove the DNA contributed by the dead cell population whilst leaving the viable cell DNA intact. We tested two published methods for differential extraction of viable and dead cell DNA; DNase treatment (Rueckert *et al.* 2005) and propidium iodide (PMA) treatment (Nocker *et al.* 2006). Both methods were first tested with bacterial cultures that had been killed by treatment with isopropanol. These cultures contain 100% dead cells and treatment with either DNase or PMA should completely remove all genomic DNA and result in no signal after Q-PCR amplification.

4.2 Method development: DNase treatment for removal of DNA from dead cells

The DNase method first described by Rueckert *et al.* (2005) is based on the ability of DNase to enter bacterial cells with poor cell membrane integrity, where the enzyme can subsequently digest the bacterial DNA. The principle of the method is that DNase enters dead cells, while viable cells, including VNC cells, remain protected. After incubation in DNase, the bacterial cells are then subjected to a genomic DNA extraction, where only the genomic DNA from viable cells should be purified as the DNA from dead cells has been digested.

A total of six experiments were performed to test the DNase method, during which a range of enzyme levels (100-500 U/sample) and enzyme incubation times (0.5-4 hours) were trialled. A representative experiment is shown in Figure 8. During this experiment the maximum enzyme amount (500 U/sample) and the longest incubation period (4 hours) were trialled. Genomic DNA was used as a positive control for the enzymatic activity of the DNase. *E. coli* O157:H7 that had been killed with 100% isopropanol was used as the target organism for testing the method at concentrations similar to those used in the microcosms (10^8-10^9 cells/ml).

As shown in Figure 8, the DNase used in this experiment was active and no DNA was detected in either of the genomic DNA controls treated with DNase, within the 50 cycle PCR amplification period, as reflected by C_t values of 50. The C_t values shown along the y-axis in Figures 8-10 is directly correlated with the amount of DNA in the sample, with decreasing C_t values indicating increasing concentrations of DNA and a C_t value of 50 indicating that no DNA was present in the sample.

In contrast, DNase treatment of dead cells did not markedly increase the C_t value in comparison to untreated samples. In two of the trials, a small increase in the C_t value occurred, suggesting some of the dead cell DNA was removed, however, complete dead cell DNA removal was not achieved in any of the three trials.

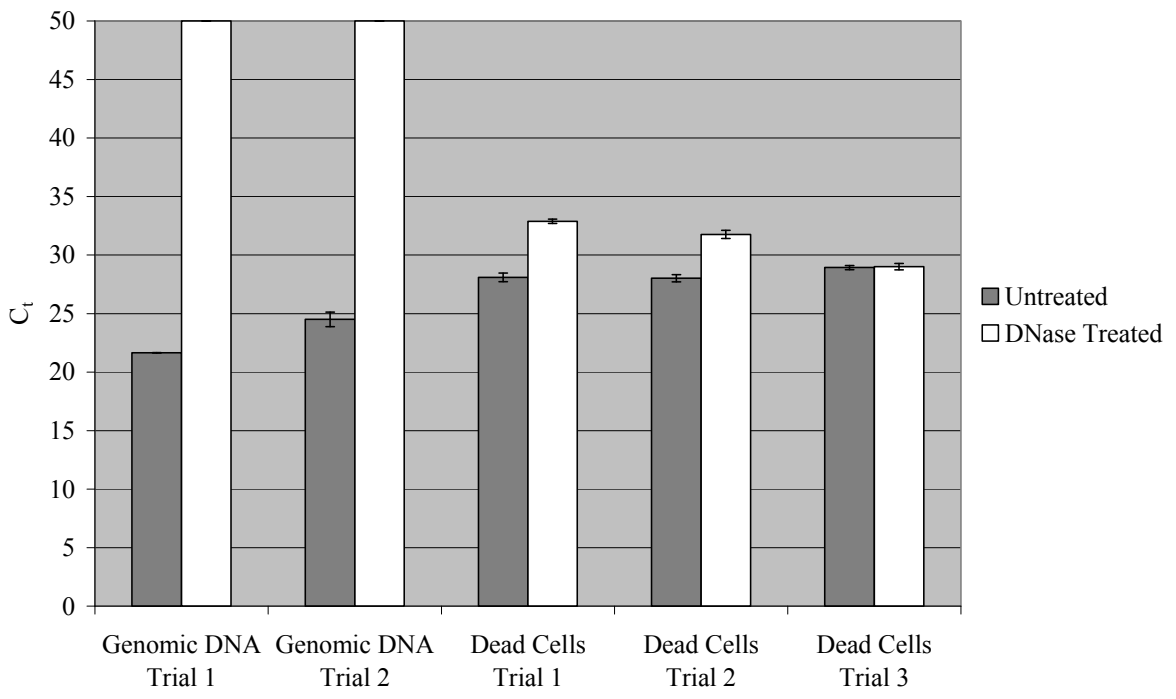

Figure 8: DNase treatment does not remove *E. coli* O157:H7 dead cell DNA

It is important to note that the C_t values of the untreated genomic DNA samples were lower than the untreated dead cell samples, indicating greater amounts of DNA in the untreated genomic DNA samples. DNase sufficiently digested the amount of DNA in the genomic DNA controls, and therefore there was sufficient enzyme present in the reaction to degrade the total amount of DNA present in the dead cell samples. Therefore, insufficient enzyme is not a probable cause of the failure of the method. Based on these results, DNase treatment was not an adequate method for removing dead cell genomic DNA, and therefore we investigated an alternative method.

4.3 Method development: PMA treatment for removal of DNA from dead cells

The PMA method first described by Nocker *et al.* (2006) is a differential DNA extraction method based on the PMA molecule entering bacterial cells with poor cell membrane integrity. PMA is a photoreactive DNA-binding dye that upon light exposure converts into a reactive nitrene radical that will permanently bind DNA. PMA should only bind DNA in dead cells, which are permeable to this small molecule, while viable cells remain protected. When bacterial cells are lysed and subsequently centrifuged in the first step of a genomic DNA extraction, some of the PMA-bound DNA is pelleted with the cell debris, reducing the amount of dead cell DNA isolated by the DNA extraction method. Also, binding of PMA to DNA inhibits PCR amplification of the PMA-bound target DNA strand, such that dead cell DNA that has been bound by PMA should not be detected by Q-PCR. Another small molecule called ethidium monoazide (EMA) also functions in a similar manner and has been used to isolate DNA from viable cells (Nocker and Camper 2006), however, we chose to use PMA because Nocker *et al.* (2006) demonstrated it is taken up by a wider range of bacterial species than EMA.

A preliminary experiment was performed to optimise PMA concentration with light exposure time for proper cross-linking of the dye to *E. coli* O157:H7 genomic DNA (Figure 9). This experiment demonstrated that 100-300 µM PMA is sufficient to remove genomic DNA, when using a 5 minute cross-linking period. However, PMA treatment of dead cells did not markedly increase C_t values in comparison to untreated samples (Figure 10). A small increase in the C_t value was observed, suggesting some of the dead cell DNA was removed, however, complete dead cell DNA removal was not achieved with any of the three concentrations of PMA trialled.

Based on these results, neither DNase nor PMA treatment was an adequately robust enough method for removing dead cell DNA, therefore, a molecular method for detecting VNC cells could not be developed within the scope of this project.

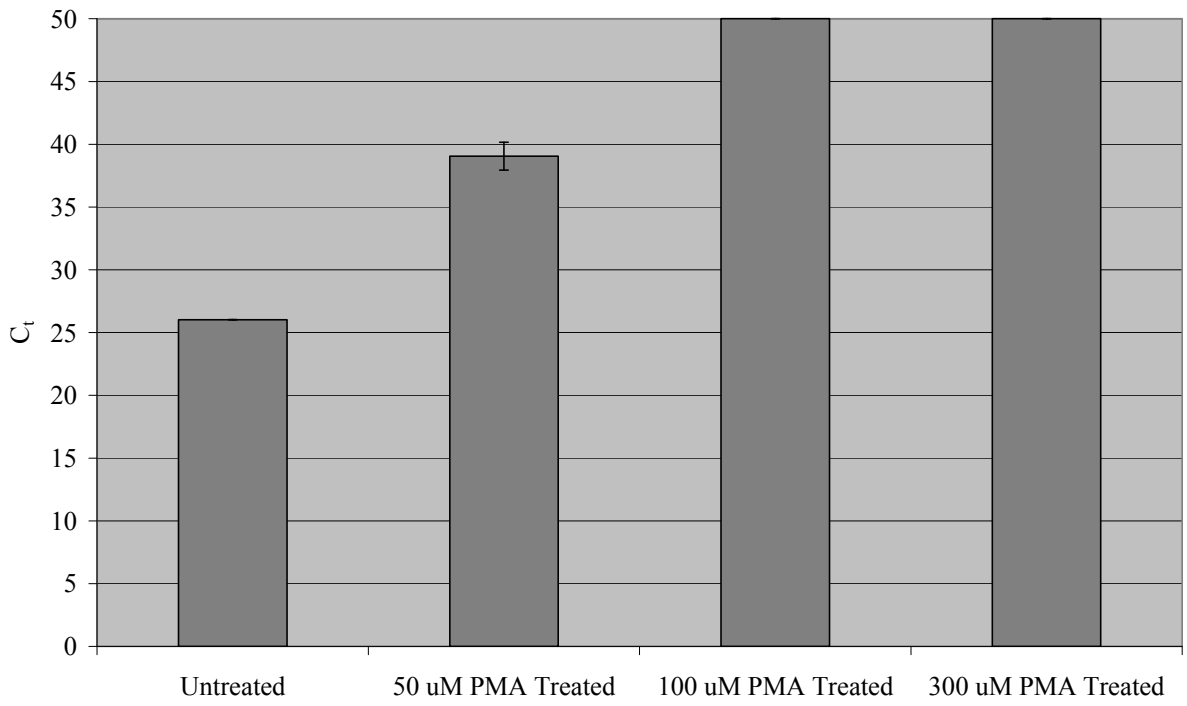

Figure 9: PMA concentration optimization with genomic DNA

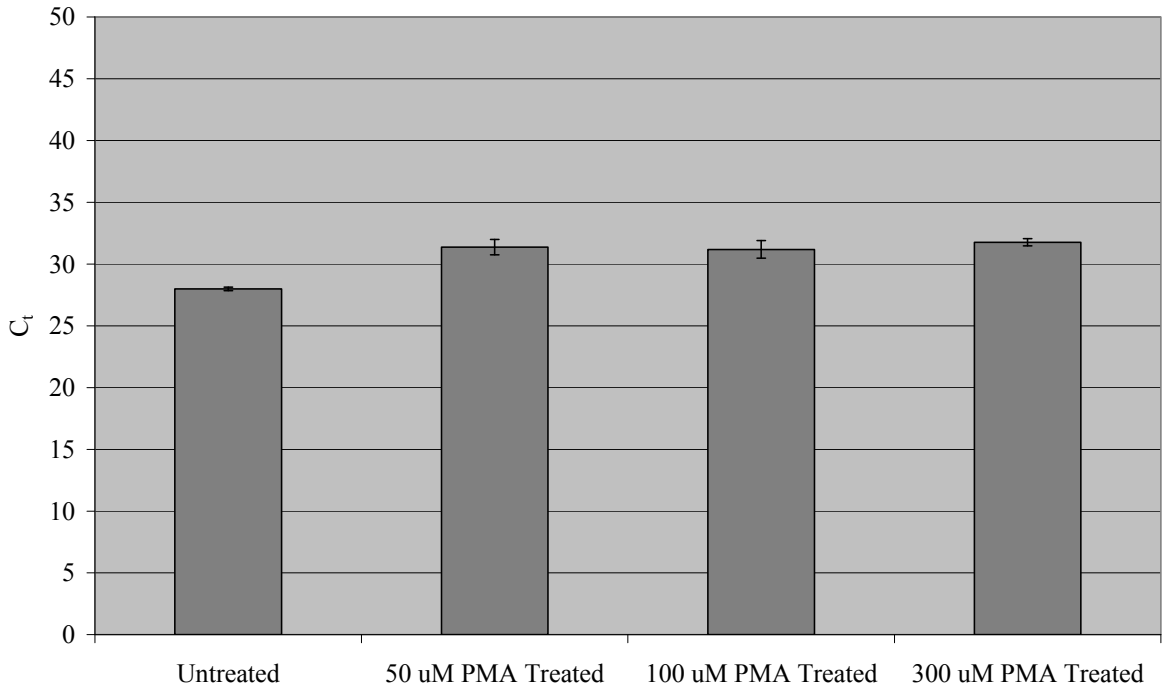

Figure 10: PMA treatment does not remove *E. coli* O157:H7 dead cell DNA

5 RESULTS: RESUSCITATING VNC CELLS

Ten different resuscitation methods for recovering VNC cells to a culturable state (Table 4) were tested. The preliminary aim of this work was to reproduce published results for resuscitation methods with the bacterial strains for which they had been successful. Hence, methods were trialled with *E. coli* O157:H7 and *S. enterica* serovars Typhimurium and Brandenburg.

Prior to resuscitation trials, VNC cells were generated in 13% w/v NaCl and incubated in a 37°C shaking waterbath until no culturable cells could be recovered from the microcosms by standard plating methods. VNC cells were subjected to the selected resuscitation method (Table 4) and culturable numbers were either assayed by standard plating in the case of heat shock resuscitation, recovery on rich media, recovery in soft agar or recovery in a microaerophillic environment, or by length of time culturable cells could be recovered by enrichment in the case of broth recovery with various growth enhancing factors.

Heat shock at 56°C or 80°C, recovery on rich media, recovery in soft agar and recovery in a microaerophillic incubator did not produce culturable cells by standard plating methods. Enrichment in M9 minimal medium was also not successful at recovering culturable cells (data not shown).

Resuscitation trials by enrichment in liquid broth containing various published bacterial growth enhancing factors, sodium pyruvate (SP), 3,3' thiodipropionic acid (TDPA), ferrioxamine E (FE), Oxyrase® and BuGro® (see Table 4 for background information), were also performed. The average results for three resuscitation trials are reported in Figure 11. Resuscitation broth enrichments were set up the first day after culturable cells could no longer be recovered from the test microcosm by standard plating methods. It should be noted that when no culturable cells are able to be recovered using plating methods it may still be possible to recover some culturable cells by broth enrichment.

If a given factor were to successfully resuscitate VNCs, the number of days culturable cells should be recoverable in broth in the presence of this factor should be longer than the number of days culturable cells should be recovered in broth in the absence of this factor. As shown in Figure 11, none of the factors tested extended the culturable period in comparison to broth alone for any of the three bacterial species tested. Therefore, none of these factors successfully resuscitated VNC cells under the experimental conditions used.

Despite the fact that none of the reported VNC cell resuscitation methods could be reproduced with bacterial species for which they had been previously published, a subset of these methods were trialed with *C. jejuni* VNC cells due to *C. jejuni*'s importance as a foodborne pathogen in New Zealand. *C. jejuni* VNC cells from microcosms that no longer produced culturable cells by standard plating or enrichment methods were used. Neither heat shock at 55°C or 80°C, or enrichment for up to two weeks in nutrient broth no. 2 containing any of the aforementioned growth enhancing factors restored culturability to *C. jejuni* VNC cells formed in 13% w/v NaCl microcosms.

A large number of empirical experiments could have been carried out to test further for the ability to resuscitate VNC cells from the 13% w/v NaCl microcosms. However, due to

financial and time constraints within the project, the focus of the work switched to examine if conditions could be established that would cause VNC cells to die.

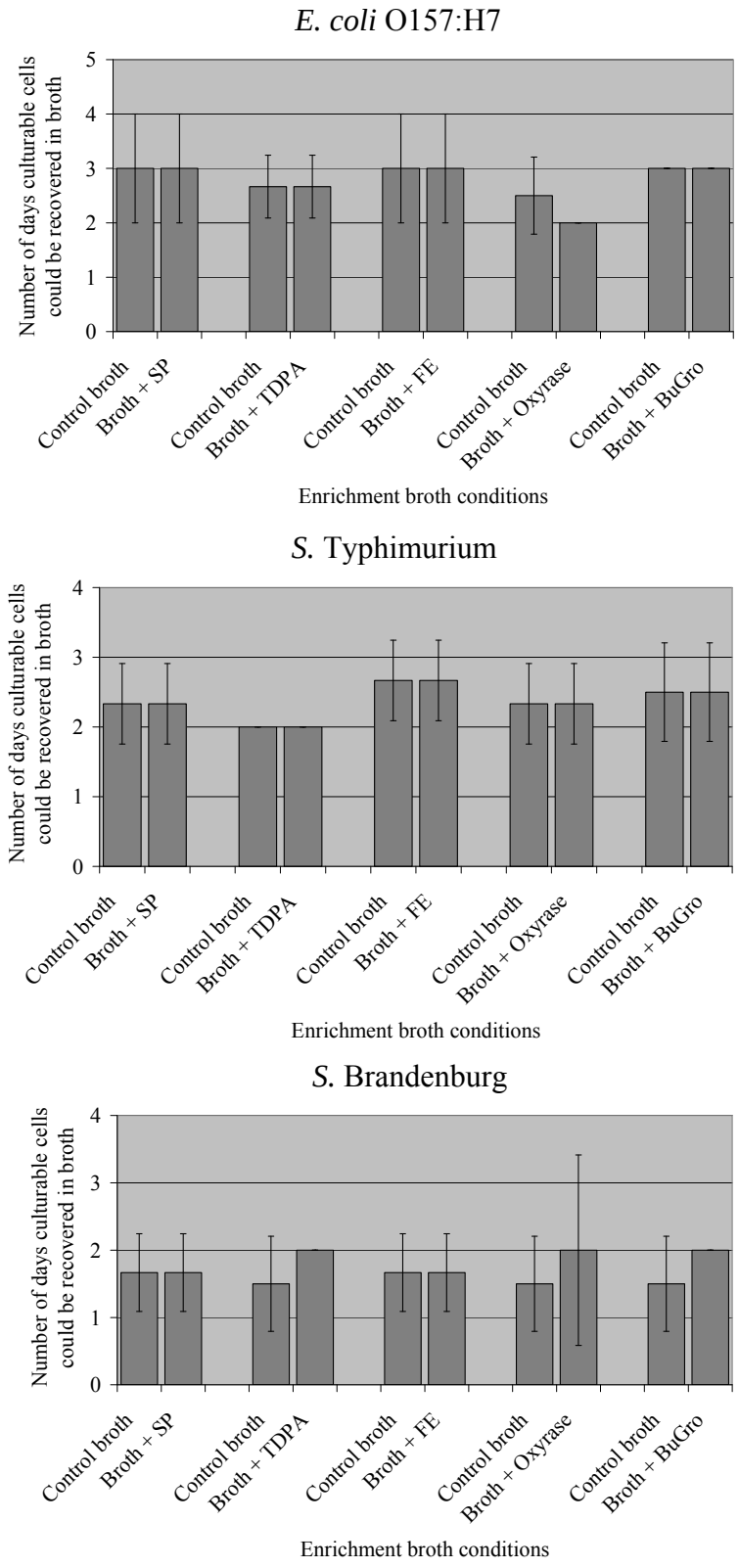

Figure 11: Resuscitation trials with enrichment broths supplemented with various bacterial growth enhancing factors

6 RESULTS: SURVIVAL OF VNC CELLS IN HUMAN FLUID ENVIRONMENTS

In order to gain a better understanding of the risk of VNC bacterial cells to humans who may unwittingly ingest them while consuming food or water, we tested the ability of VNC bacteria to survive various simulated human fluid environments. We chose to examine simulated human saliva, gastric fluid and intestinal fluid, as these would likely be encountered by ingested enteric pathogens prior to when they could colonize the host and cause disease.

6.1 Generating VNC cells for human fluid experiments

VNC bacterial cells were generated in 13% w/v NaCl microcosms incubated in a 37°C shaking water bath or, in the case of the two *C. jejuni* isolates, at room temperature (standing on the laboratory bench top). Three microcosms were set up for each isolate of interest on three different dates and from three different starting cultures. Each microcosm was monitored daily for culturable bacterial cells by standard plating methods and monitored every other day for viable bacterial cells using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) over an 8 day period (Figure 12).

Culturable bacterial cells declined in the *E. coli* O157:H7, *C. jejuni, and L. monocytogenes* microcosms as expected based on previous experiments (Figure 5). After 2-4 days in 13% w/v NaCl, culturable bacterial cells could not be recovered from the *E. coli* O157:H7 or *C. jejuni* microcosms. Bacterial cells could be cultured from *L. monocytogenes* microcosms throughout the experiment with cell numbers stabilizing after an approximately 4 $\log_{10}$ cfu/ml decline in the first three days in 13% w/v NaCl.

Unexpectedly, culturable bacterial cells could also be recovered from the *Salmonella* microcosms throughout the duration of the 8 day experiment (Figure 12). In previous experiments, after 2-4 days culturable cells could not be recovered from *S.* Typhimurium and *S.* Brandenburg 13% w/v NaCl microcosms (Figure 5). However, during these experiments both *Salmonella* spp. isolates behaved like the *Listeria* isolates, where an initial 5 $\log_{10}$ cfu/ml decline in the microcosms was observed in the first 4 days followed by culturable numbers stabilizing and remaining unchanged for the duration of the experiment. It is unclear why the *Salmonella* spp. isolates behaved differently in these experiments.

In 13% w/v NaCl microcosms, all isolates produced VNC cells in sufficient numbers (10^6 – 10^8 VNC cells/ml) for subsequent experiments. Therefore, between days 4-8, samples containing viable but non-culturable cells were removed from the microcosms and exposed to various simulated human fluids.

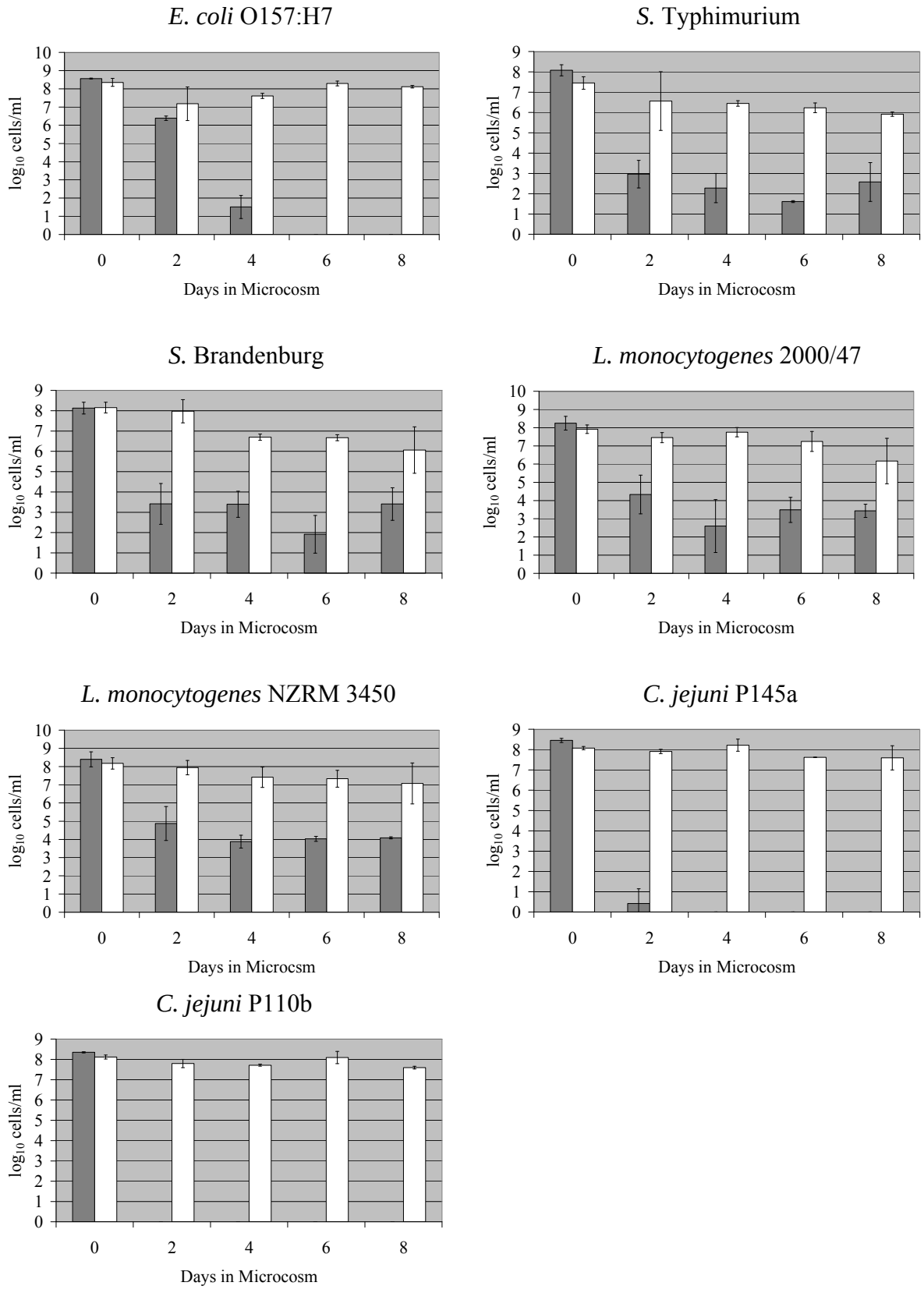

Figure 12: VNC cells generated for human fluid experiments; dark bars = culturable cells, white bars = viable cells

6.2 VNC cell survival in simulated human saliva

The first human fluid encountered by food during ingestion is saliva produced by the salivary glands in the mouth. Saliva contains hydrolytic enzymes which begins the process of breaking down the food for digestion and has been shown to contain several antimicrobial factors (Weinberg 2007). While in the mouth, food is chewed and broken apart, exposing internal portions of the food to saliva; this process however may also inadvertently release pathogens, including those that may have entered a VNC state, from internal locations within the food that are protected from washing and cooking procedures.

We tested whether viable but non-culturable bacterial cells formed by enteric pathogens could survive exposure to saliva. Initial experiments were performed using simulated human saliva as previously described (Davis *et al.* 1971). The simulated human saliva is of neutral pH and contains α-amylase, a glycoside hydrolase enzyme that breaks down starch into maltose, and mucin, a glycosylated protein often found on mucosal surfaces and in saliva.

Food remains in the mouth for minutes depending on the toughness of the food being ingested. VNC bacterial cells were exposed to simulated human saliva for five minutes at human physiological temperature (37°C) and survival was measured using the LIVE/DEAD® *Bac*Light™ Live/Dead Bacterial Viability Kit (Invitrogen Corporation) (Figure 13).

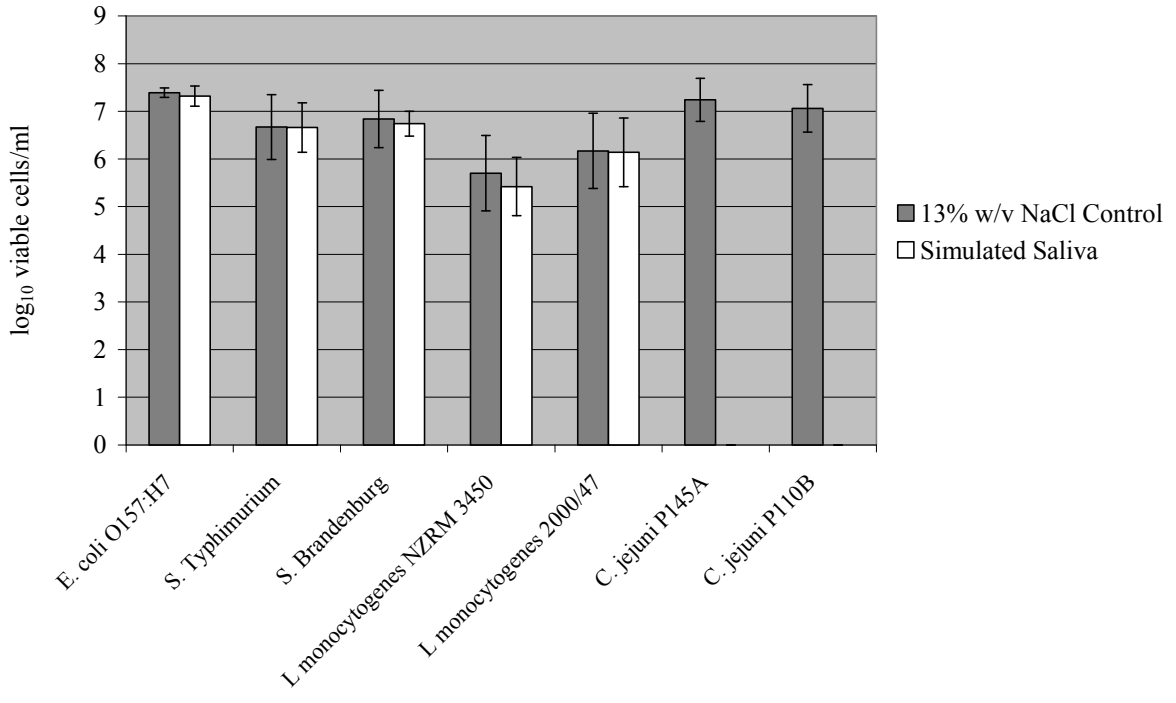

Figure 13: VNC cell survival in simulated saliva

We found that *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. VNC cells were unchanged in comparison to control samples and so were unaffected by exposure to simulated human saliva. In contrast, both isolates of *C. jejuni* were sensitive to simulated

human saliva. No viable *C. jejuni* cells could be detected after exposure to simulated human saliva and all cells became permeable to the red propidium iodide dye in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation), suggesting that the cells were killed by exposure to simulated human saliva.

To further test the sensitivity of *C. jejuni* VNC cells to human saliva, the cells were exposed to human saliva collected from a volunteer (Figure 14). The saliva was collected during the half-hour after the volunteer had eaten, to a volume of approximately 3-4 mls, and used immediately. The cells were exposed for five minutes (as described for the treatment with simulated human saliva). VNC cells of *C. jejuni* were sensitive to human saliva, but not to the same degree as to the simulated human saliva.

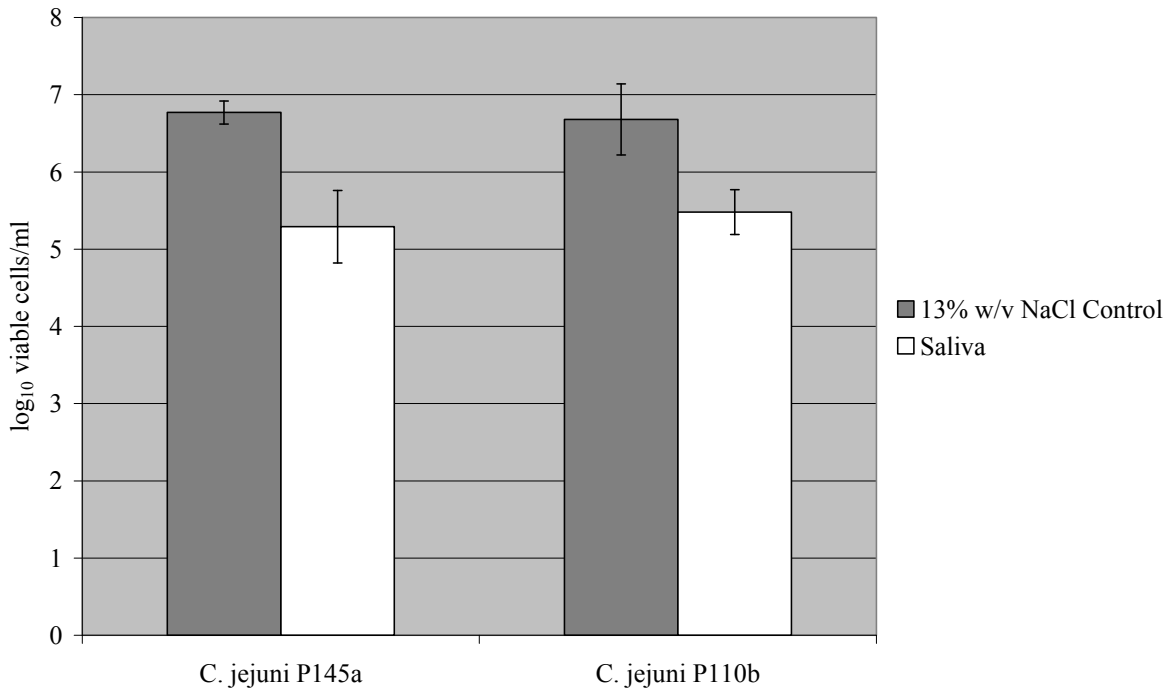

Figure 14: *C. jejuni* VNC cell survival in human saliva

6.3 VNC cell survival in simulated human gastric fluid

The acidic nature (pH 1.2-2.0) of human gastric fluid, encountered in the stomach during digestion, is the body's most effective defense against ingested enteric pathogens. However, successful enteric pathogens, such as those of interest to this study, have evolved mechanisms to survive this harsh host environment and subsequently travel to the intestine where they can colonize and cause intestinal disease.

We tested whether VNC bacterial cells formed by these enteric pathogens can survive exposure to simulated gastric fluid. Experiments were performed using simulated gastric fluid as outlined by the 30th edition United States Pharmacoepia. With pH 1.2, the simulated gastric fluid is very acidic, and contains predominantly pepsin. Pepsin is a protease released

by cells in the stomach and functions to degrade proteins into peptides that can then be absorbed by the intestine.

Food is digested within the stomach in the order of hours (Gotthard *et al.* 1986). Therefore, VNC bacterial cells were exposed to simulated human gastric fluid for one hour at human physiological temperature (37°C) and survival measured using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) (Figure 15).

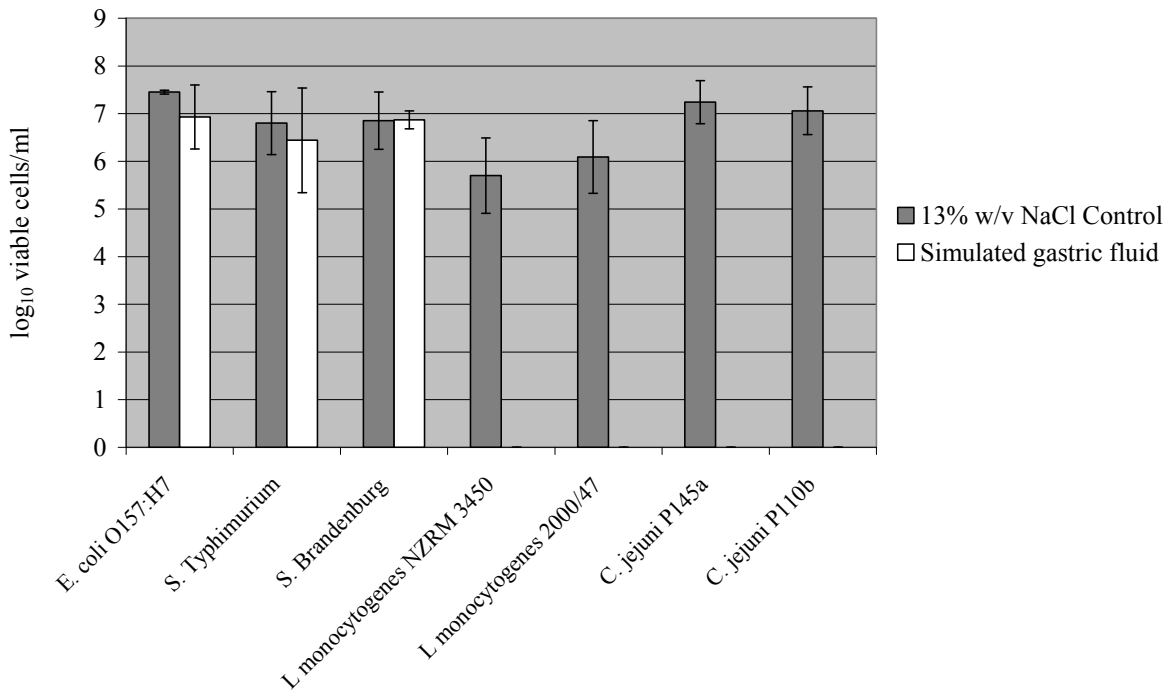

Figure 15: VNC cell survival in simulated gastric fluid

We found that VNC cells of *E. coli* O157:H7 and *Salmonella* spp. were unaffected by exposure to simulated human gastric fluid as viable cells numbers were unchanged in comparison to control samples. In contrast, VNC cells of both isolates of *Listeria* spp. and *C. jejuni* were sensitive to simulated human gastric fluid. No viable *Listeria* spp. or *C. jejuni* cells could be detected after exposure to simulated human gastric fluid and all cells became permeable to the red propidium iodide dye in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation), suggesting that the cells were killed by exposure to simulated human gastric fluid.

6.4 VNC cell survival in simulated human intestinal fluid

The final body fluid encountered by enteric pathogens prior to host cell attachment and colonization is intestinal fluid which is neutral pH, and consists mainly of various digestive enzymes produced by the pancreas.

We tested whether VNC bacterial cells formed by enteric pathogens could survive exposure to simulated intestinal fluid. Experiments were performed using simulated human intestinal fluid as outlined by the 30th edition United States Pharmacoepia. The simulated human intestinal fluid is of neutral pH and contains pancreatin, which is a mixture of several digestive enzymes (trypsin, amylase and lipase) that function to convert complex proteins, sugars and fats into simpler molecules that can be more easily absorbed by the intestine.

Food transits slowly through the intestine to allow for proper absorption of nutrients. Estimated intestinal transit time is 5-15 hours (Glober *et al.* 1977; Shils and Young 1988). For experimental ease, and to minimize replication of any contaminating culturable cells in the sample, VNC bacterial cells were exposed to simulated human intestinal fluid for one hour at human physiological temperature (37°C). Survival was measuring using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) (Figure 16).

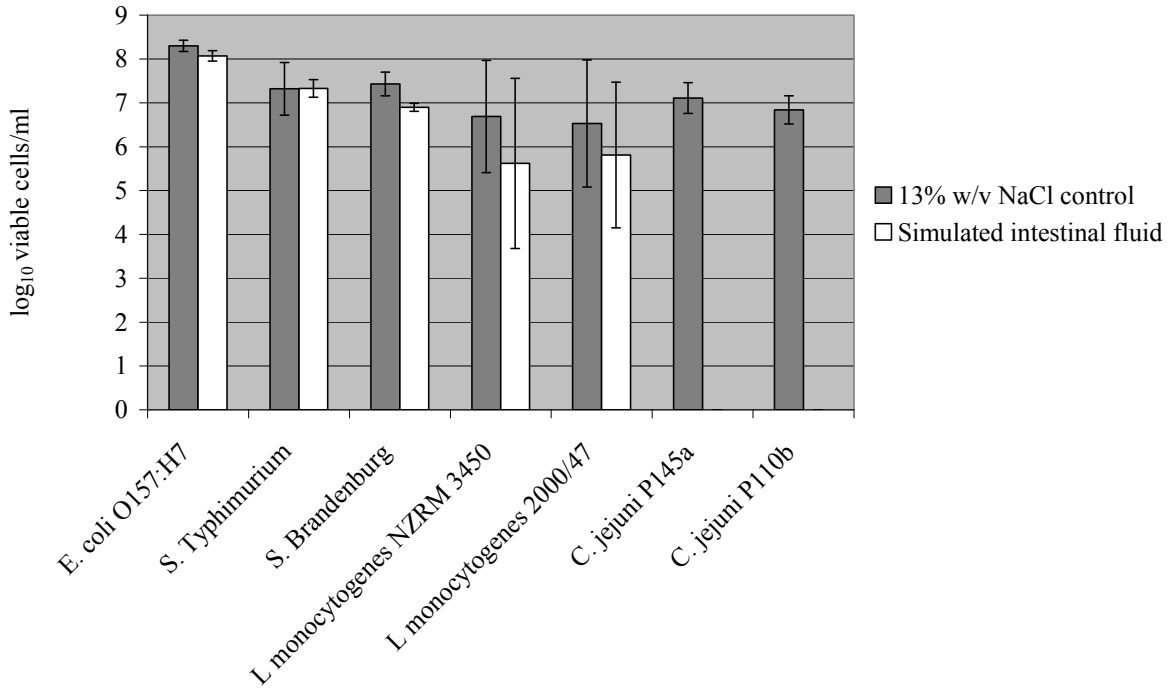

Figure 16: VNC cell survival in simulated intestinal fluid

We found that VNC cells of *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. were unaffected by exposure to simulated intestinal fluid as viable cells numbers were unchanged in comparison to control samples. In contrast, both isolates of *C. jejuni* were sensitive to simulated intestinal fluid. No viable *C. jejuni* cells could be detected after exposure to simulated intestinal fluid and all cells became permeable to the red propidium iodide dye in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation), suggesting that the cells were killed by exposure to simulated intestinal fluid.

7 DISCUSSION

Generating VNC cells

The first aim of this research was to determine if New Zealand strains of foodborne pathogens can enter the VNC state in response to adverse environmental conditions. In order to address this question, microcosms were prepared modelling typical environmental stresses that foodborne pathogens might be subjected to outside of the host.

Low temperature and starvation are commonly used laboratory stress models to induce VNC cell formation (Warner and Oliver 1998; Reissbrodt *et al.* 2002; Arana *et al.* 2007). Therefore, microcosms were prepared in water or 0.85% w/v NaCl and incubated at 4°C. The particular New Zealand strains of *E. coli* O157:H7, *Salmonella enterica* spp., and *L. monocytogenes* used in this study were reasonably robust under these conditions; culturable cells declined little after one week and only between 1-3 $\log_{10}$ cfu/ml after 2.5 months.

In general, culturable cells declined at similar rates whether in water or 0.85% w/v NaCl microcosms, except for the two New Zealand isolates of *L. monocytogenes*, which declined faster and 1 $\log_{10}$ cfu/ml further in water microcosms in comparison to 0.85% w/v NaCl microcosms. This might suggest that *L. monocytogenes* is more sensitive to hypo-osmotic environments in comparison to the other foodborne pathogens examined.

Extrapolating from these results, bacterial isolates examined in this study may take months to years to become completely non-culturable in low temperature, nutrient deprived microcosms. This time-frame was not conducive to achieving the objectives of this research within the specified time period of the project and would not reflect practical food storage situations. Given this and the fact that viable cells were declining at the same rate as culturable cells in the microcosms that were examined, an alternative 'model system' based on hyperosmotic stress was established. This method produced VNC cells in a rapid and reliable manner for all foodborne pathogens of interest to this study. .

The 'model system' that induced VNC cell formation by all strains tested was microcosms prepared in 13% w/v NaCl. These were initially incubated at 4°C to reflect storage conditions in a domestic refrigerator; however, culturable cells did not decline significantly faster than in water and 0.85% w/v NaCl microcosms incubated at this temperature. When 13% w/v NaCl microcosms were then incubated at higher temperatures, as described by Makino *et al.*, 2000, 10^7-10^8 VNC cells/ml were generated within 2 days at 37°C by New Zealand strains of *E. coli* O157:H7, *Salmonella enterica* spp., and *L. monocytogenes*, or at room temperature by *C. jejuni*. This work demonstrated that New Zealand strains of important foodborne pathogens can form VNC cells under certain conditions.

Interestingly, *L. monocytogenes* and, in some experiments, *S. enterica* spp. cell populations stressed in 13% w/v NaCl did not become completely non-culturable. In these microcosms, approximately 0.01% of the starting cell number remained culturable after a period of rapid culturable cell loss. Why these microcosms stabilized is unclear, but it is interesting to speculate upon. Perhaps a small percentage of the starting population is innately resistant or develops resistance to the stress applied (in this case osmotic stress), and thereby remains culturable; perhaps the population of bacteria in the microcosm can work in concert to reduce the strength of the osmotic stress over time through an active metabolic process that

detoxifies the environment, such that a percentage of cells survive the adverse conditions. Perhaps the growing dead cell population in the microcosm provides adequate sustenance, allowing a percentage of the population to survive. This latter speculation is interesting to ponder as proponents of the 'stochastic VNC cell model' espouse that VNC cell formation evolved for exactly this purpose (Bogosian and Bourneuf 2001).

In summary, the microcosms established in 13% w/v NaCl demonstrated that New Zealand strains of important foodborne pathogens are capable of producing VNC cells. This work does not address whether these particular strains can or do produce VNC cells in the New Zealand environment or within a given New Zealand food matrix; however, this work strongly supports this possibility Addressing this issue would require the development of new techniques to detect and quantify species-specific VNC cells directly from environmental or food samples. Furthermore, if, by future work, it is established that VNC cells contribute to foodborne illness in New Zealand, detection methods would be necessary to screen for their presence in food and ensure the safety of the food supply. For these reasons, methods for VNC cell detection by molecular means or via entry out of the VNC state by resuscitation to culturability were pursued.

Detecting VNC cells by quantitative real-time PCR

The first step towards validating a molecular method for detecting VNC cells was to compare cell numbers determined by Q-PCR for all four bacterial species of interest to this study with cell numbers determined by standard plating methods. Cell numbers determined by Q-PCR were categorically higher than those quantified by plating, by 2-7 fold. As the bacteria used in this comparison were sampled from mid-exponential growth cultures grown in optimal medium under optimal conditions, the vast majority of cells would be expected to be culturable, however, this discrepancy could be the result of Q-PCR detecting VNC cells or, alternatively, a dead cell population along with culturable cells.

These results highlight a general issue with using molecular methods for detecting and quantifying bacterial cells in a sample. A robust and reliable method for removing DNA contributed by a dead cell population in a sample is necessary to be confident that Q-PCR values accurately represent the viable cell number in the sample and do not overestimate cell quantities or produce false-positives. Until this methodology is developed, it is prudent to perform classical microbiological quantification methods alongside quantitative molecular methods when screening environmental and food samples for the presence of pathogens of interest.

To this end, two methods for removing dead cell DNA from a bacterial cell population were attempted, such that only viable cell DNA would be amplified and quantified by subsequent real-time PCR. Neither the DNase method, as described by Rueckert *et al.* (2005), or the PMA method, as described by Nocker *et al.* (2006), effectively removed dead cell DNA from a 100% isopropanol-killed bacterial cell culture. This is an essential positive control for the efficacy of the method because without removing the dead cell DNA, viable cell numbers would be overestimated and VNC cells could be falsely identified in a sample.

Both methods effectively removed 'naked' genomic DNA prior to quantitative real-time PCR, suggesting that the methods could be successful with the experimental conditions used given the DNase or PMA could gain access to the genomic DNA in dead cells. As these

methods are dependent on dead cells becoming permeable to the DNase or PMA it might be the case that isopropanol-treated cells, although dead, are not permeable to these agents. DNase treatment was also attempted with heat-killed *E. coli* O157:H7 and produced similar unsuccessful results (data not shown), suggesting that the manner by which the bacterial cells were killed was likely not responsible for the failure of the methods. Alternatively, the dead cells may have been permeable to the DNase and PMA, but the intracellular environment of the dead cells reduced the activity of the reagents or neutralized their activity altogether. Rueckert and co-workers demonstrated that DNase improved the correlation between cell quantities obtained by real-time PCR and cfu obtained by standard plating for *Anoxybacillus flavithermus*, but did not, however, perform the essential control of testing the method on a 100% dead cell population (Rueckert *et al.* 2005). Since environmental and food samples are likely to contain significant percentages of dead cells, this was an important starting point for validation of the method for practical purposes.

The initial report describing the use of PMA for removing dead cell DNA from a bacterial culture for subsequent cell quantification by real-time PCR demonstrated that, in comparison to a 100% viable cell culture, PMA did reduce the cell quantities detectable in 100% dead cell samples (Nocker *et al.* 2006). However, these researchers did see a significant amount of amplification during the real-time PCR reaction with 100% dead cell samples, perhaps because of the high sensitivity of the PCR method, and therefore the PMA treatment did not effectively remove all dead cell DNA. This finding is consistent with the results presented in this report.

Interestingly, a recent paper trialed the PMA method for detecting viable cells of *S. enterica*, *L. monocytogenes* and *C. perfinges* in diluted fermentor sludge to evaluate the efficacy of the method in an environmental matrix (Wagner *et al.* 2008). The authors found that the PMA method reduced cell numbers by 93% in PMA-treated samples of heat-killed cultures; however, this equated to at best a 1 $\log_{10}$ cell reduction in the PMA-treated samples in comparison to untreated samples containing 5-6 $\log_{10}$ cells. This work demonstrated that PMA was also not effective at removing the DNA of all nonviable cells in a model sludge matrix, and supports the findings presented in this report. Hence, the work presented in this report that demonstrated that PMA and DNase are not robust enough, using previously published methods, for accurately quantifying samples containing large numbers of dead cells by such a highly sensitive DNA detection method as Q-PCR is an important finding. The practical use of these two methods for quantifying viable bacterial cells in environmental and food samples is questionable.

If either the DNase or PMA method had robustly removed dead cell DNA, Q-PCR would have been evaluated alongside viable counts obtained by LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) staining. As neither method was adequate for removing dead cell DNA, this evaluation was not performed. Given the remainder of the objectives of the project could be met using standard culturing methods and the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation), establishment of molecular methods for detecting VNC cells was not necessary to pursue further.

Resuscitating VNC cells

There is no published, coherent picture of what triggers exit of bacterial cells from the VNC state. Several groups have reported resuscitation of VNC cells by a simple temperature

increase. *S. enterica* VNC cells, which had formed after incubation at low temperatures (4-5°C) became culturable after exposure to 56°C for 15 seconds (Gupte *et al.* 2003); *Vibrio* spp. also became culturable after a temperature upshift (Oliver *et al.* 1995; Wai *et al.* 1996). In this study attempts were made to reproduce this resuscitation phenomenon initially with *S. enterica* serovar Typhimurium, *S. enterica* serovar Brandenburg and *E. coli* O157:H7 and then with *C. jejuni* without success. In fact, if culturable cells were still present in the microcosms, not only were VNC cells not resuscitated, but culturable cells were killed by the temperature increase.

An explanation for why temperature increase was not sufficient to resuscitate our 'model' VNC cells may be that VNC cells were formed under different conditions by Gupte *et al.*, who induced VNC cells of *S. enterica* serovar Typhimurium by incubation at low temperature. Therefore removing this stress by temperature increase, along with a brief heat shock to stimulate the cells to activate stress response survival pathways, was an effective resuscitation method. In this study, culturable cells in microcosms placed at low temperatures, with an additional nutrient deprivation stress, did not decline nearly as rapidly as other groups have previously reported and viable cells declined at the same rate, thereby indicating that VNC cells were not forming. Therefore, an alternative method using nutrient deprivation and hyperosmotic shock in 13% w/v NaCl was used to generate VNC cells. Although this method was tested at low temperatures, VNC cell formation was most efficient at 37°C. Because the 'model' VNC cells used in these experiments were generated at human physiological temperature in a high salt environment, the changes to the cell physiology resulting in VNC cell formation may be different than those produced by the method used by Gupte *et al*., thereby making a simple temperature increase no longer effective to resuscitate the cells.

Another reported resuscitation method that was tested was the addition of purified autoinducer (a signalling molecule involved in bacterial cell to cell communication) to enrichment medium. Autoinducers are secreted by a number of enterobacterial species in the presence of the mammalian neuroendocrine hormone norepinephrine, which can stimulate bacterial growth under stress conditions (Lyte *et al.* 1996; Freestone *et al.* 1999). A purified autoinducer called BuGro® (formerly called Baxcell) was kindly supplied by Dr. Primrose Freestone at the University of Leicester. BuGro® is a purified extract from a non-pathogenic bacterial species, which has been shown to elongate the period of time culturable cells can be isolated from stressed microcosms of *S. enterica* serovar Typhimurium and *E. coli* O157:H7 prepared in water and incubated at room temperature (Reissbrodt *et al.* 2002).

Resuscitation of *S. enterica* Typhimurium, *S. enterica* Brandenburg, *E. coli* O157:H7, and *C. jejuni* VNC cells was attempted using enrichment broths supplemented with BuGro®, however, we did not observe an improvement in the end-point (days) for detection of culturable cells with any of the bacterial strains tested. Perhaps resuscitation was not observed because BuGro® has no effect on these particular New Zealand strains or perhaps the autoinducer has no effect on VNC cells generated under our specific model conditions. This begs the question of whether VNC cells formed under certain conditions cannot be resuscitated. That is, if VNC cell formation results from a non-deterministic series of events on the road to cell death, is there a point of no return?

Several other resuscitation approaches were tested without success with *S. enterica* Typhimurium, *S. enterica* Brandenburg, *E. coli* O157:H7 and *C. jejuni* VNC cells. These

included: (i) altering the nutrient content of recovery medium (minimal vs. optimal vs. rich medium), (ii) increasing the water content of recovery medium to aid in rehydration of cells from the hyperosmotic environment of the 13% w/v NaCl microcosms, (iii) recovery in lower oxygen environments (recovery in a microaerophillic incubator and recovery in medium supplemented with Oxyrase®), and (iv) recovery in the presence of antioxidants (sodium pyruvate, TDPA and Oxyrase®) or a siderophore (ferrioxamine E) to minimise oxidative stress during emergence from a stressed state to regrowth (McDonald *et al.* 1983; Reissbrodt *et al.* 2000; Reissbrodt *et al.* 2002).

If indeed *S. enterica* serovar Typhimurium, *S. enterica* serovar Brandenburg, *E. coli* O157:H7 and *C. jejuni* VNC cells can be resuscitated after exposure to 13% w/v NaCl at 37°C, then none of these methods as they were performed in this study, were sufficient to induce resuscitation. Resuscitation of VNC cells is a contentious area of research. The work presented in this report joins a growing body of experimental evidence demonstrating inability to resuscitate VNC cells using *in vitro* methods (Arana *et al.* 2007; Dreux *et al.* 2007). However, it must be noted that this work does not prove that these methods are not able to resuscitate VNC cells generated in other controlled model laboratory conditions or in the environment or in food.

Entry into the VNC state is not a very well understood phenomenon. Whether the cellular physiology of VNC cells is the same, regardless of the stress trigger, is not known, and whether VNC cells formed by different bacterial species share common physiological characteristics that would render all VNC cells responsive to a single resuscitation method is also not known. It may not be possible to develop a global resuscitation method. Further work is necessary to gain a better understanding of the physiology of the VNC cell of each of the important foodborne pathogens, formed in different model environmental and food matrix conditions, in order to design plausible *in vitro* resuscitation methods. It may be the case that if resuscitation is used to screen for the presence of VNC cells in foods, a cohort of distinct resuscitation procedures would need to be used for different foodborne pathogens in a specific food matrix.

Survival of VNC cells in human fluid environments

This work has demonstrated that New Zealand strains of important foodborne pathogens are capable of forming VNC cells in an experimental model system. Given exposure to an appropriate stress in the environment these strains may also be capable of forming VNC cells, which are, by definition, not detectable by standard culture methods that are used to screen for the presence of pathogens in the food supply. In the absence of being able to establish an accurate quantitative molecular method to detect VNC cells, or a reliable and robust procedure for VNC cell resuscitation, the focus of the project shifted from the original objective of applying these methods to VNC cells on meat to conducting experiments to better understand if VNC cells could pose a potential risk to human health.

Establishing if VNC cells are infectious and can cause disease if ingested by people is a difficult and ethically-challenging question to address. One approach by a group in the Netherlands was to feed healthy human volunteers *C. jejuni* VNC cells. This group did not observe disease (Beumer *et al.* 1992). Several other groups have passaged *C. jejuni* VNC cells through mouse intestines (Baffone *et al.* 2006) or the yolk sacs of embryonated chicken eggs (Cappelier *et al.* 1999), and observed resuscitation of *C. jejuni* VNC cells to a culturable

state. Conducting such *in vivo* experiments was not within the scope of this project. Instead, our approach to assess the infection potential of VNC cells was to determine the ability of VNC cells to survive in human body fluids that would be encountered after ingestion and prior to bacterial colonisation of the host. If, *in vivo*, VNC cells are killed by the constituents of these fluids, then VNC cells may be less likely to cause disease. The body fluids examined were simulated versions of human saliva, gastric fluid and intestinal fluid, which serve as a model for what may occur *in vivo*.

The results of these experiments were interesting as VNC cells from different bacterial species displayed different sensitivities to the three simulated body fluids, as assayed by the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation). *C. jejuni* VNC cells were killed by all three simulated body fluids and, of the four bacterial species examined, were the most sensitive to exposure to simulated human body fluids. VNC cells of *L. monocytogenes* were killed by simulated gastric fluid only, while in comparison, *E. coli* and *S. enterica* spp. VNC cells were unaffected by any of the three simulated body fluids. Multiple strains of the same bacterial species behaved similarly to each other in a given simulated fluid, suggesting that VNC cells of different strains of the same bacterial species may share similar cellular physiologies that render the cells sensitive or resistant to exposure to such fluids. However, further work with a larger cohort of bacterial strains would be necessary to establish this definitively. Also, since the bacterial cell surface is often an important determinant of survival in different conditions, it would interesting to compare and contrast changes in the cell wall and membrane composition during VNC cell formation in an effort to identify common modifications associated with sensitivity or resistance to exposure to simulated human body fluids.

C. jejuni VNC cells were the most sensitive to exposure to simulated human body fluids. If, *in vivo*, these body fluids do indeed effectively kill *C. jejuni* VNC cells, this might explain the results of Beumer *et al.* (1992), who reported that *C. jejuni* VNC cells did not cause disease when fed to human volunteers. However, further work would be necessary to establish whether *C. jejuni* VNC cells formed in the environment or on foods are also sensitive to contact with simulated human body fluids.

The results of these experiments may also be characteristic of the particular adverse conditions used to generate the 'model' VNC cells in this study. It may be that VNC cells formed under other conditions might respond differently in the simulated human body fluids. Stress resistance can often result in a trade-off where resistance to one stress can render bacteria sensitive to another. The results presented in this report are reflective of the response of 'model' VNC cells generated under specific experimental conditions and may not be representative of what may occurs in the environment or in the human *in vivo* situation. However, this work does demonstrate that *C. jejuni* and *L. monocytogenes* VNC cells can be killed by simulated human body fluids and supports the possibility that VNC cells could be killed after ingestion and thereby would not cause disease.

8 CONCLUSIONS

Work in this project covered three broad areas related to VNC bacterial cells of foodborne pathogens:

1. Development of a model system to generate VNC cells of New Zealand strains of foodborne pathogens important to human health.
2. Investigation of a quantitative molecular method to detect VNC cells.
3. Investigation of methods to remove cells from the VNC state, either by resuscitation of cells and by killing cells.

The bacteria selected for the study were *Salmonella enterica* serovar Brandenburg (one strain) and serovar Typhimurium (one strain), two strains of *Listeria monocytogenes*, two strains of *Campylobacter jejuni* and one strain of Shiga-toxin producing *Escherichia coli*. These bacteria are all of importance to New Zealand public health and the strains chosen were from NZ or Australian isolates.

VNC cells are, by definition, unable to be cultured on standard microbiological medium. It is therefore necessary to utilize an alternative method to detect VNC cells. In this study a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) was used to detect VNC cells. This method relies on the integrity of the membrane of 'live' bacterial cells and its ability to exclude a red fluorescent dye. Live cells are detected by staining with a green fluorescent dye. Dead cells, with compromised membranes, are able to take up the red fluorescent dye and stain red. All VNC results in this study therefore rely on the premise that the VNC state reflects the continued ability of the bacterial membrane to exclude the red fluorescent dye.

A model, laboratory-based, system for generation of VNC cells for all of the bacteria of interest was successfully established. Methods for generation of VNC cells were trialled that could mimic stress conditions that the bacteria may encounter either directly in the environment or during food processing. The successful system utilized for ongoing studies involved incubation of cultures in 13% w/v NaCl. This puts the bacteria in a situation of osmotic stress, analogous to dehydration. VNC cells could be established at 4^{o}C (analogous to refrigerator storage conditions) however the development of VNC cells at this temperature was slow and the time frame unlikely to be representative of a food storage condition. To facilitate a more rapid experimental system a physiological temperature of 37^{o}C was used. VNC cell formation was rapid for all bacterial strains tested, although the actual time taken was bacterial genera dependant. Overall the results suggest that VNC cells of the bacteria of interest may be generated using a variety of stress conditions. The time taken for bacteria to enter the VNC state may be quite variable depending on the conditions used. The conditions used in the model system in the laboratory are unlikely to be the only conditions to which the particular bacteria can respond. The results of the in vitro studies outlined in this report suggest that if VNC of the bacteria of interest are established in the environment it may be in response to a range of conditions and occur over a range of time periods.

The study of VNC cell prevalence in the environment and during food processing would be greatly facilitated by the availability of a rapid, high throughput, quantitative molecular detection method. Reports in the literature suggested the use of a quantitative-PCR method to specifically detect 'live' bacteria in a background of dead bacterial cells. We tested two methods for removal of dead cell DNA from an assay to allow the selective detection of only

live bacterial cells using bacterial cell cultures that had been 100% killed and found neither method was robust enough to selectively remove all dead bacterial DNA background from an assay. Therefore neither of these methods was considered robust enough for us to be able to utilize it in our ongoing studies on VNC cells. This was an important finding as a number of reports are available in the literature using these methods and our work would suggest that results from these reports may be unreliable. Further work needs to be done to establish a quantitative molecular detection method robust enough to be able to be employed in the detection of VNC cells in environmental or food samples.

Little is known about the conditions required for VNC bacterial cells to exit that state – either by resuscitation to a culturable state, or by death. To investigate these issues ongoing work in this project focussed on the ability to resuscitate and/or kill VNC cells in culture. VNC cells were established using our model system. A range of both published and empirical methods were tested to see if the VNC cells could be resuscitated to a culturable state. None of these methods was successful for any of the bacteria of interest. These results concur with a growing body of experimental evidence from the literature demonstrating inability to resuscitate VNC cells using *in vitro* methods. Due to the specificity of our model system for VNC generation, however, this result cannot be taken to indicate that VNC cells are generically unable to be resuscitated. Resuscitation ability may be a combination of the specific conditions used to establish the VNC cells, the resultant cellular physiology of the bacteria and the resuscitation method itself.

The importance of VNC foodborne pathogenic bacteria to human health lies in whether or not bacteria in this state are, or can become, infectious. In a preliminary study to address this question we investigated the ability of VNC cells generated in our model system to survive incubation in human fluids likely to be encountered by the bacteria during ingestion. Interestingly the VNC cells from different bacterial species showed different sensitivities to the fluids tested (simulated human saliva, gastric fluid and intestinal fluid), as assessed by LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen Corporation) staining. VNC cells of *C. jejuni* were the most sensitive to the human fluids, being killed by all three. *L. monocytogenes* VNC cells were only sensitive to simulated gastric fluid, while *S. enterica* spp. and *E.coli* VNC cells were unaffected by any of the human fluids. These results indicated that it is certainly possible to kill VNC cells and some are sensitive to simulated human fluids, which may impact on their ability to cause disease. The sensitivity of the VNC cells tested in this system is likely to relate to the conditions used in our model system to generate the VNCs and the environment these cells were exposed to in the fluid exposure experiments. These results are interesting to speculate on with regards to the potential for VNC bacterial cells to survive ingestion and go on to cause disease. The result cannot, however, be directly extrapolated to VNC cells that may be established in an environmental or food processing situation or to the human *in vivo* ingestion situation, as a whole range of additional factors may influence VNC cell sensitivity in these systems. Further studies would be required to elucidate the VNC state and the influence of exposure systems before any implication to human health from ingestion of VNC pathogenic bacterial could be determined.

Overall this study has confirmed the ability of certain foodborne pathogenic bacteria of interest to the NZ food industry to become VNC under certain conditions. The model system for VNC establishment developed in this study is unlikely to be the only conditions under which these bacterial strains can become VNC. An inability to establish a method to

resuscitate cells in this state to one where they could be cultured using standard methods supports the contention that there may be no universal system for resuscitation of VNC cells and the phenomenon, if real, may be highly variable and bacterial species specific. VNC cells of the bacteria of interest to this study were shown to have variable sensitivity to human fluid environments likely to be encountered if the bacteria are ingested. That some of the species were sensitive to simulated human fluids may have implications for the ability of VNC cells of these bacteria to cause disease.

9 REFERENCES

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