Fate of key food-borne pathogens associated with intensive pig and poultry farming environments

Helene Nalini Chinivasagam, B. Sc. (Hons), M. Phil., M.A.S.M

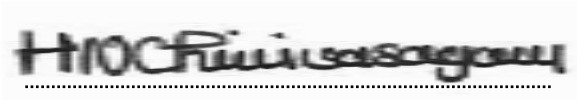
School of Environmental and Life Sciences

Ph.D. Thesis

February 2013

Declaration

"I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University is the result of my own investigations, and all references to all ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree and is not being currently submitted in candidature for any other degree.



Declaration of co-author roles

Monday, 4 March 2013

To Whom It May Concern

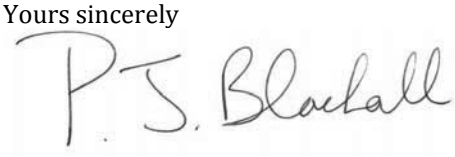
I have been requested to provide a formal allocation of roles of all authors on a series of scientific publications in which I have been an author.

The roles of each of the authors is provided in the attached document.

I can confirm that the roles are an accurate reflection of the contribution of each of the named authors.

If any further information regarding this matter, please do not hesitate to contact me on telephone +61 7 3255 4298 or email p.blackall@uq.edu.au. .

Yours sincerely



Pat Blackall (B.Sc., Ph.D., F.A.S.M., P.S.M.)

Principal Research Fellow
Research Manager, Poultry CRC
Editor, *Journal of Veterinary Diagnostic Investigation*

Publications and Author Roles

Publication 1

H.N. Chinivasagam, R.J. Thomas, K. Casey, E. McGahan, E.A. Gardner, M. Rafiee and P.J. Blackall (2004) Microbiological status of piggery effluent from 13 piggeries in the south east Queensland region of Australia. *Journal of Applied Microbiology* **97**, 883-891.

Author roles:-

Chinivasagam – responsible for all experimental design, performed all bench work, prepared publication, responsible for editing of publication through publication process

Thomas – supervised aspects of the bacteriology work, assisted with publication

Casey – assisted with access to piggeries

McGahan - assisted with access to piggeries

Gardner – assisted with industry contacts

Rafiee – provided the PCR techniques for *Erysipelothrix*

Blackall – responsible for funds for work, assisted with publication.

Publication 2

H.N. Chinivasagam and P.J. Blackall (2005) Investigation and application of methods for enumerating heterotrophs and *Escherichia coli* in the air within piggery sheds. *Journal of Applied Microbiology* **98**, 1137-1145.

Chinivasagam – responsible for all experimental design, performed all field sampling and laboratory work, prepared publication, responsible for editing of publication through publication process

Blackall – responsible for funds for work, assisted some field sampling, assisted with publication.

Publication 3

H.N. Chinivasagam, B.G. Corney, L.L. Wright, I.S. Diallo and P.J. Blackall (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland. *Journal of Applied Microbiology* **103**, 418-426.

Chinivasagam – responsible for all experimental design, performed all laboratory work (except sequencing), prepared publication, responsible for editing of publication through publication process

Corney – supervised PCR method development, supervised sequencing work

Diallo - supervised PCR method development, supervised sequencing work

Wright – performed bench work associated with DNA sequencing work

Blackall – responsible for funds for work, assisted some field sampling, assisted with publication.

Publication 4

H. N. Chinivasagam, T. Tran, L. Maddock, A. Gale and P. J. Blackall (2009) Mechanically Ventilated Broiler Sheds: a Possible Source of Aerosolized *Salmonella*, *Campylobacter*, and *Escherichia coli*? *Applied and Environmental Microbiology* **75**, 7417-7425.

Chinivasagam – responsible for all experimental design, assisted with field sampling and laboratory work, prepared publication, responsible for editing of publication through publication process

Tran – assisted in field sampling and laboratory work

Maddock – assisted in field sampling and laboratory work

Gale – assisted in field sampling and laboratory work

Blackall – responsible for funds for work, assisted some field sampling and some laboratory work, assisted with publication.

Publication 5

H.N. Chinivasagam, T. Tran, L. Maddock, A. Gale and P.J. Blackall (2009) The aerobiology of the environment around mechanically ventilated broiler sheds. *Journal of Applied Microbiology* **108**, 1657-1667.

Chinivasagam – responsible for all experimental design, assisted with field sampling and laboratory work, prepared publication, responsible for editing of publication through publication process

Tran – assisted in field sampling and laboratory work

Maddock – assisted in field sampling and laboratory work

Gale – assisted in field sampling and laboratory work

Blackall – responsible for funds for work, assisted some field sampling and some laboratory work, assisted with publication.

Publication 6

H. N. Chinivasagam, M. Redding, G. Runge and P. J. Blackall (2009) Presence and incidence of food-borne pathogens in Australian chicken litter. *British Poultry Science* **51**, 311-318.

Chinivasagam – responsible for all experimental design (except litter sampling design), assisted with field sampling; performed laboratory work, prepared publication, responsible for editing of publication through publication process

Redding – responsible for design of litter sampling

Runge – responsible for industry liason and field sampling

Blackall – responsible for funds for work, assisted some field sampling and some laboratory work, assisted with publication.

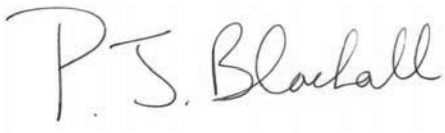
Publication 7

H.N. Chinivasagam, T. Tran, P.J. Blackall (2012) Impact of the Australian litter re-use practice on *Salmonella* in the broiler farming environment. *Food Research International* **45**, 891-896.

Chinivasagam – responsible for all experimental design, responsible for funds for work; assisted with field sampling and laboratory work, prepared publication, responsible for editing of publication through publication process

Tran – assisted in field sampling and laboratory work

Blackall – assisted some field sampling and some laboratory work, assisted with publication.



Pat Blackall (B.Sc., Ph.D., F.A.S.M., P.S.M.)

Principal Research Fellow
Research Manager, Poultry CRC
Editor, *Journal of Veterinary Diagnostic Investigation*

Acknowledgements

My sincere thanks to Professor Karen Gibb (Charles Darwin University) for accepting me to present a thesis by publication at CDU and for her overall support and guidance given during the preparation of this thesis

My sincere to thanks to Dr. Ala Lew-Tabor (QAAFI, University of Queensland) for her overall support and guidance given during the preparation of this thesis

The financial support of Charles Darwin University and the Department of Agriculture, Fisheries and Forestry was an essential part of my undertaking and then completion of this thesis.

I acknowledge Dr. Pat Blackall (QAAFI, University of Queensland and formally the Department of Primary Industries) for the opportunity to work in this area, his support and guidance which was an integral part of these studies.

I acknowledge the key role of Dave Jordan, my supervisor at Department of Agriculture, Fisheries and Forestry, for his work in ensuring that my research was given full organisational support across many years.

I also would like to acknowledge the skills and professional input of all my co-authors of the published papers (scientists and my technical staff from the Department of Primary Industries currently the Department of Agriculture Fisheries and Forestry) that form the core of this thesis.

In addition, the financial support of the funding bodies (Australian Pork Limited, The Australian Poultry CRC and the Rural Industries Research and Development Corporation) was essential and deeply appreciated. Due to their financial support I have had the opportunity to combine the outcomes from both poultry and pigs to address the key issues of pathogen transfer in the environment through this thesis.

The poultry companies who allowed me access to their company farms and the many pig and poultry farmers who allowed my work to be undertaken on their properties are greatly appreciated.

I finally acknowledge my family and in particular my mother and sister for their support provided during the preparation of this thesis.

Abstract

Intensive pig and poultry farming in Australia can be a source of pathogens with implications for food-safety and/or human illness.

Seven studies were undertaken with the following objectives:

- Assess the types of zoonotic pathogens in waste
- Assess the transfer of pathogens during re-use both *within* the shed and *externally* in the environment
- The potential for movement of pathogens via aerosols

In the first and second studies the extent of zoonotic pathogens was evaluated in both piggery effluent and chicken litter and *Salmonella* and *Campylobacter* were detected in both wastes.

In the third study the dynamics of *Salmonella* during litter re-use was examined and results showed a trend for lower *Salmonella* levels and serovar diversity in re-used litter compared to new litter. Thus, re-use within the poultry farming system posed no increased risk.

The fourth study addressed the direct risks of pathogens to farm workers due to re-use of piggery effluent within the pig shed. Based on air-borne *Escherichia coli* (*E. coli*) levels, re-using effluent did not pose a risk.

In the fifth study high levels of *Arcobacter* spp. were detected in effluent ponds and freshly irrigated soils with potential food-safety risks during the irrigation of food-crops and pasture.

The sixth and seventh studies addressed the risks from aerosols from mechanically ventilated sheds. Staphylococci were shown to have potential as markers, with air-borne levels gradually dropping and reaching background levels at 400 m distance. *Salmonella* was detected (at low levels) both inside and outside the shed (at 10 m). *Campylobacter* was detected only once inside the shed during the 3-year period (at low levels). Results showed there was minimal risk to humans living adjacent to poultry farms

This is the first comprehensive analysis studying key food-safety pathogens and potential public health risks associated with intensively farmed pigs and poultry in Australia.

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Chapter 1

Review of Literature

1.0 Food-safety and public health

The issue of food-safety has been the focus of both the developed and the developing world, with the main aim to protect public health. The growth in intensive animal farming and agriculture has pressures not only in environmental issues but also in the movement and adaptation of the associated food-borne pathogens. These key pathogens ultimately either have a direct or indirect impact on the human food chain (Pell 1997).

In 1998, the Pathogen Reduction and HACCP (Hazard Analysis Critical Control Point) Rule was passed by the USDA to both reduce pathogens in food processing plants as well as clarify industry and federal roles on food-safety (Torrence 2003). Pathogen reduction performance standards for *Salmonella* were adopted and preventative controls were established (Torrence 2003). Thus the prevention of all food-safety pathogens is largely a "farm to fork" process. Whilst focus at the level of farming is to control and manage key pathogens such as *Salmonella* and *Campylobacter*, the environment can also be a source of indirect transfer (Spencer & Guan 2004). The food standards Australia and New Zealand (FSANZ) discuss HACCP relevant to Australian situations.

Zoonotic pathogens (transferred from animals to humans) are responsible for key illnesses such as Salmonellosis, *Campylobacter*iosis and Listeriosis. Rivers/creeks, air, soil, and feed can become contaminated by animal waste due to inadequate waste management practices and thus contribute to the epidemiology of zoonotic diseases at the pre-harvest level, (Hensel & Neubauer 2002). Thus the intensive animal farming industries (pigs, poultry and cattle) can play a contributory role in food-borne illnesses and would need to adopt a proactive approach at all levels of farming (Hill 2003). There is also the need to develop improved methods to control the colonisation of these pathogens in food animals (Torrence 2003), such as pig and poultry.

The global demands on both food supply and security means many countries, both developed and developing, are moving towards intensive animal agriculture. Pigs and particularly poultry are fast becoming a more affordable meat source to the average consumer. This means that responsibility in delivering food-safety will continue to be a major focus in countries such as Australia. Practices such as reuse

in agriculture will be an increasing focus as Australian agriculture adapts to the challenges of food security in a changing world (Chinivasagam & Blackall 2009). There is thus the need to better understand the ecology and movement of these key pathogens in such farming environments, (Torrence 2003), i.e. both the immediate farm and beyond. This will assist in the development of suitable on and off-farm guidelines and codes of practice. Such hygiene regulations need to be cost effective to the farmer plus be acceptable to both the animal production facilities and the surrounding environment (Heinonen-Tanski *et al.* 2006).

1.1 Poultry and pig farming

The gross value of Australian farm production for pig and poultry was estimated at being 865 million (2009/2010) (Australian Pig Annual 2010) and 1.9 billion dollars (2010/2011) (Australian Chicken Meat Federation 2012) respectively. The majority of this is intensively farmed and production will increase based on future consumer demands. The poultry industry is set to grow due the lower retail price of chicken compared to beef, lamb and pork (Australian Chicken Meat Federation 2012). Both industries (pigs and poultry) are of significant size and capacity, with the bird numbers slaughtered in 2009/10 estimated at 465.7 million (Australian Chicken Meat Federation 2012) and pig carcasses estimated at 4,606,000 in 2009/10, (Australian Pig Annual 2010).

Most intensive pig and poultry operations are large, concentrated (particularly poultry) and can be close to urban populations due to the need for rapid market access. This means that large volumes of concentrated waste can be of environmental concern (O'Connor *et al.* 2005) due to its proximity to sensitive locations (e.g. rivers and creeks) (Ma 2002). There is thus the potential for surface and ground water as well as food crops becoming contaminated by such wastes due to the transfer pathogens of concern to humans (Hill 2003).

Used poultry bedding (litter) can be re-used (Macklin *et al.* 2006) either within the operation or in the external environment (Das *et al.* 2002). Such bedding can be a source of pathogens originating from poultry faeces (SchockenIturrino *et al.* 1996). Similarly piggery operations also could be a source of pathogens (Chandler &

Craven 1981) from effluent or solid bedding material. Both poultry and pig wastes contain faeces, the main source of pathogens. Thus these by-products (waste, including faeces) are of concern due to the potential presence of zoonotic pathogens such as *Salmonella* and *Campylobacter* (Hutchison *et al.* 2005a).

1.1.1 The Pig industry

The piggery industry has traditionally adopted responsible waste management strategies for the disposal of waste from intensive piggery operations. According to the Australian Bureau of Statistics (ABS) and the Australian Bureau of Agricultural and Resource Economics (ABARE) the industry is estimated to have in 2007-08 produced approximately 5.4 million pigs (385,000 tonnes carcase weight) with a gross value of production of $880 million, (Anon 2009a). Negative effects of swine production in the environment have already led to new legislation that limits the use of animal manure and expansion of pig operations in some countries (Jongbloed & Lenis 1998). In Australia the National Environmental Guidelines in Piggeries (Australian Pork Limited 2010) stipulate effluent re-use in the environment and is based more on safe nutrient loads. However the impact of pathogen movement in the environment is an emerging issue.

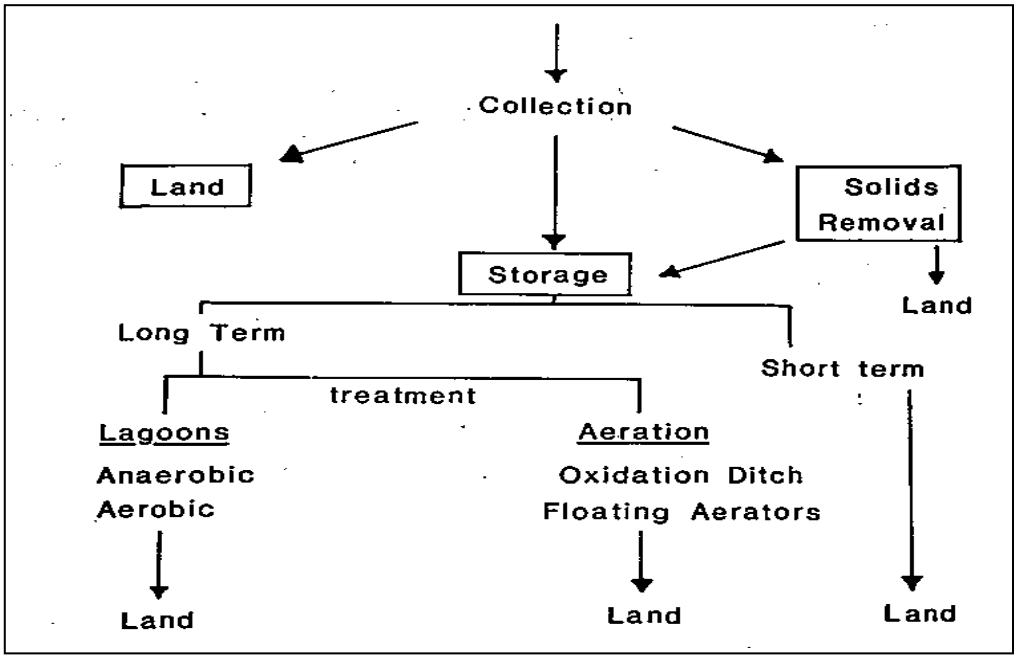
Pigs may be raised in slatted sheds (floor with slats to collect waste material below) or alternatively raised on straw. Where pigs are raised in slatted sheds effluent is the waste by-product (water is used to clean the sheds) and when straw is used (as bedding), litter is the waste by-product. Both effluent and litter could be a source of pathogens. Alternatively pigs (and poultry) could also be raised free-range and in such situations the external environment can be a direct source of pathogen transfer. Effluent is stored onsite and treated in a series (usually two) effluent treatment ponds. The treatment process is anaerobic and sedimentation of solids occurs. After a period of time (i.e. the resident time) the effluent is pumped into the piggery sheds for “flushing” and cleaning. This practice can generate aerosols that potentially contain food-safety pathogens and be of risk to humans due to ingestion.

Piggery effluent is a by-product of pig farming (Wang *et al.* 2004) and in Australia a typical farm can have 1 – 3 effluent ponds. These series of effluent ponds sequentially transfer effluent from one to another as a means of nutrient reduction but bacterial “die-off” can also occur (Polprasert *et al.* 1983). These ponds are

anaerobic ponds and the retention time within each pond along with factors such as the UV in sunlight can aid in pathogen "die-off" with time (Sinton *et al.* 2002). Studies have been carried out to model "die-off" of organisms such as *Escherichia coli* in ponds (Saqqar & Pescod 1992). It is thus necessary to understand pathogen "die-off" in piggery effluent ponds as effluent is used for on-farm irrigation (Redding *et al.* 2002), and this can include food crops or pasture for cattle.

The treatment methods adopted for effluent ponds mainly target the reduction of nutrients, oxygen demand, odours and solid build-up (Payne 1990). Farren (1979) describes a system in a large Victorian piggery to deal with water catchment quality (and odour) which was achieved by the use of a screen separator, land treatment, runoff collection and recycling of the liquid fraction (Figure 1). In this system both the effluent and solids were the normal waste products. It is possible that either effluent or screened solids from ponds can all be a source of pathogens. The pond or lagoon systems commonly used in Australia are cost effective and mainly deal with nutrient reduction though not necessarily pathogen reduction.

Figure 1 Typical waste from a piggery waste management system (Farran 1979)



Some previous work on pathogens in piggery effluent in Queensland has been carried out, (Henry *et al.* 1995a, Chandler *et al.* 1981). These studies looked at the presence of *Salmonella* in piggery wastes (Henry *et al.* 1983, Henry *et al.* 1995a),

though there is no knowledge on the extent (or levels) of this organism (or *Campylobacter*) in piggery waste. The assessment of the levels of pathogens can aid in the understanding and development of risk assessment processes for effluent re-use in food agriculture supported by relevant guidelines.

The use of effluent and waste for crop irrigation could result in direct transmission of pathogens from effluent or "cross contamination" of crops from soil irrigated with effluent (Islam *et al.* 2004). Similarly there could be indirect transmission of pathogens following irrigation via "run-off" during heavy rain and flooding into adjacent creeks and water-ways (Chandler *et al.* 1981). The number of organisms (pathogens) available for rainfall run-off (or transport via the soil environment) would depend on the number of surviving organisms in soil. Bacterial die-off in soil has been shown to occur following the first order kinetic reaction (Reddy *et al.* 1981) with the levels of organisms decreasing with time.

On farm pig mortalities (or carcasses) can be composted (Morrow *et al.* 1995) and treatment of pig litter can be part of an overall waste management strategy. While it is possible to remove and/or bury solid waste, innovative solutions such as composting can provide environmentally friendly solutions that still meet the need for the economic realities of pig production. A well-managed controlled composting process can destroy the pathogenic microorganisms present in wastes resulting in environmentally friendly end products that have value for agriculture (Cekmecelioglu *et al.* 2004).

Common pig waste on farm could include litter i.e. partially composted pig waste and sawdust bedding (pigs raised on litter) or sludge from slurries settled at the bottom of the primary sedimentation effluent tank (pigs from slatted sheds) (Tiquia & Tam 2000). Increasingly, on-farm waste management strategies are focussed on processes that allow either effluent or litter to be fully utilised for soil amendment in agricultural applications (Tishmack & Jones 2003). Whilst the composting process has been used for a long time, larger herd sizes have increased the need for environmentally sound and safe composting systems.

Work is being carried out in countries such as the USA to address pathogens and aerosols associated with both pig and poultry farming operations. Bioaerosols from swine confinement buildings in Canada were analysed for selected human pathogens and antimicrobial resistant bacteria to assess worker exposure to such

pathogens (Létourneau *et al.* 2010). A similar study has also been carried out within poultry houses in the US (Brooks *et al.* 2010).

Pig effluent can be re-used on farm for various purposes. The movement of pathogens in aerosols from human effluent used for spray irrigation have been carried out in other countries such as the USA (Brenner *et al.* 1988). Mathematical models have been developed to predict the dispersion of microorganisms from the source during irrigation using human effluent (Canamm 1980). It is thus possible to predict the levels of microorganisms transferring in aerosols originating from spray irrigation (EPA 1982) and their subsequent survival in the surrounding environment (Sorber & Sagik 1979). However there is a lack of understanding of the survival (and transport) of the levels of key pathogens in open aerosol environments.

More so, there is an inability to accurately predict the health risks associated with bioaerosolized pathogens (originating from animal waste operations) (Pillai & Ricke 2002). To fulfil such needs mathematical models have also been developed to predict the risk of infection arising from bacteria and viruses (from bioaerosols) to residences downwind to waste operations (during the land application of biosolids) (Brooks *et al.* 2005a). These issues are of importance with both pig and poultry, where the farming operations adjacent human settlements and thus be a source and subsequent transfer of contaminated aerosols.

Thus there is increasing pressure from legislators requiring in understanding (and managing) the risks to human settlements (and farm workers) as a consequence of the transfer of zoonotic pathogens via aerosols either directly (farming) or indirectly pathways (e.g. use of animal manures). Thus there is a need for Australian data to support both the farming operations and the legislators of such operations.

1.1.2 The broiler industry

The poultry industry typically is made up of broiler farming for meat production and layer farming for egg production. The farming systems are different with broilers commonly raised in mechanically ventilated sheds though free-range within both systems is becoming more common. Broiler production depends largely on the availability of wood-shaving as bedding for each cycle and re-use of bedding material does occur in Australia. The sourcing of quality shavings (and disposal), together with the cost of both material is becoming a pressing issue. Approximately 1.6 million m^3 of spent litter is produced annually (Runge *et al.* 2007). Re-use of

chicken litter is common in the USA (Macklin *et al.* 2006) as well as Australia (Runge *et al.* 2007). Litter has traditionally been used as manure for horticulture, pasture and vegetable crops due to the nutrients present (Redding 2011).

Bedding or the commonly used resource wood shavings is a renewable natural resource. The constraints faced by the industry in sourcing good quality litter for each production cycle has resulted in the need to adopt the practice of re-using litter during the production cycle. Both *Salmonella* (some serovars) and *Campylobacter* are generally normal commensals of chickens. Thus there is the possibility that these organisms can be present in used bedding (litter), and re-enter the next cycle. However other factors such as husbandry practices, farm characteristics and general hygiene (Le Bouquin *et al.* 2010) as well as the parent breeder flock (Sasipreeyajan *et al.* 1996), also can have an impact on the *Salmonella* status. *Salmonella* serovars from a previous flock do have also the potential to re-infect future flock across broiler farming cycles.

There is the potential for food-borne pathogens such as *Salmonella* (Payne *et al.* 2002) and *Campylobacter* (Shanker *et al.* 1990) to be present in poultry litter and thus pose a food-safety challenge. Both *Campylobacter* (Montrose *et al.* 1985), and *Salmonella* (Williams & Benson 1978) have shown the ability to survive in re-used litter under experimental conditions.

In Australia, excess poultry litter is stock piled in the environment in some states such as South Australia. This could be of concern due to potential pathogen run-off (Bicudo & Goyal 2003) during heavy rain or flooding. Composting is a simple process used to treat livestock waste (Imbeah 1998). Not all solid waste undergoes treatment due to the cost associated with practices such as composting and thus can be stock-piled in the environment. In contrast, poultry litter has been used as a nutrient supplement for vegetable growing in Australia (Jaeger *et al.* 2003). It is also possible that pathogens associated within the poultry waste can contaminate soil/crops when used for agriculture (Bolan 2004).

Re-use of chicken litter across sequential poultry farming cycles has been adopted in Australia, with the possibility of pathogen transfer to birds in subsequent farming cycles. In-house composting of poultry litter does occur overseas and studies have looked at pathogen survival in litter piles intended to be re-used for the next farming cycle (Macklin *et al.* 2006). They have shown that in-house composting of covered litter piles can reduce bacterial counts. Solid waste is not the sole concern for pathogen transfer, the farming practices themselves can be a source of pathogen movement to the surrounding environment

Litter is the key by-product of poultry farming and could be a valuable resource for agriculture. In a comprehensive review to the Australian Rural Industries Research Development Corporation following a workshop on litter re-use in the State of Queensland in 2007, (Dorahy 2007) identified issues linked to re-use of litter as a fertilizer. Dorahy 2007 identified the key users of litter across Australia, being food/horticultural crops (617,000 m^3), followed by pastures (410,000 m^3) and broadacre (213,500 m^3) as listed in table 1. In addition, litter is also used for food crops, thus the issue of pathogens is a primary concern. Across the country composting litter is seen as expensive and thus the litter can be used either directly or aged for 3 – 12 months. In addition some horticultural products such as dynamic lifter (a commercial litter by-product) also use poultry litter as a base.

Table 1 Estimates of area (ha) treated with poultry litter; rates at which it is applied (m^3/ha); typical prices ($/$m^3$) end users pay for poultry litter; and maximum distance (km) poultry litter is usually transported in broadacre, pasture and horticultural/ food crop markets. (Dorahy 2007)

State	Area treated (ha)	Rates applied (m^3/ha)	Quantity (m^3)	Value ($/$m^3$)	Value ($)	Transport limit (km from source)
Food/ horticultural crops						
Qld	3,500	60	200,000	20	4,000,000	150
NSW	10,000	30	300,000	15	4,500,000	50
SA	Unknown		77,000	18	1,386,000	100
WA	1,000	25	40,000	20	800,000	200
Total			617,000		10,686,000	
Pastures						
Qld	20,000	5 to 80	200,000	20	4,000,000	120
NSW	14,000	15	210,000	18	3,780,000	100
SA	Negligible					
WA	Negligible					
Total			410,000		7,780,000	
Broadacre						
Qld	Negligible					
NSW	14,000	7	90,000	12.5	1,125,000	150
SA	23,000	4	73,500	9.5	698,250	100
WA	8,000	6	50,000	18	900,000	
Total			213,500		1,598,250	
Total across all market segments			**1,240,500**		**20,064,250**	

Notes: (Qld – Queensland, WA – Western Australia, SA – South Australia)

Qld:

- Macadamias, avocados, viticulture, strawberries, rhubarb, vegetables, turf are the main horticultural markets
- Approximately, 50% litter is applied in South East Queensland
- Lockyer Valley water is an issue and soils v. fertile. 1 grower is using composted litter but he produces the compost himself
- Value of raw litter is $0-20/m3 whilst composted litter ranges from $20-40/ m3
- Limit of transport inversely proportional to application rates i.e. the closer to source the higher the application rate
- Negligible amounts going into broadacre

- In Condamine Catchment 250,000 ha are treated with cattle manure (220,000 t) so is there potential for substitution (main barrier is transport and distance from source.

WA:

- Weed seeds in litter is an issue
- Stopped using raw litter in potatoes because it rots the crop. Litter is applied to soils during the rotation crop in potato production.

SA:

- Viticulture and oranges are the key horticultural crops.
- Vegetable producers applied litter at high rates and overloaded the soils which lead to yield decreases and imbalanced C/N ratios. Consequently, litter isn't applied to vegetables in SA any longer.
- The proportion of litter going into broadacre is growing.

The use of litter in the environment has the potential for the spread of pathogens to contaminate water courses via run-off. Similarly during the land application of biosolids aerosolised microorganisms were observed (Brooks *et al.* 2005b). In Australia, litter has to leave the farm within short period time (a few days) prior to the next cycle and thus tends to be stockpiled in the environment. This is a biosecurity issue from both a perspective of storage and future use.

In Australia, the majority of the poultry operations are mechanically ventilated and this can result in large volumes of air (with biological material) (Vaicionis *et al.* 2006) transferring to the immediate environment. Additionally aerosols can also be a source of odours (Schiffman 1998) and settled dust (causing respiratory concerns) (Banhazi *et al.* 2008b). These sheds are concentrated close to urban areas and dispel large quantities of air to the neighbouring surroundings causing concern from neighbouring populations. Poultry are a reservoir for key food-borne pathogens such as *Salmonella*, *Campylobacter* and also dust (Clark *et al.* 1983) there is thus a potential for these pathogens to travel via dust particles to the surrounding aerosol environment (Patterson & Adrizal 2005). There is an urgent need to provide pathogen data to assist regulators and councils to address the challenges associated with the growth of the industry in urban areas. There is also a need for risk reduction and management of such bioaerosols originating from intensive animal operations (Millner 2009).

Modern poultry sheds are mechanically ventilated (to create air movement in sealed environments) to create a climate-controlled atmosphere and can contain around 40 – 50 thousand birds per shed (with four to eight sheds on a typical farm). Thus

large volumes of air are transferred to the external environment. Pathogens linked with litter also can be transferred via aerosols generated during farming.

1.1.3 The egg industry

The Australian egg industry consisted of a flock size of 22,522 million (pullets and layer hens) that produced 392 million dozens of eggs in 2011, either by cage, free range or barn production systems (Anon 2012). This overall production system generates waste in the forms of faeces and egg waste and carcasses (spent hens and daily mortalities). These wastes are composted within layer farms. Composting has shown to eliminate or reduce pathogens such as *Salmonella* and *E. coli* in poultry waste (Das *et al.* 2002). Composting of layer manure (and litter) to reduce microbes is a more efficient and shorter practice than aging (of waste) (6 weeks compared to 6 months) (McGahan *et al.* 2006). Bioaerosols arising from the use of animal manures can also contain aerosolized microbial pathogens (and other pollutants) (Pillai 2007).

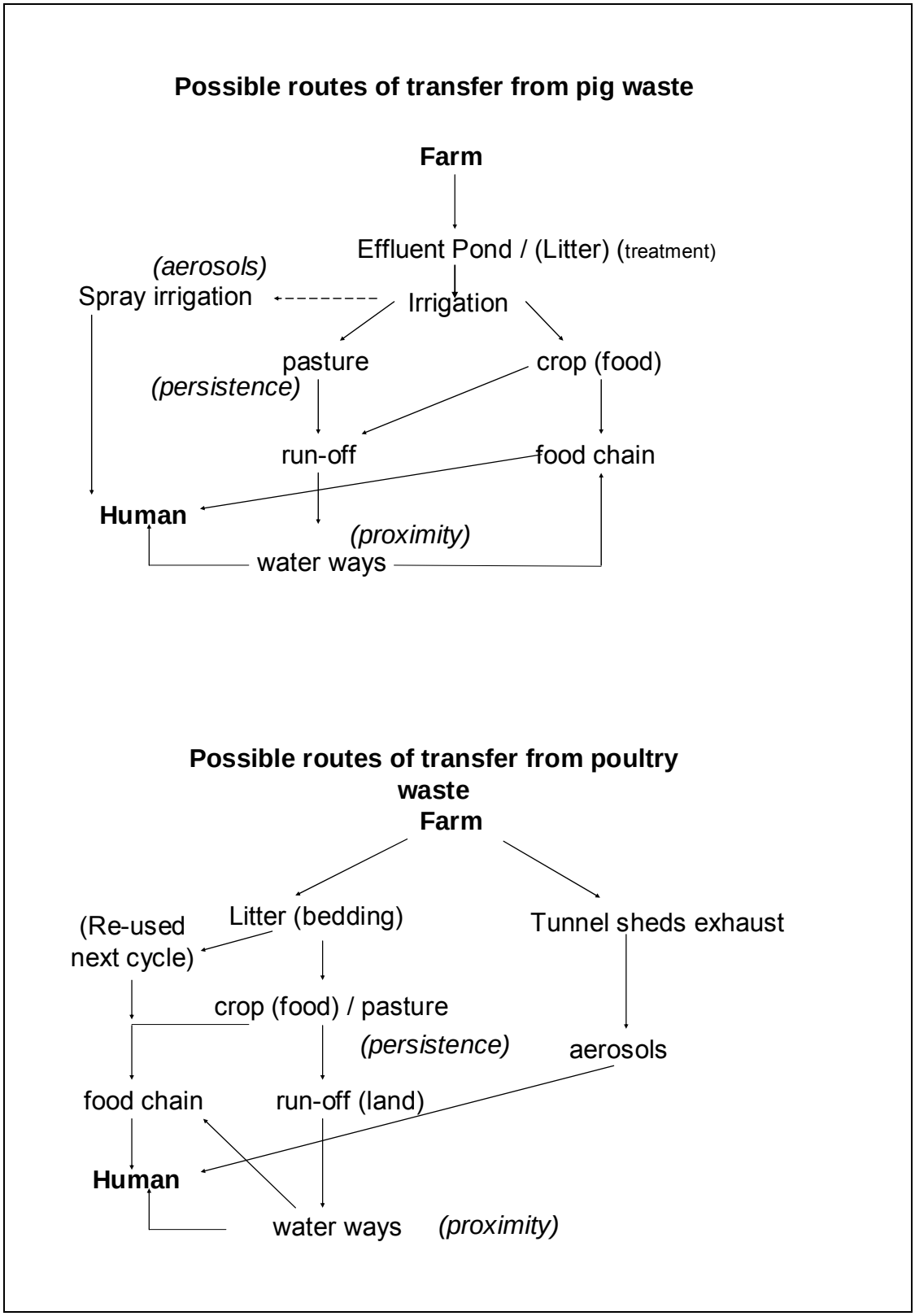
1.2 The food chain and intensive farming

The proximity of urban population to farms, the treatment of animal waste (either on or off-farm), and the use of animal waste in agriculture can all have impact on the movement of pathogens to the food process chain via various routes (Spencer & Guan 2004). These microbes can also adapt to surviving in the environment, grow, and produce toxic compounds that impact on human health (Havelaar *et al.* 2010). *Salmonella* can survive through various points in the food is chain due to its ability to respond effectively to environmental challenges and thus unlikely to ever be eradicated (Humphrey 2004). Thus when *Salmonella* transfers from animal waste to the environment the risk of further contamination largely depends on its ability to survive in manure, soil, fresh water and agricultural produce (such as both in /on plants) (Jacobsen & Bech 2012).

In contrast *Campylobacter* genus with several species is a relatively new zoonotic pathogen and as a consequence there is still the need to understand the behaviour (and pathogenicity) from a food industry/food-safety perspective of this key organism (Humphrey *et al.* 2007). Thus the movement of food-safety pathogens can have both a direct impact on humans as a result of transfer via various sources.

Figure 2 illustrates the possible pathways to the food chain originating from pigs and poultry farms.

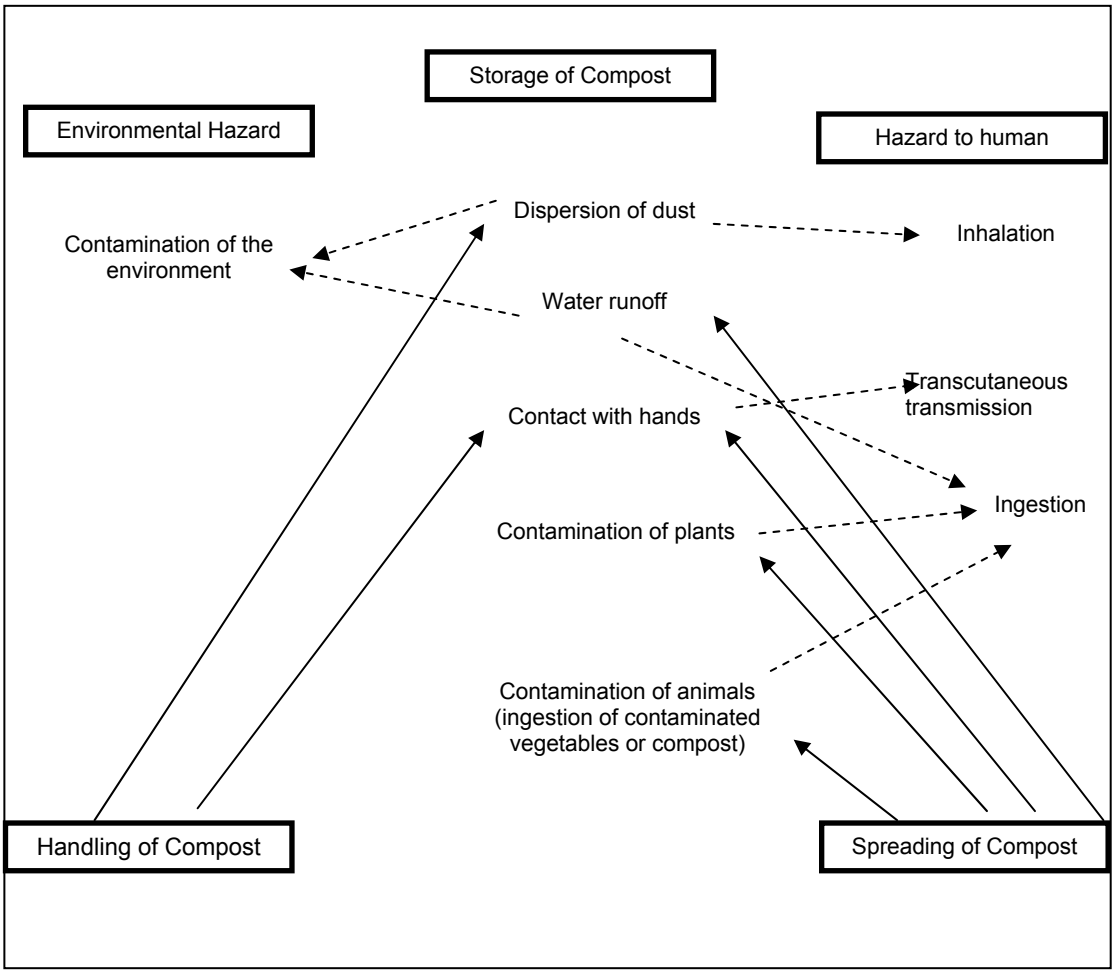
Figure 2 Movement of pathogens within pig and poultry farming operations



1.2.1 Pathways of entry and pathogen survival

There are a number of environmental pathways pathogens can gain entry to the food chain. Figure 3 illustrates potential environmental transmission routes from a composted waste product (Déportes *et al.* 1995). There are both "direct" and "indirect" pathways for possible pathogen entry into the food chain. A comprehensive review by Jamieson *et al.* (2002) identified the application of animal manures to tilled drained land, the subsequent transport of pathogens to both subsurface drainage and to surface water systems, to be the major pathogen transport pathway. The environment factors that can influence faecal bacterial survival are moisture, soil type, temperature, pH, manure application rate, nutrient availability, and microbial competition with optimal bacterial survival in cool, moist environments (Jamieson *et al.* 2002).

Figure 3 Possible pathogen pathways – adapted from Déportes *et al.* 1995



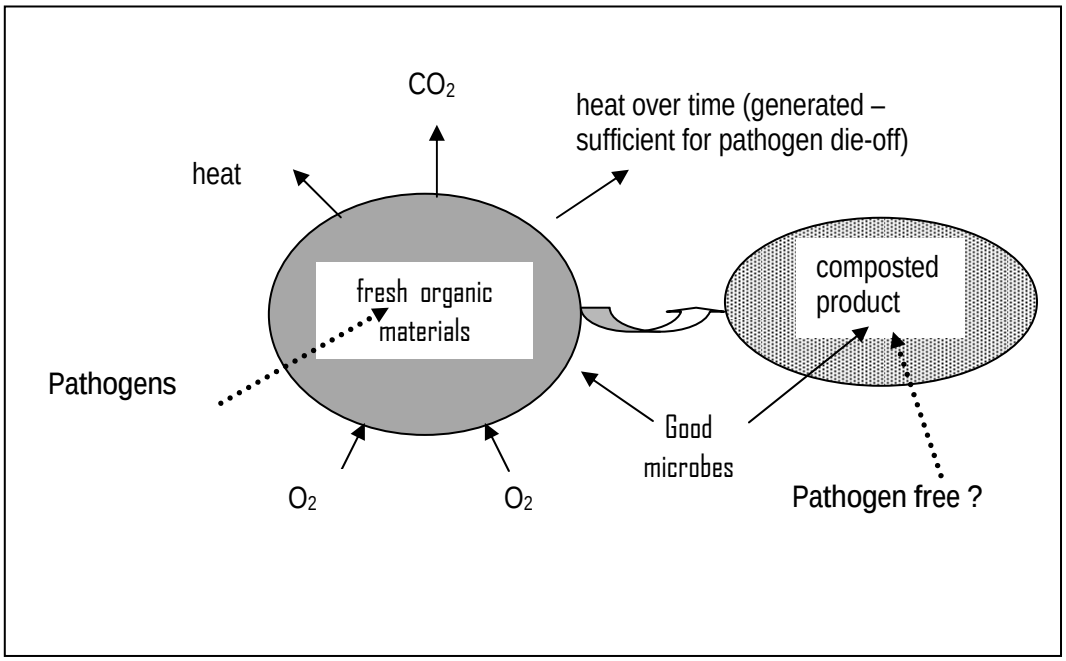
Treatment practices aimed at reducing pathogen levels can contribute to reduced levels (or numbers) of organisms transporting via different pathways to the food chain. Sobratee *et. al*. (2008), used a risk scenario based approach to assess the quantitative exposure of root crops to indicator enterobacteria originating from composted spent broiler litter. The study emphasised the fact that different treatments or treatment stages contributed to the extent of pathogens (or i.e. numbers of organisms) surviving during the various treatment stages prior to re-use of such waste in the environment (Sobratee *et al.* 2008).

1.1.2 Composting pig or poultry waste and pathogens

Several studies have provided a comprehensive understanding on composting. These studies (Tiquia *et al.* 1996, Imbeah 1998, Tiquia and Tam, 2000) have discussed with the key composting parameters that are associated with normal microbial activity that drives successful composting of piggery waste. Among these parameters, the temperatures achieved (i.e. around 55^{o}C) in a compost pile and the duration of such temperatures influence pathogen die-off (Wichuk & McCartney 2008). In addition, competitive exclusion of the pathogens driven by microbial interactions (or diversity) within such piles also plays a contributory role (Ryckeboer *et al.* 2003, Déportes *et al.* 1995).

Composting can deal with either direct waste such as litter, or mortalities that occur from time to time in piggery or layer facilities. Kelleher *et al.* (2002), in a comprehensive review on poultry waste disposal strategies, discussed various co-composting options (i.e. the use of other material along with poultry waste) which can result in a successful composted product. Whilst the composting process has been used for a long time, the on-going waste generated at a producer level has highlighted the need for environmentally sound and safe composting systems. Poultry carcasses resulting from death by natural occurrences represent a large amount of organic matter that requires environmentally and biologically safe disposal (Blake 2004). Poultry waste can be fully utilised as valuable resources for soil amendment in agricultural applications. Composting is a microbe driven process and contributes to the elimination of pathogens (Figure 4).

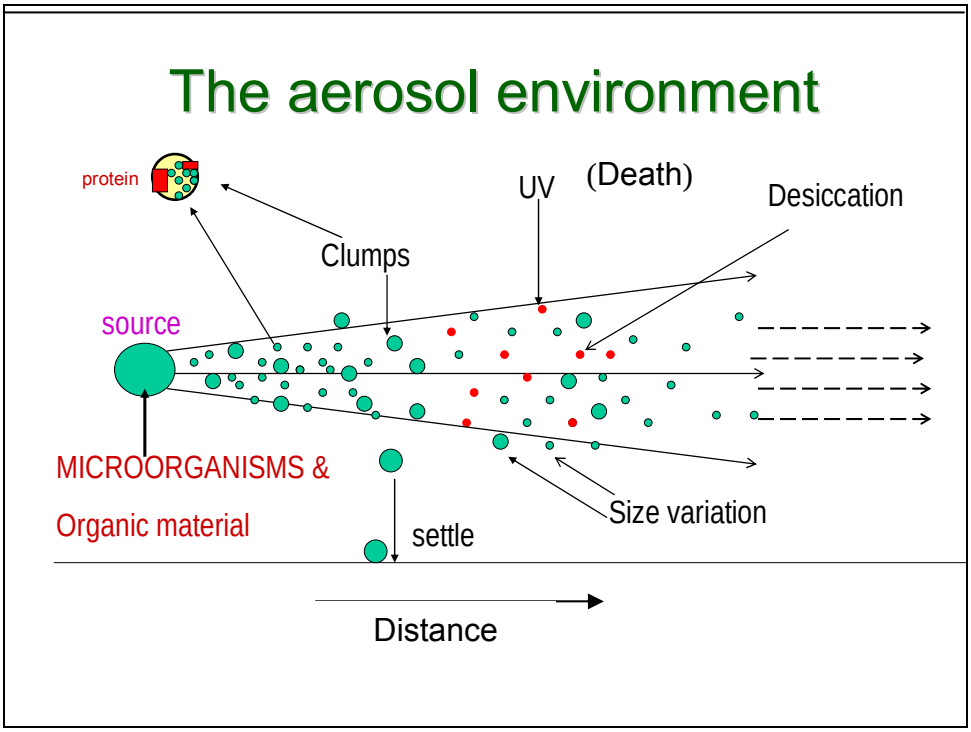
Figure 4 Summary of the composting process for animal wastes



1.1.3 Aerosols, transport and survival of pathogens

Various types of aerosols from farming can be a source (fans, dust or the farm environment) of pathogens. Pathogen survival in different types of aerosols is dependent upon pathogen concentration as well as meteorological conditions at the time (Camman 1980). Bacteria travel within particles in the atmosphere (Lighthart 2000) and such particles can originate from either effluent or litter. Microorganisms under go an initial shock immediately after aerosolisation that contributes to microbial decay over time (Lighthart & Frisch 1976). Survival relates to the degree of aerosolization (of the source material) and other factors such as wind velocity, relative humidity and temperature when travelling in the atmosphere (Lighthart 1999). Several mathematical models (such as the Gaussian plume model) predict bacterial die-off in the atmosphere (Lighthart & Kim 1989, Lighthart & Mohr 1987). Studies have been carried out to determine microorganism survival in aerosols following spray irrigation (Donnison *et al.* 2004) and in aerosols generated by land application of sewage sludge (Pillai *et al.* 1996). Figure 5 illustrates pathogen movement and survival in an aerosol environment.

Figure 5 – Movement and survival of pathogens in the aerosol environment



Aerosols can also be produced and spread during spray irrigation. Aerosolized coliforms were detected when their levels were 10^3/ml or more in waste water (Teltsch & Katzenelson 1978). Thus the pathogen levels in waste water are also a vital factor in relation to spray irrigation. A study estimating pathogen levels in aerosols found faecal coliforms levels from sprinkler irrigated waste water were above background bacterial levels at distances of 730m at night (in 100% of the samples) and daytime (in 67% of the samples), (Shuval *et al.* 1989). Teltsch *et al.* (1980) determined the bacterial die-away constant λ using labelled *E.coli* where a λ of $6.6 \times 10^{-2} s^{-1}$ was observed in the afternoon compared to $8.8 \times 10^{-3} s^{-1}$ for early morning indicating that the time of the day influenced the levels of in *E.coli* aerosols. Along with bacteria, viruses could also be present in wastewater. Enteric viruses were found in 10% of the samples with counts ranging from 0.03-1.94pfu/m^3, (Shuval *et al.* 1989). Such observations can contribute to the risk management of wastewater spraying regimes. The presence of bacteria and viruses in aerosols could be a result of them surviving best at high relative humidity.

1.3 Food-safety pathogens and the environment

Farming at large scale can have an impact on the survival and movement of key food and water-borne pathogens such as *Campylobacter* and *Salmonella* spp. In Australia the number of notifications for salmonellosis for 2010 was 10,666 cases and campylobacteriosis was 14,872 cases, (Table 2) (Anon 2011b), though not all of these cases can be attributed to poultry. However, the poultry industry is viewed as the major contributor due to the close association of *Salmonella* and *Campylobacter* with poultry.

Table 2 Number of notifications for all diseases by year, Australia, 2000 - 2010

Gastrointestinal diseases	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Botulism	2	2	0	1	1	3	1	1	0	1	0
*Campylobacter*iosis	13674	16110	14712	15367	15579	16488	15434	17004	15533	15978	14872
Cryptosporidiosis	NN	1627	3263	1221	1676	3211	3202	2809	2003	4623	1357
Haemolytic uraemic syndrome (HUS)	17	3	13	15	16	20	14	19	31	13	8
Hepatitis A	809	538	391	430	319	327	281	165	276	564	251
Hepatitis E	12	14	12	10	30	30	23	19	44	33	34
Listeriosis	67	64	62	69	67	54	61	50	68	91	63
STEC, VTEC	37	46	59	52	49	86	70	106	106	154	70
Salmonellosis	6189	7035	7868	6999	7841	8424	8244	9536	8304	9526	10666
Shigellosis	488	566	503	442	520	729	546	600	828	623	504
Typhoid Fever	58	74	70	50	74	52	77	90	105	116	88

NN – non-notifiable

1.3.1 Key food-borne pathogens poultry and pigs

These pathogens can be present in poultry (or pig) waste and can transfer via improperly treated manure or waste to the food chain. Food-borne pathogens such as *Campylobacter, Clostridium perfringens* and *Salmonella* are all associated with poultry and poultry waste (Shane 1992, Limawongpranee, *et al* 1999, Chalmers, *et al.* 2008). Pig waste can also be a source of both *Salmonella* (Henry *et al.* 1995) and *Campylobacter* (Weijtens *et al.* 1997).

Listeria monocytogenes, commonly linked with the environment, can also be associated with the free range poultry production systems (Esteban *et al.* 2008). *Arcobacter* spp., considered as emerging pathogens are linked to both poultry (Wesley & Baetz 1999) and pigs (Van Driessche *et al.* 2004). This section discusses the importance of these organisms from a public health point of view. The common *Campylobacter* spp. associated with poultry are *C. jejuni* and *C. coli.*

Clostridium botulinum, though of concern for humans is more of concern in cattle in Australia, due to ingestion of improperly composted poultry source material (Bongers *et al.* 1988). There are reported cattle mortalities in Australia as a result of consuming poultry litter (Trueman *et al.* 1992) due to toxin formed.

A summary of pathogens of primary and secondary concern have been categorised in Table 3.

Table 3 Food-safety pathogens of environmental concern

Organisms of primary importance

Organism	Reason for consideration
Salmonella	• Ability to survive in the environment • Common association with poultry • One of the major food-borne pathogens • Key serovars linked with egg related outbreaks in Australia
Cl. botulinum (Cattle)	• Good survivor in the environment • Spore former • Cattle mortality – chicken waste, associated with previous toxin linked outbreaks in Australia • Chicken – chicken waste link
E. coli	• A common indicator organism

Organisms of secondary importance

Organism	Reason for consideration
Campylobacter	• Poor survivor in under different environmental conditions • Poor survival during composting • Common association with poultry • One of the major food-borne pathogens • Prevalent in farmed barn, cage, free range birds
Cl. perfringens	• Good survivor in the environment • Spore forming ability contributes to good survival • Chicken – chicken waste link • A food-borne pathogen
Listeria monocytogenes	• Present in the environment • Possible link with free range poultry • A food-borne pathogen
Arcobacter	• An emerging food borne pathogen • Prevalent in broilers (Houf *et. al.* 2002)

1.3.2 *Salmonella*

The genus *Salmonella* is widely distributed in nature and is associated with the intestinal tract of mammals, rodents and reptiles. Salmonellosis is linked to both public and animal health and the organism can be isolated from the intestines of both humans and animals. *Salmonella* are motile bacteria and belong to the family Enterobacteriaceae along with *E. coli*. Currently, there are two recognized species:

S. enterica and *S. bongori*, with six main subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). (Brenner *et al.* 2000). Serovars such as *Salmonella enterica* subsp*. enterica* serovar Typhi are only linked to humans (Jay 1978) whilst *Salmonella enterica* subsp*. enterica* serovar Gallinarum and *Salmonella enterica* subsp*. enterica* serovar Pullorum (Callaway *et al.* 2007) are associated with poultry.

The *Salmonella* terminology through the rest of this thesis will be referred to based on the following example: *Salmonella enterica* subsp*. enterica* serovar Pullorum will be as follows: The genus (*Salmonella*) and the serovar (e.g. Pullorum), i.e. *Salmonella* Pullorum for the first time and from then onwards will be referred to as *S*. Pullorum.

There are around 2252 *Salmonella* serovars which can be catogorised as "pathogenic" in man and animals, "rarely pathogenic" i.e. those infrequently associated with infection but isolated from various sources, or "environmental", i.e. those that are found in the environment but rarely in animals (Murray 1991). Amongst all food producing animals, poultry is a common source of the organism (rather than pigs). Whilst some of these serovars are asymptomatic in poultry they can be of significance to humans causing illness. *Salmonella* is also closely linked with poultry farming environments (Davies & Breslin 2001).

The organism is shed in faeces and can contaminate soil, pasture, streams and lakes, which serve as the source of organisms to colonise other animals (Jay *et al.* 2003). The organism also can survive in soil for months (Jay *et al.* 2003). *Salmonella enterica* subsp*. enterica* serovar Newport survived for 184, 332, and 405 days in manure, manure-amended non sterilized soil and manure-amended sterilized soil, respectively, under experimental conditions (You *et al.* 2006). From an Australian perspective the dominant serovar isolated from human outbreaks in 2005 was *S. enterica* subsp*. enterica* serovar Typhimurium (Owen *et al.* 2007). Table 4 lists the top ten serovars isolated from outbreaks and some serovars such as *S.* Typhimurium, *S.* Virchow and *S.* Chester are associated with poultry.

Table 4 Top 10 human isolates of *Salmonella*, Australia, 2005, by state or territory (Owen *et al.* 2007)

Organism	State or territory								Total
	ACT	NSW	NT	Qld	SA	Tas	Vic	WA	%
S. Typhimurium 135	14	188	1	135	23	175	198	68	16.6
S. Typhimurium 197	1	113	0	140	5	2	280	4	11.3
S. Typhimurium 170*	14	328	0	48	3	6	64	9	9.8
S. Saintpaul	3	42	48	271	13	2	24	33	9.0
S. Typhimurium 9	11	155	5	33	57	10	124	11	8.4
S. Virchow 8	2	28	10	182	6	1	7	12	5.1
S. Typhimurium 44	6	67	0	59	28	6	53	9	4.7
S. Birkenhead	0	85	0	128	0	0	6	1	4.5
S. Chester	1	30	14	87	14	1	10	29	3.8
S. Hvittingfoss	5	23	5	129	1	0	19	3	3.8
Sub-total	57	1059	83	1212	150	203	785	179	77
Other isolates	6	217	35	370	90	63	134	197	23

Source: National Enteric Pathogenic Surveillance System.
* Reported as *Salmonella* Typhimurium phage type 108 in some states and territories.

Thus, *Salmonella* is of concern during the re-use of animal waste such as composts. Whilst thermophilic temperatures achieved during composting contribute to *Salmonella* reduction, the organism can grow in litter, manure and adjust to dry environments (Eriksson De Rezende *et al.* 2001). Due to its ubiquitous nature, *Salmonella* is not likely to be eradicated from the food chain (Humphrey 2004). Thus simple measures, such as improved on-farm biosecurity and the proper storage and use of animal excreta as fertiliser could greatly improve animal health and food-safety (Humphrey 2004).

1.3.3 *Salmonella*, the broiler and layer industry

Salmonella is linked to both broilers and layers. In broilers the most important source of contamination is the resident *Salmonella* of the flock i.e., the strain isolated on chicks' first day in the poultry house rather than the serovars isolated during the rest of the rearing period (Lahellec *et al.* 1986). The serovar dominance in broilers can vary from country to country. For example in the European union, (27 states) the most common serovars in 2009 reported for broilers were *S. enterica* subsp*. enterica* serovar Infantis *(*29%), *S. enterica* subsp*. enterica* serovar Enteritidis (13.6 %), and *S. enterica* subsp*. enterica* serovar Kentucky (6.2 %). *S.* Typhimurium (4.4), *S. enterica* subsp*. enterica* serovar Bredeney (4.3%) and *S. enterica* subsp*. enterica* serovar Virchow (4.1%) respectively (Anon 2011a). In Australia the dominant serovar isolated from broilers in 2009 was *S. enterica* subsp*.*

enterica serovar Sofia (36.6%) followed by *S*. Typhimurium (13%) and from layers it was *S*. Typhimurium (28.3%) followed by *S*. Infantis (18.3%) (Anon 2009b).

Salmonella outbreaks are likely to occur from consumption of undercooked contaminated broilers or undercokked or raw eggs. Studies have shown that that the likelihood of *Salmonella* contaminated carcasses entering the processing stage was associated with the higher contamination of birds and shed (at harvest and prior placement of chicks) (Volkova *et al.* 2009). In Australia, *Salmonella* Typhimurium has been reported in both layers and eggs. One of the largest egg-associated outbreaks of food borne illness in Australia for many years occurred between June and December 2005. Five outbreaks of *S.* Typhimurium phage type 135 were identified in Tasmania, leading to 125 laboratory-confirmed cases. Foods containing raw egg or contaminated foods were the possible vehicles for infection. Such outbreaks are common (Anon 2011b).

Table 5 lists the *Salmonella* serovars associated with broiler and egg layers in Australia.

Table 5 Most common *Salmonella* serovars in Broiler and Layers (2009) Source – Australian *Salmonella* Reference Centre – 2009 Annual Report,

Chicken broilers		**Chicken layers**	
S. Sofia	36.6 (%)	*S*. Typhimurium	28.3 (%)
S. Typhimurium	13 (%)	*S*. Infantis	18.3 (%)
S. Kiambu	12.5 (%)	*S*. Mbandaka	5.4 (%)
S. Agona	4.8 (%)	*S*. Singapore	5.4 (%)
S. Infantis	3.3 (%)	*S*. Kiambu	4.7 (%)
S. Muenchen	2.7 (%)	*S*. Agona	4.4 (%)
16:1v:-	2.7 (%)	*S*. Ohio	3.7 (%)
S. Montevideo	2.3 (%)	*S*. Zanzibar	3.5 (%)
S. Ohio	2.2 (%)	*S*. Cerro	2.3 (%)
S. Singapore	2.1 (%)	*S*. Virchow	2.3 (%)

(% = percentage of total isolates submitted)

Salmonella's route to waste from broilers is largely via the bedding or litter which is re-used. The prevalence and levels of *Salmonella* associated with the production environment (i.e. farm) will influence the extent of contamination of the waste.

Contamination by *Salmonella* can occur from layer flocks or eggs) (Poppe *et al.* 1992), manure (Himathongkham *et al.* 1999b) and mortalities or spent hens (Liebana *et al.* 2003). All of these waste materials are finally destined for on-site composting with the potential risk for *Salmonella* to re-enter the sheds/farm due to its survival. Survival of environmental stress is *Salmonella* strain dependent both in the slaughter house and the external environment (Someya *et al.* 2005). This suggests that some serovars may be selected over the others in the waste with potential for further transmission via the environment.

1.3.4 *Salmonella* on-farm biosecurity

There is potential for *Salmonella* to re-enter the sheds or transfer to the environment. Rodents (Lapuz *et al.* 2007, Kinde *et al.* 2005), litter beetles (Skov *et al.* 2004), wild birds (Davies & Breslin 2001), ground beetles and centipedes (Davies & Breslin 2003) have been associated with *Salmonella* in operations such as cage layer flocks, barn egg production, free range flocks and egg processing facilities. Various *Salmonella* serovars were also isolated from different fly species from cage and layer facilities, while *S*. *enterica* subsp*. enterica* serovar Mbandaka was isolated from a beetle species from manure a pit beneath the house (Olsen & Hammack 2000) Free-range chickens have access to outside soil and water, which could provide exposure to additional vectors of infection (Wales *et al.* 2007). Thus, there is a need for biosecurity measures to be adopted with on-farm composting. Failure to manage wildlife vectors is likely to negate even the most effective cleaning and disinfection regimes in layer and broiler houses (Wales *et al.* 2006).

1.3.5 *Campylobacter*

Campylobacter species are found in the intestinal tracts of a wide range of both domestic and wild animals that show no sign of disease (Wallace 2003). *Campylobacter* species colonise the intestines of poultry and are responsible food-borne enteritis in humans (Shane 1992). *C. jejuni* is the most significant of the three thermophilic Campylobacters with poor growth under aerobic conditions (Wallace 2003). Birds and in particular, poultry are known to be the primary reservoir for *C. jejuni* with the organism generally being considered to be a commensal that has evolved to grow at 42^{o}C due to the long association with the avian gut (not 37^{o}C as in the mammalian gut) (Park 2002). Campylobacters are ubiquitous in nature and large environmental reservoirs of *Campylobacter* are present in avian populations as a part of their commensal microflora (Carrillo *et al.* 2007). A common vehicle for

infection is contaminated poultry meat and carcasses can carry more than 10^8 *Campylobacter* cells per carcass (cited in Humphrey *et al.* 2001).

Park (2002) provides a succinct summary of the nature of this major food and water borne pathogen, *Campylobacter*.

- has fastidious growth requirements
- has an unusual sensitivity to environmental stress compared to other food borne pathogens,
- lacks many of the well characterised adaptive responses associated with resistance to stress in other bacteria
- has a minimal capacity for recognising and responding to stress
- lacks the ability to grow below 30^{o}C
- is sensitive to heat (though able to grow at 42^{o}C)
- is very sensitive to dessication
- is readily inactivated by pasteurization treatments and domestic cooking processes

In spite of all these factors, *Campylobacter* can persist in the food chain and in doing so remains the most common cause of bacterial food-borne illness (Park 2002). However the belief that *Campylobacter* is a sensitive organism is based on laboratory studies and *Campylobacter*s may be more robust than previously thought representing a greater challenge to food-safety (Humphrey *et al.* 2007).

1.3.6 *Campylobacter* and chickens

The incidence of *Campylobacter* species in farmed poultry can vary from 0 -100%, depending on such factors as the age of the bird, natural resistance and hygiene (Wallace 2003). An epidemiological investigation into Austrian broiler flocks over a 3 year period demonstrated a dominance of *C. jejuni* (88%) over *C. coli* (12%) (Neubauer *et al.* 2005). Generally broilers show infection at 2-3 weeks of age and within a week, all birds within a flock are infected and remain infected until slaughter. Layers show a similar pattern of infection but the isolation rate from the birds begins to fall from around 12 weeks of age to about 30% after a year (Wallace 2003).

1.3.7 Biosecurity measures for *Campylobacter*

The biosecurity measures in terms of vectors adopted for *Salmonella* can also be effective for control of *Campylobacter*. There is a potential for manure / compost piles to contribute to pathogen transfer via vectors, depending on the role played by

the various vectors in pathogen transmission. More specifically the following play a role in the management of *Campylobacter* in a production system:

- Litter beetles may harbour *Campylobacter;* however, this were considered not to have the ability to transfer from one flock to another (Skov *et al.* 2004)
- Flies have been shown to be a source of *Campylobacter* (Gregory *et al.* 1997)
- Cattle in surroundings are a source of *Campylobacter* (Gregory *et al.* 1997)
- Manure heap location; A study assessing the risk factors (and prevalence) of *Campylobacter* in chicken flocks raised on-farm (amongst other risk factors) showed a 5.2 higher risk for the odds of *Campylobacter* colonisation when a manure heap was located >200m from the poultry farm (Arsenault *et al.* 2007).

Overall, measures adopted for vector control is to create barriers or minimise odours.

1.3.8 *Campylobacter* and pigs

Pigs excrete *Campylobacter* at levels of 10^3 -10^7 CFU/g) faeces) from the age of 8-13-weeks of age (Jensen *et al.* 2006). *Campylobacter* spp. (both *Campylobacter jejuni* and *Campylobacter coli*) were isolated from outdoor organically raised pigs and *C. coli* was isolated in the outdoor paddocks (Jensen *et al.* 2006). *Campylobacter lanienae* is also associated with pigs (Sasaki *et al*. 2003)

1.3.9 *Clostridium botulinum* – general background

The organism was first isolated in 1895 by Van Ermengen who named the organism *Bacillus botulinus* (Jay 1978). *Clostridium botulinum* is responsible for botulism, a fatal illness in both humans and animals (Jay 1978). *Cl. botulinum* is widely distributed in environmental sources such as soil (Smith 1975, Wobeser *et al.* 1987) and aquatic environments (Segner *et al.* 1971, Huss 1980).

Cl. botulinum is an anaerobic spore-forming organism with spores that are able to survive in the environment for decades (Mitscherlich and Marth 1984, cited in Böhnel *et al.* (2002) and under most environmental circumstances e.g. dry heat (Critchley 1991). The ingestion of a highly toxic, soluble exotoxin produced by the organism is responsible for the symptoms of food-borne botulism (Jay 1978). This exotoxin, also known as botulinum neurotoxin, differs depending on the different

serotypes. Toxins formed within the organisms release during autolysis. These toxins are among the most toxic substances known to man with a mouse LD_{50}/mg of 30,000,000 mouse units (Jay 1978).

1.3.10 *Clostridium perfringens* in poultry

Food poisoning strains of *Cl. perfringens* are present in soil, water, foods, dust, the intestinal tract of humans and other animals (Jay 1978). *Cl. perfringens* is also a spore-forming organism and is able to withstand conditions such as drying and heating. The organism plays a role in various disease conditions, such as necrotic enteritis of chickens (McCrea & Macklin 2006). However the organism has also been isolated from healthy broiler chickens (Chalmers *et al.* 2008).

The organism also caused wound infections and food poisoning outbreaks in humans (Niilo 1980). The optimal growth temperature is between 37 – 45^{o}C with the ability to grow at pH range of 5.5 - 8.0 (Jay 1978). *Cl. perfringens* has been isolated from 96% of the composts in Greece (Lasaridi *et al.* 2006), indicating the organism has the ability to survive the composting process.

1.3.11 Biosecurity measures for *Cl. perfringens*

Cl. perfringens is a faecal indicator and has been linked to dirty transport containers and thus can potentially contaminate subsequent poultry flocks (McCrea & Macklin 2006). This observation can be related to any surface areas or equipment that is used in a composting operation. The use of pressure washing, with sodium hypochlorite spray and quaternary ammonium spray with 48-h drying showed a 2 to 3 log10 cfu/mL, bacterial reduction after 48 h of drying (McCrea & Macklin 2006).

1.3.12 *Listeria monocytogenes*

Listeria monocytogenes is an environmental organism commonly found in moist and cool environments. Widely found in nature, *L. monocytogenes* is carried by wild and domestic animals and is commonly associated with soil (cultivated, uncultivated soils, mud and moist soils) (Sutherland *et al.* 2003). *L. monocytogenes* was able to grow for a period of 2 days in fresh chicken manure at 20^{o}C and continued to survive when the storage time was prolonged to 6 days (Himathongkham & Riemann 1999). These changes were accompanied by an increase in pH and an accumulation of ammonia in the manure (Himathongkham & Riemann 1999), both of which can have antibacterial properties.

Listeria survived for up to three months in stored animal slurries and dirty water and less than one month in solid manure heaps at temperatures greater than 55^{o}C. Following manure spreading to land, *Listeria* survived in sandy arable and clay loam grassland soils for more than one month (Nicholson *et al.* 2005a). In composted rural sewage sludge, *Salmonella* decayed at a rate greater than *Listeria* (Pourcher *et al.* 2005). *Listeria* thus appears to be more stable than *Salmonella* in some environments.

L. monocytogenes was present in 4-week-old seeded in-vessel biowaste composts (under experimental conditions) and absent in older composts suggesting that proper composting may prevent long-term survival (Lemunier *et al.* 2005). Faecal wastes (poultry manure and dirty water) inoculated with *L. monocytogenes* (indicative of normal levels) and spread on to grass pasture resulted in the organism surviving for 42 (in poultry) and 128 (dairy cattle) days in farm yard manures (Hutchison *et al.* 2005b).

1.4 Pathogens and intensive farming, the concerns

Both chicken litter and piggery effluent tend to be re-used both within the farming system and in the external environment for various purposes. Both chicken litter and piggery effluent are used either as manures (litters) or as a source of irrigation for pasture/food crops. Such practices have resulted in on-going concerns of potential direct risks to the human food chain. Such risks could include waste coming into direct contact with food crops and to farm workers (during activities such as spray irrigation). There also could be potential indirect risk with the possibility of pathogens entering the food chain as a result of run-off to adjacent creeks and rivers during heavy rain and flooding or spread via aerosols from large mechanically ventilated operations.

Studies on the re-use of animal waste in general have been carried out in the UK (Hutchison *et al.* 2004, Hutchison *et al.* 2005c, Hutchison *et al.* 2005, Hutchison *et al.* 2005a, Hutchison *et al.* 2008) and the USA (Erickson *et al.* 2010, Stringfellow *et al.* 2010) and the work continues to progress in the USA. There is thus a need for Australian research carried under local farming conditions relevant to the farming practices adopted in this country. This study addresses such a need with the understanding that the key food-safety pathogens *Salmonella* and *Campylobacter* are closely associated with both pig and poultry farming.

Both poultry litter and piggery effluent are valuable nutrient rich resources with potential for use in agriculture. These benefits need to be balanced against the potential risks linked to pathogens that may be present in waste by-products. There is thus a need for understanding the types (and levels) of key pathogens to be able to quantify such risks via appropriate risk assessments or other means of management. Such data would thus be of use not only to the various intensive animal industries but also to the related agricultural industries that store and/or use the waste.

1.4.1 The questions

Intensive farming can be a source for the on-going transmission of pathogens and thus raises many questions relevant to food-safety and/or human illness, (irrespective of the different on and off farm pathways) in Australia. Due to a lack of Australian research in these emerging areas, these questions continue to be raised by food-safety regulators, environmentalists, the general consumer and the farming community. Answers to these questions will provide options for pathogen management in both farming and the use of farm waste in the environment. This study was designed to provide both background knowledge and contribute to the responsible re-use of animal waste, resulting in minimising impacts on food-safety, food agriculture and the environment. The environment is both the source (water and bedding material such as wood shavings) and ultimately receipient of the potentially contaminated waste material. This continuous cycle occurs in both pig and poultry farming.

In the past, the answers to these questions and concerns were dependent on the available data from studies undertaken in other countries. However, such international data may not be relevant to Australian conditions or more specifically may not be available to address current questions associated with both Australian pig and poultry farming. Australian studies also have the potential to form the basis for future more specialised research in this area of growing importance. Overall, the current study was designed to enhance an understanding of food-safety and human illness consequence of intensively farmed pigs and poultry.

Thus, the key questions addressed in this thesis are:

- What are the types (and levels) of zoonotic pathogens present in Australian pig and poultry waste (addressed in Chapters 2 and 3)?
- What is the potential for movement of key food-safety pathogens during the re-use of both poultry and pig waste *within* the farming systems (addressed in Chapters 4 and 5)?
- What is the potential for movement of pathogens, during re-use *external* to shed environments, such as soil (addressed by studying an emerging food-safety pathogen in Chapter 6)?
- What is the potential for movement of key food-safety pathogens via aerosols because of mechanically ventilated intensive farming operations (addressed in Chapters 7 and 8)?

These questions are addressed through on-farm studies at commercial pig and poultry operations across various pig and poultry farms in the South-East State of Queensland region in Australia.

Chapter 2

Microbiological status of piggery effluent from thirteen piggeries in the South East Queensland Region of Australia

This Chapter is published in the *Journal of Applied Microbiology*

Citation:

H.N. Chinivasagam, R.J. Thomas, K. Casey, E. McGahan, E.A. Gardner, M. Rafiee and P.J. Blackall (2004). Microbiological status of piggery effluent from thirteen piggeries in the South East Queensland Region of Australia, *Journal of Applied Microbiology,* **97**: 883-891;

2.1 Summary

Aims: To assist in the development of safe piggery effluent re-use guidelines by determining the level of selected pathogens and indicator organisms in the effluent ponds of 13 south-east Queensland piggeries.

Methods and Results: The numbers of thermotolerant coliforms, *Campylobacter jejuni/coli, Erysipelothrix rhusiopathiae, Escherichia coli, Salmonella* and Rotavirus were determined in 29 samples derived from the 13 piggeries. The study demonstrated that the 13 final effluent ponds contained an average of 1.2 X 10^5 colony forming units (CFUs) 100 ml^{-1} of thermotolerant coliforms and 1.03 X10^5 CFUs 100 ml^{-1} of *Escherichia coli*. The *Campylobacter* level varied from none detectable (2 of 13 piggeries) to a maximum of 930 Most Probable Number (MPN) 100 ml^{-1} (2 of 13 piggeries). *Salmonella* was detected in the final ponds of only 4 of the 13 piggeries and then only at a low level (highest level being 51 MPN 100 ml^{-1}). No rotavirus and no *Erysip. rhusiopathiae* were detected. The average log_{10} reductions across the ponding systems to the final irrigation pond were 1.77 for thermotolerant coliforms, 1.71 for *E. coli* and 1.04 for *Campylobacter*.

Conclusions: This study has provided a base-line of knowledge on the levels of indicator organisms and selected pathogens in piggery effluent.

Significance and Impact of the Study: The knowledge gained in this study will assist the development of guidelines to ensure the safe and sustainable re-use of piggery effluent.

2.2 Introduction

In Australia, piggery effluent has been traditionally treated by removal of solids by means of screens followed by holding of the effluent in one or more anaerobic ponds (Kruger *et al.* 1995). The use of this system has developed as it is regarded as the simplest and most reliable method for biological stabilisation of piggery waste (Kruger *et al.* 1995). The treated piggery effluent is then disposed to agricultural land (Payne 1990). This practice of disposal to agricultural land is also common in other countries such as the UK (Nicholson *et al.* 2000).

There is increasing community concern about animal effluent disposal and the re-cycling of animal effluent. This increasing community concern is demonstrated by such activities as the issue of a UK government report on the risks and hazards associated with recycling effluent to land (Carrington *et al.* 1988) and the issue of a US Food and Drug Administration report on guidelines to minimise the risk of pathogen contamination of fresh fruit and vegetables (Anon 1998).

Pig faeces and urine can contain a range of pathogens (Strauch 1991). Even in a pig herd which displays no clinical signs of an illness, animals within that herd may be excreting pathogens, e.g. *Salmonella*s (Jones *et al.* 1976). Despite the increasing importance of pathogens in the re-use of piggery effluent, there is little published information on the levels of various pathogens in Australian piggery effluent (Kruger *et al.* 1995).

We selected to study the two major bacterial causes of human enteritis in Australia - *Salmonella* spp and *Campylobacter* spp. In the year 2002, there were 14,619 notified cases of *Campylobacter* infection in humans in Australia and 7,787 cases notified cases of salmonellosis, by far the two most common notifiable gastro-intestinal infections in the Australian population (http//www.health.gov.au/cda). There is knowledge that both of these pathogens are present in Australian pigs e.g. (Henry *et al.* 1995a) have reported the presence of *Salmonella* in all three piggery effluent ponds they examined. However, there is no knowledge of the levels of these two major pathogens in piggery effluent. We also selected to include the traditional indicator organisms of effluent quality – thermotolerant coliforms and *Escherichia coli*. A further two other pathogens – *Erysipelothrix rhusiopathiae* and rotavirus – were selected for study. *E. rhusiopathiae* was selected as the organism

is well recognised as a pathogen of humans (Reboli & Farrar 1989) and can be detected for up to four years in the effluent after a herd becomes free of swine erysipelas (Chandler & Craven 1980). While viruses are not likely to play a major role in human disease outbreaks linked to piggery effluent, we selected porcine rotavirus as the only virus of potential relevance.

This paper reports the current and detailed knowledge of the level of key pathogens in 13 south-eastern Queensland piggeries. We believe that effective effluent re-cycling guidelines that minimise the risk to human health due to pathogen contamination of the food chain or the general environment can only be developed if there is a detailed knowledge of the change in levels of key pathogens in the effluent being re-cycled.

2.3 Materials and Materials

2.3.1 Effluent Samples

A total of 13 piggeries were selected for inclusion in this study. The piggeries were selected to represent the overall variety in south-east Queensland pig industry. The piggeries consisted of nine farrow-to-finish piggeries (the most common style of piggery in Queensland), one was a breeder only unit, one a weaner only unit and two were grow-out only units. Brief details of the 13 piggeries are given in Table 1. Wherever possible, effluent samples were taken from the sump (ie before entry into the ponding system) secondary pond and final pond at each piggery. All treatment ponds were anaerobic ponds. All samples were collected as 5 l volumes and were transported on ice to the laboratory. The processing of the samples for the bacterial side of the work was completed within 8 h of the sample being taken. The samples were then held at 4^0C for later processing in the rotavirus work. As the rotavirus testing did not involve the use of detection test that required viable rotavirus the storage at 4^0C had no effect on this work. One piggery was sampled over time, as a means of observing any changes which may occur due to seasonality.

Table 1 Details of the 13 piggeries included in this survey

Piggery	Production System	Number of Sows	Number of SPUs*
A	Farrow to Finish	210	
B	Farrow to Finish	90	
C	Farrow to Finish	1,200	13,365
D	Farrow to Finish	1,000	9,596
E	Farrow to Finish	430	
F	Breeder Facility	2,700	4,900
G	Grow Out Facility Only	NA	9,212
H	Farrow to Finish		13,487
J	Farrow to Finish		15,477
K	Farrow to Finish	1,200	14,992
L	Farrow to Finish	1,000	12,497
M	Weaner Facility	NA	2,900
N	Grow Out Facility Only	NA	2,699

*SPU = Standard Pig Unit

2.3.2 Isolation and enumeration methods

Thermotolerant coliforms and E. coli The effluent samples were examined for the presence and number of thermotolerant coliforms and *E. coli* using the Australian Standard Method AS 4276.7 –1995 (Anonymous 1995). In brief, 1 ml volumes of appropriate serial dilutions of the effluent, in phosphate buffered saline (PBS, pH 7.2) were filtered and the membranes each placed on the surface of Membrane Lauryl Sulphate agar. The filter/medium was then incubated for 5 h at 30^0C, followed by overnight incubation at 44.5^0C. The thermotolerant coliform count was obtained by counting the number of typical colonies present on the filter. Representative colonies (the square root of the thermotolerant coliform count) were confirmed as *E. coli* using EC Broth (for gas production) and tryptone water (for indole production) simultaneously, both incubated at 44.5°C.

Salmonella A most probable number (MPN) method was used to estimate the numbers of *Salmonella*e (Hussong *et al.* 1985, Yanko *et al.* 1995). Ten 1 ml aliquots of each effluent were each inoculated into a 10 ml tube of 0.1% buffered peptone water (BPW) (Oxoid). The tubes were incubated at 36^0C for 24 h. After incubation each tube was vortexed and 1 ml was transferred to 10 ml of tetrathionate brilliant green broth (Hussong *et al.* 1984). The broths were then incubated at 43°C for 24 h. Each tube was then inoculated onto xylose-lysine-brilliant green agar (Hussong *et al.* 1985) and the agar plate incubated at 36°C for 24 h. Presumptive colonies (at least one per plate) were confirmed using Triple Sugar Iron agar (Oxoid), Lysine Iron Agar (Oxoid) and agglutination with *Salmonella* O antiserum (Polyvalent for groups A to I and Vi - Difco). The number of positive enrichments for each dilution was used to calculate the MPN / 100 ml for each sample.

Campylobacter The presence and number of *Camp. jejuni/coli* was determined using an MPN method (Wallace *et al.*, 1997). For each effluent sample triplicate serial dilutions were prepared in 0.1% BPW. One ml aliquots of the dilutions were added to 5.5 ml of Preston Enrichment Broth and enriched by incubation at 37°C for 4 h followed by 44 h at 42°C (all incubation under microaerobic conditions). Following enrichment, the tubes were inoculated onto modified *Campylobacter* charcoal deoxycholate agar-Preston (Oxoid). The inoculated plates were incubated at 37°C for 48 h under microaerobic conditions, before being examined for growth of *Campylobacter*s. Confirmatory identification as *Camp. jejuni/coli* was performed on the basis of catalase and oxidase tests and the typical motility of *Campylobacter*s.

The number of positive enrichments for each dilution was used to calculate the MPN 100 ml^{-1} for each sample.

Erysipelothrix. The MPN method of Chandler and Craven (1980) was used for determining the presence and number of *Erysip. rhusiopathiae*. Initially a 10 tube MPN, for piggeries A-D (minimum detection of 11 organisms 100 ml^{-1}) was then followed by a 3 tube MPN for piggeries E-N (minimum detection of 30 organisms 100 ml^{-1}), based on the levels encountered in the first piggeries.

For each effluent sample, appropriate serial dilutions were made in 0.1% peptone, buffered at pH 7.4. From each dilution tube, 1 ml was inoculated into the selective medium of Wood (1965). The Wood (1965) selective medium was modified such that the azide concentration was that recommended by (Stephenson & Berman 1978) for Packer's Medium (Packer, 1943). The selective broth was incubated at 37^{o}C for 48 h. After enrichment, each tube was inoculated onto Packer's medium (Packer 1943) modified as described by (Stephenson & Berman 1978) and the inoculated plates incubated at 37^{o}C for 48 h. Presumptive identification as either *Erysip. rhusiopathiae* or *Erysipelothrix* was performed by polymerase chain reaction (PCR). Colony sweeps were examined in two PCR tests – the *Erysipelothrix* genus specific PCR described by (Makino *et al.* 1994) and the *Erysip. rhusiopathiae* species-specific PCR described by (Shimoji *et al.* 1998). The number of enrichments that gave a positive in both the *Erysip. rhusiopathiae* species-specific PCR and the *Erysipelothrix* genus-specific PCR for each dilution was used to calculate the MPN of *Erysip. rhusiopathiae* 100 ml^{-1} for each sample. The number of enrichments that gave a positive in the *Erysipelothrix* genus-specific PCR but a negative in *Erysip. rhusiopathiae* species-specific PCR for each dilution was used to calculate the MPN of *Erysipelothrix (*not *rhusiopathiae)* 100 ml^{-1} for each sample.

Rotavirus The presence and number of rotavirus particles in each effluent sample was determined using a modification of a previously described method (Mehnert *et al.* 1997). A 4 l volume of piggery effluent was filtered initially through cotton gauze (3 layers), under vacuum, to remove larger particles such as floating mosquito larvae, sticks etc. Next the effluent was filtered using cross–flow membrane filtration with a Sartorius Sartocon Mini SM 17521 filtration unit fitted with a 0.22 μm membrane. The effluent was processed by recirculating it through the membrane at 300 rpm and the filtrate was retained for the filtration of viruses. The volume of the final filtrate was a little less than 4 l and was recorded for each sample. The pH of

the filtered effluent was adjusted to 6.5 using concentrated HCl. The effluent was then filtered using a 90 mm Zeta Plus 60S microporous positively charged filter (Cuno Filter Systems Sydney, Australia) in a pressure filter holder (Cuno Filter Systems Sydney, Australia). The flow rate was maintained at 50 ml min^{-1} using a peristaltic pump (Masterflex Console Drive, Cole Parmer Instrument Company, USA). At pH 6.5, virus particles are absorbed onto the membrane. The filtrate was discarded. The filter-bound viruses were eluted with 70 ml of sterile 3% Beef Extract (Oxoid) in 0.05 mol l^{-1} glycine solution, pH 9.0. The eluted virus suspension was then neutralised to pH 7.0. The eluted virus suspension was further concentrated by ultra-centrifugation at 180,000 x g (Beckman Ultra-centrifuge and Beckman 70 l Ti rotor) for 2 h at 4^{o}C. The resultant sediment was resuspended in 1.3 ml of the diluent provided in the rotavirus ELISA detection kit (Dako IDEIA Rotavirus kit). As the starting material for this concentration method was a 4 l sample and the end result was a 1.3 ml suspension, the overall result was a 3,000 fold concentration. During the development and validation of this concentration technique, the efficacy of the method was determined by spiking piggery effluent with a known concentration of simian rotavirus (kindly provided by the Institute for Medical and Veterinary Sciences, Adelaide, Australia).

2.4 Results

2.4.1 Validation of rotavirus detection methodology

The ELISA kit used in this work was shown, using a known concentration range of simian rotavirus, to produce a standard curve (r^2=99.7%) with a minimum detection level of 18,000 virus particles in the 100 μl sample examined in the ELISA kit. The calculation formula developed in the creation of this standard curve was as follows:-

$X = [(Y - 0.127)/98.5] \times 6 \times 10^8$, where X is the predicted rotavirus concentration and Y is the absorbance.

This formula was then used to determine the level of simian rotavirus detected in a 4 l piggery effluent that was initially spiked with a total of 4.8 X 10^7 plaque forming units (pfu) (a log cycle of 7.68) (average of two experiments). The level of virus detected by the concentration method used in this work was 1.0 X 10^7 pfu (a log

cycle of 7.64). Based on log cycle calculations, this means that the virus recovery efficiency was 91%.

Hence, the method we have developed uses a total concentration of 3,000 times, a recovery efficiency of 91% and a detection system with a minimum limit of 18,000 pfu. This means that for the 4 l sample used in this work, the minimum detection level in the initial effluent is 64 pfu ml^{-1}.

2.4.2. Enumeration results

Thermotolerant coliforms and E. coli Most effluent samples contained high levels of thermotolerant coliforms (with *E. coli* making up the vast majority of these organisms) (Table 2). The range in results for the final pond samples varied from 1.2 X 10^2 cfu to 5.3 X 10^5 100 ml^{-1} for thermotolerant coliforms and 1.2 X 10^2 to 4.2 X 10^5 cfu 100 ml^{-1} for *E. coli.*

Table 2 Detailed Results of Examination of Effluent Samples from Thirteen Piggeries in southern Queensland

Piggery/ Sample type	**Thermotolerant coliforms (cfu 100 ml^{-1})**	***Escherichia coli* (cfu 100 ml^{-1})**	***C. jejuni/coli* (MPN 100 ml^{-1})**	***Salmonella* spp. (MPN 100 ml^{-1})**	***Erysipelothrix rhusiopathiae* (MPN 100 ml^{-1})**	***Erysipelothrix* spp. (MPN 100 ml^{-1})**	**Rotavirus (pfu ml^{-1})**
Piggery A							
No. 1 – Anaerobic pond inlet	2.4 X 10^5	2.4 X 10^5	4600	<11	<11	22	< 64
No. 2 – Anaerobic pond	1.9 X 10^5	1.9 X 10^5	11000	<11	<11	161	< 64
No. 3 - Effluent lagoon	3.3 X 10^4	3.3 X 10^4	930	<11	<11	36	< 64
Piggery B							
No. 4 – Surface water	1.7 X 10^5	1.7 X 10^5	230	<11	<11	120	< 64
Piggery C							
No. 5 – Secondary pond	1.1 X 10^5	1.1 X 10^5	430	11	<11	161	< 64
No. 6 – Primary pond	5.2 X 10^5	5.2 X 10^5	930	<11	<11	51	< 64
Piggery D							
No. 7 – Final pond	1.2 X 10^5	1.2 X 10^5	230	51	<11	<11	< 64
No. 8 – Primary pond	2.8 X 10^6	2.8 X 10^6	>11000	230	<11	230	< 64
No. 9 – Sump	3.7 X 10^7	Not done-	>11000	92	<11	<11	< 64

Piggery/ Sample type	**Thermotolerant coliforms (cfu 100 ml^{-1})**	***Escherichia coli* (cfu 100 ml^{-1})**	***C. jejuni/coli* (MPN 100 ml^{-1})**	***Salmonella* spp. (MPN 100 ml^{-1})**	***Erysipelothrix rhusiopathiae* (MPN 100 ml^{-1})**	***Erysipelothrix* spp. (MPN 100 ml^{-1})**	**Rotavirus (pfu ml^{-1})**
Piggery E							
No. 10 – Pond effluent	2.6 X 10^5	1.9 X 10^5	430	<11	<30	<30	< 64
No. 11 – Sump	2.1 X 10^8	1.1 X 10^8	>11000	<11	<30	11000	< 64
Piggery F							
No. 12 – Final pond	5.3 X 10^5	4.2 X 10^5	230	22	<30	<30	< 64
No. 13 – Primary pond	5.2 X 10^6	5.2 X 10^6	4600	120	<30	<30	< 64
Piggery G							
No. 14 – Tertiary pond	6.9 X 10^4	6.9 X 10^4	430	<11	<30	430	< 64
No. 15 – Inlet of primary pond	4.3 X 10^7	3.6 X 10^7	>11000	<11	<30	4600	< 64
Piggery H							
No. 16 – Tertiary pond	4.4 X10^4	3.5 X10^4	230	<11	<30	<30	< 64
No. 17 – Primary pond	4.6 X10^5	4.6 X10^5	930	<11	<30	90	< 64
Piggery J							
No. 18 – Primary pond	1.1 X 10^6	1.1 X 10^6	1500	<11	<30	430	< 64
No. 19 – Recycle pond	5.4 X 10^4	5.4 X 10^4	40	<11	<30	90	< 64
Piggery K							
No. 20 –Tertiary pond	2.7 X 10^4	2.7 X 10^4	<30	<11	<30	<30	< 64
No. 21 – Primary pond	6.1 X 10^5	6.1 X 10^5	230	<11	<30	<30	< 64

Piggery/ Sample type	**Thermotolerant coliforms (cfu 100 ml^{-1})**	***Escherichia coli* (cfu 100 ml^{-1})**	***C. jejuni/coli* (MPN 100 ml^{-1})**	***Salmonella* spp. (MPN 100 ml^{-1})**	***Erysipelothrix rhusiopathiae* (MPN 100 ml^{-1})**	***Erysipelothrix* spp. (MPN 100 ml^{-1})**	**Rotavirus (pfu ml^{-1})**
Piggery L							
No. 22 - Final pond	3.1 X 10^4	3.1 X 10^4	40	<11	<30	<30	< 64
No. 23 – Primary pond II	7.0 X 10^5	7.0 X 10^5	230	<11	<30	<30	< 64
No. 24 – Primary pond I	2.3 X 10^6	2.3 X 10^6	930	120	<30	230	< 64
Piggery M							
No. 25 - Final pond	1.2 X 10^2	1.2 X 10^2	<30	<11	<30	<30	< 64
No. 26 – Primary pond	2.7 X 10^4	2.7 X 10^4	430	<11	<30	150	< 64
No. 27 – Primary pond inflow	9.6 X 10^5	9.6 X 10^5	2400	<11	<30	230	< 64
Piggery N							
No. 28 – Sump	1.0 X 10^6	1.0 X 10^6	2400	<11	<30	90	< 64
No. 29 - Final pond	9.1 X 10^4	8.0 X 10^4	90	<11	<30	90	< 64

Campylobacter. *Camp. jejuni/coli* was detected in all but two samples (Table 2). The sole negative samples were from two final ponds (Piggeries K and Piggery M). In another two samples (final ponds for Piggeries J and L), the numbers of *Camp. jejuni/coli* were very low (40 MPN 100 ml^{-1}). Levels ranging from 230 MPN 100 ml^{-1} to >11,000 MPN 100 ml^{-1} were present in the sump/primary pond samples. The final pond levels of *Camp. jejuni/coli* varied from 30 MPN to >930 MPN 100 ml^{-1}.

Salmonella. Only seven samples, representing four of the 13 piggeries, were positive for *Salmonella* spp (Table 2). The levels of *Salmonella* were low, with the worst case being the primary pond of Farm D which had a level of 230 MPN 100 ml^{-1}. In terms of the final ponds that would be the source of any effluent that would be re-used, only three farms were positive, with the highest level in such a pond being 51 MPN 100 ml^{-1}.

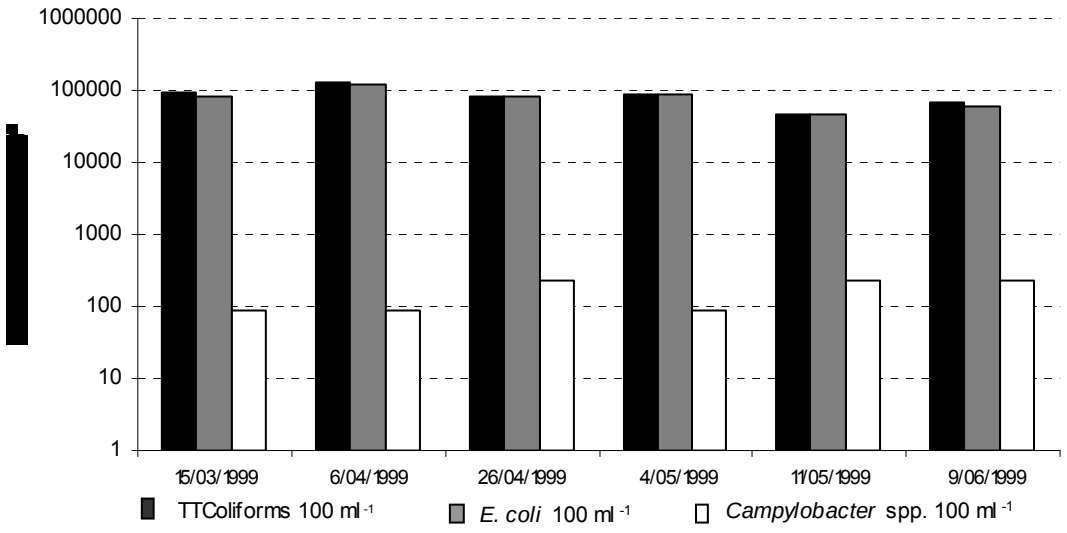
Erysipelothrix. We experienced some difficulties in the *Erysip. rhusiopathiae* testing. We found that the selective enrichment of *Erysip. rhusiopathiae* was resulting in most samples yielding *Erysipelothrix*-like organisms. In the light of the difficulty in separating *Erysip. rhusiopathiae* from *Erysip. tonsillarum* (Takahashi *et al.*, 1987), we established the *Erysipelothrix* genus-specific PCR test described by Makino *et al.* (1994) and the *Erysip. rhusiopathiae* species-specific PCR of Shimoji *et al.* (1998). Of the 120 suspect isolates examined in these two PCR tests, 118 were positive in the *Erysipelothrix* genus-specific PCR while all 120 were negative in the *Erysip. rhusiopathiae* species-specific PCR. These results mean that all the effluent samples had a level of *Erysip. rhusiopathiae* below the minimum level of detection of the MPN method (ie < 30 MPN 100 ml^{-1} for Piggeries E to N and <11 MPN 100 ml^{-1} for Piggeries A to D). We defined those organisms that were positive in the *Erysipelothrix* genus-specific assay and negative in the *Erysip. rhusiopathiae* species-specific assay as *Erysipelothrix* spp *(*not *rhusiopathiae*). The MPN results for the *Erysipelothrix* spp are shown in Table 2.

Rotavirus. None of the samples examined in the survey were positive for rotavirus, meaning that all samples had a level of rotavirus of < 64 pfu ml^{-1}.

2.4.3. Changes over time

To gain some insight into the fluctuations that may occur over time, we repeatedly sampled the final pond of Piggery N over a three-month period. This period covered late summer, autumn and winter. Fig. 1 shows the results for thermotolerant coliforms, *E. coli* and *Camp. jejuni/coli* tests over this time of the final pond samples. There was only minor variation for both organisms over the three month testing period.

Figure 1 Levels of thermotolerant coliforms (cfu 100 ml^{-1}), *E. coli* (cfu 100 ml^{-1}) and *Campylobacter* spp. (MPN 100 ml^{-1}) in Piggery N from March (late summer) to June (winter).



2.4.4. Reduction of bacteria through the ponding system

To gain insight into the effectiveness of the ponding systems used by thee piggeries, we calculated the reduction in the viable count of the three bacteria that were at high enough levels to allow this calculation - thermotolerant coliforms, *E. coli* and *Campylobacter.* For this analysis, we calculated the reduction in viable count in log numbers (termed log_{10} reduction) from the initial sump (if a sump sample was not available, we used the initial pond result) to the final pond. Piggery B could not be included in this analysis as we had only a final pond sample from this piggery. The results are summarised in Table 3. The average log_{10} reduction for thermotolerant coliforms was 1.77 – with piggery M achieving the highest reduction (3.9) and Piggery C the lowest (0.67). The results were very similar for *E. coli* - the average log_{10} reduction was 1.71 – with piggery M achieving the highest reduction

(3.9) and Piggery C the lowest (0.67). Fewer piggeries could be used in calculating the *Campylobacter* $\log_{10}$ reduction as there were several instances where the counts were above the maximum dilution or below the minimum detection level. Using the data from the eight piggeries that were in range, we found an average $\log_{10}$ reduction for *Campylobacter* of 1.04 – with the largest value being 1.57 at piggery J and the lowest 0.34 at Piggery C.

Table 3 Details of the log_{10} reductions in selected organisms recorded for the13 piggeries included in this survey. The log_{10} reduction figures have been calculated by subtracting the log_{10} count of the relevant organism in the final pond from the log_{10} count in the sump/initial pond.

Piggery	**Log_{10} Reduction in**		
	Thermotolerant coliforms	***E. coli***	***Campylobacter***
A	0.86	0.86	0.69
C	0.67	0.67	0.34
D	2.49	Not done[A]	>1.68[B]
E	2.91	2.76	>1.41[B]
F	0.99	1.1	1.3
G	2.79	2.72	>1.41[B]
H	1.02	1.12	0.61
J	1.31	1.31	1.57
K	1.35	1.35	>0.88[B]
L	1.87	1.87	1.37
M	3.9	3.9	>1.90[B]
N	1.04	1.1	1.43
Average	**1.77**	**1.71**	**1.04[C]**

[A] Not Done = Data not available

[B] The $\log_{10}$ reduction figure is a minimum estimate as one of the *Campylobacter* counts was outside the countable range (ie above maximum detection level for sumps on Piggeries D, E and G or below minimum detection level in the final pond for Piggeries K and M)

[C] The estimated $\log_{10}$ reduction values for Piggeries D, E, G, K and M were NOT used to calculate the average $\log_{10}$ reduction figure for *Campylobacter*.

2.5 Discussion

Despite the clear importance in terms of human and animal health, there is little definitive knowledge on the actual levels of pathogens in Australian piggery effluent. (Chandler *et al.* 1981) surveyed 92 effluent samples for the level of the indicator organism – faecal (thermotolerant) coliforms. (Chandler & Craven 1980) examined forty effluent samples for presence of *Erysip. rhusiopathiae* with 12 of these 40 samples being subjected to quantification. (Henry *et al.* 1995a) have reported the presence but not the level of *Salmonella* in the three piggery effluent ponds.

The level of thermotolerant coliforms we found in the final effluent of the 13 piggeries was an average of 1.2 X 10^5 CFU 100 ml^{-1} (varying from 1.2 X 10^2 to 5.3 X 10^5). This is around 100 times lower than that reported by (Chandler *et al.* 1981) who found that, for the 92 effluent samples they examined, the average thermotolerant coliform count was 3.1 X 10^7 CFU 100 ml^{-1} (varying from 2.0 X 10^5 to 7.3 X 10^8). Our results are in agreement with work from the United States where an anaerobic piggery effluent pond was found to have a faecal coliform concentration of 3.3 X 10^5 CFU 100 ml^{-1} (Hill & Sobsey 1998).

The *E. coli* levels we have found in the final ponds (mean of 1.03 X 10^5 CFU 100 ml^{-1} – varying from 1.2 X 10^2 to 4.2 X 10^5) match closely those reported by Hill and Sobsey (1998) for an anaerobic pond in the United States (2 X 10^5 CFU 100 ml^{-1}). It is notable that both our study and the (Hill & Sobsey 1998) study found that, for piggery effluent, the thermotolerant coliform count is a very close match for the *E. coli* count.

There appear to have been no previous studies on the levels of *Campylobacter* in piggery effluent. There is knowledge on the levels of *Campylobacter* in pig faeces – with levels varying from 2,500 to 5,500 MPN 100 g^{-1} faeces (Weijtens *et al.*, 1993). We found similar levels in the primary ponds and or sumps sampled in this survey – with levels varying from 930 to >11,000 MPN 100 ml^{-1}. In the final effluent, the levels of *Campylobacter* were lower as we found a mean $\log_{10}$ reduction of 1.04. Hence, we found levels of *Campylobacter* that varied from below the detectable minimum (< 30 MPN 100 ml^{-1}) to a maximum of 930 MPN 100 ml^{-1}. Our finding that *Campylobacter* was detectable in all primary effluent/sump material examined is further evidence of the high prevalence of this organism. Others have reported the high prevalence of *Campylobacter* in pigs eg (Weijtens *et al.* 1993) found that more

than 85% of sampled Dutch porkers were intestinal carriers at all stages of fattening with no marked difference in this rate across farms.

We found only a few effluent samples to be positive for *Salmonella.* Only four of the 13 piggeries gave at least one positive sample with only three of the 13 piggeries being positive in the final pond – a positive percentage of 23%. Surveys of effluent ponds in England in the 1970s reported positive rates that varied from 22% to 36% (Jones & Hall 1975, Jones *et al.* 1976). The only available Australian data is the study of Henry *et al.* (1995) who reported that all three piggeries examined yielded *Salmonella* in the final pond. It should be made clear that our data reflects a single sampling of a relatively small number of piggeries. Nevertheless, our data is consistent with the larger studies performed in the UK – where 54 and 33 piggeries were examined (Jones & Hall 1975, Jones *et al.* 1976).

In both the UK studies and the previous Australian study, there was no evidence of clinical salmonellosis in any of the piggeries examined (Henry *et al.* 1995b, Jones & Hall 1975, Jones *et al.* 1976). Indeed, in the study of Jones *et al.* (1976) minimal disease piggeries showed a higher prevalence of *Salmonella* than conventional piggeries – 44% compared with 13%. Jones *et al.* (1976) speculate that this difference is due to the fact that the minimal disease herds were larger units that use more feed and thus have a greater chance of acquiring *Salmonella* via the feed. Interestingly, the only piggeries positive for *Salmonella* in our study had a sow herd size of a 1,000 or higher (piggeries C, D and F) or were very large grow out facilities (piggery J with an standard pig unit equivalent of 15,477). All three piggeries with a sow herd size of less than 1,000 (piggeries A, B and E) and the small grow out and weaner piggeries (piggeries M and N) were negative for *Salmonella*. The three piggeries reported by Henry *et al.* (1995b) as being positive for *Salmonella* all had sow herd sizes of 1,000. The association between large herd size and *Salmonella* is only a relative association as three of the piggeries in the current study with sow herd sizes of 1,000 to 1,200 (piggeries H, K and L) and one relatively large grow out facility (piggery G) were negative for *Salmonella*.

There have been few attempts to quantify the levels of *Salmonella* present in piggery effluent. In the English survey of the 1970s, 12 farms were positive but only three yielded counts high enough for the technique used to quantify *Salmonella* to function – with all three positives yielding counts of between 2 – 5 X 10^3 CFUs ml^{-1} (Jones *et al.* 1976). None of these farms were experiencing any recorded problems

with salmonellosis. In a Finnish herd known to be contaminated with *Salm. typhimurium*, the count in the slurry was 210 MPN g^{-1} slurry (Heinonen-Tanski *et al.*, 1998). The levels we have found for *Salmonella* are much lower than those recorded in these overseas studies. The highest level of *Salmonella* we found was 230 MPN 100 ml^{-1} in a primary pond and 51 MPN 100 ml^{-1} in a final pond (both at Piggery D). The highest level we found was around 10^2 times lower than the Finnish study (Heinonen-Tanski *et al.* 1998) and 10^3 times lower than the English study (Jones *et al.*, 1976). The prior study of Henry *et al.* (1995b) did not use a formal enumeration technology such as MPN but did use a technique in which both 5 ml and 50 ml of effluent were sampled. Of the 15 samples that were positive five were positive in the 5 ml sampling – with the remaining being positive only in the 50 ml sample (Henry *et al.* 1995a). This is evidence that the level of *Salmonella* was less than 0.5 CFUs 100 ml^{-1} in 10/15 positive samples recorded by Henry *et al.* (1995b).

The results of the PCR testing have shown that the isolates we found commonly in piggery effluent were not *Erysip. rhusiopathiae*. Based on the positive reaction in the *Eryspipelothrix*-genus PCR and the negative reaction in the *Erysip. rhusiopathiae*-species PCR, our isolates appear to be other members of the genus *Erysipelothrix* – possibly either *Erysip. tonsillarum* (Takahashi *et al.* 1987) or *Erysipelothrix* species 1 or 2 (Takeshi *et al.* 1999). All that can be concluded from our current results is that the organisms we detected commonly in piggery effluent are not *Erysip. rhusiopathiae*. This is an important finding as only *Erysip. rhusiopathiae,* and not *Erysip. tonsillarum* and *Erysipelothrix* spp. 1 and 2, is known to be pathogenic for pigs (Takeshi *et al.* 1999).

Chandler and Craven (1980) have reported that "*Erysip. rhusiopathiae"* is common in Australian piggery effluent – reporting that 15 of 40 effluents were positive for the organism. However, the study of Chandler and Craven (1980) was performed before the understanding of the multi-species nature of the genus *Erysipelothrix* was gained.

We found that 6 of the 13 (46%) piggeries were positive for *Erysipelothrix* in the final effluent ponds with the levels varying from 430 to 36 MPN 100 ml^{-1}. In the study of Chandler and Craven (1980), a similar level of positive samples was encountered (15 of 40 - 37.5%). Chandler and Craven (1980) only attempted quantification on a

limited number of samples, finding levels that varied from 1,000 to >24,000 MPN 100 ml^{-1} in the five samples and levels of < 1,000 MPN 100 m^{-1} in seven samples.

Overall, our results indicate that while piggery effluent may indeed contain members of the genus *Erysipelothrix*, these isolates may be the non-pathogenic species, rather than the pathogenic *Erysip. rhusiopathiae*.

The absence of detectable levels of rotavirus in piggery effluent is not surprising. Rotavirus excretion has been shown to not occur in pigs less than one week of age or over two months old (Fu & Hampson 1987). Even in actively excreting pigs, rotavirus is shed in the faeces for only an average of 7.4 days (Fu and Hampson, 1987). Hence, in a piggery undergoing active infection only a very small percentage of the overall pig population is likely to actively shedding the virus. Hence, while Fu *et al.* (1989) were able to detect virus in the immediate environment around the actively shedding pigs and in weaner and farrowing houses, they failed to detect virus in the environment of the fattening and sow sheds. Hence, our finding that the levels of rotavirus in the effluent ponds was below the detectable level supports and extends the earlier findings of Fu *et al.* (1989).

We calculated $\log_{10}$ reduction for thermotolerant coliforms, *E. coli* and *Campylobacter* for 12 of the 13 ponding systems investigated in this study. In waste treatment studies, the calculation of the $\log_{10}$ reduction is a standard method of assessing the efficiency of bacterial reduction and has been used by others to assess treatment efficiency e.g. Hill and Sobsey (1998). The average $\log_{10}$ reduction for *E. coli* found in our study (1.71) is similar to that recorded by Hill and Sobsey (1998) who reported a figure of 2.1. Our study appears to have been the first study to record the $\log_{10}$ reduction for *Campylobacter* that occurs in standard anaerobic pond waste treatment of piggery effluent. The $\log_{10}$ reduction for *Campylobacter* (1.04) was lower than the figure we recorded for *E. coli* (1.71). It is important to note that our calculated $\log_{10}$ reduction of 1.04 is a conservative estimate. For five piggeries (Piggeries D, E, G, K and M) we could not calculate a $\log_{10}$ reduction figure as one of the *Campylobacter* counts for each of these piggeries was out of range. For these piggeries, we could only estimate a $\log_{10}$ reduction figure. We calculated the average $\log_{10}$ reduction excluding these piggeries where only an estimated $\log_{10}$ reduction figure was possible. For four of the piggeries not included in the calculation, the estimated $\log_{10}$ reduction for *Campylobacter* was above the calculated average of 1.04. This is strong evidence that our calculated average

log_{10} reduction figure for *Campylobacter* of 1.04 is a very conservative figure. A further point to note is that there was considerable variation from piggery to piggery in the log_{10} reduction figures. Piggery M achieved a log_{10} reduction of 3.9 for both thermotolerant coliforms and *E. coli* and a log_{10} reduction figure of at least 1.9 for *Campylobacter*. This is evidence that anaerobic ponding systems can achieve marked reduction in indicator and pathogenic bacteria levels.

We found very little variation in the levels of thermotolerant coliforms, *E. coli* or *Campylobacter* using intermittent sampling over a 3 month time period on a single effluent pond. The climate in South-East Queensland lacks any dramatic difference between summer and winter. Typical summer temperatures are a maximum of 29^0C and a minimum of 20.6^0C while typical winter temperatures are a maximum of 20.4^0C and a minimum of 9.5^0C (http://www.bom.gov.au). Hence, we cannot be sure if other localities with more extreme variations between summer and winter may yield greater fluctuations in pathogen and indicator organism numbers. It is interesting to note that a study over time showed the human sewage effluent from treatment plants in the same broad geographical region showed a marked variation over time in the levels of indicator organisms as well as pathogens (Thomas *et al.* 2000). The results from the sewage effluent work suggest temperature variations are not a key issue in why human sewage bacteria show considerable variation.

This work provides a basis of solid information on the presence and level of key pathogens in Australian piggery effluent. This information can now be used in the development of guidelines for the safe and sustainable use of piggery effluent.

2.6 Acknowledgements

I acknowledge my co-authors R.J. Thomas, K. Casey, E. McGahan, E.A. Gardner, M. Rafiee and P.J. Blackall

I acknowledge Australian Pork Limited provided funding for this study. The co-operation of the owners of the 13 piggeries is gratefully acknowledged. The assistance of G.W. Blight in the creation of the standard curve for the enumeration of rotavirus is gratefully acknowledged.

Chapter 3

Presence and incidence of food-borne pathogens in Australian chicken litter

This Chapter is published in *British Poultry Science*

Citation:

H.N. Chinivasagam, M. Redding, G. Runge, P. J. Blackall (2010). Presence and incidence of food-borne pathogens in Australian chicken litter. *British Poultry Science,* **51**(3): 311-318.

3.1 Summary

1. Litter samples were collected at the end of the production cycle from spread litter in a single shed from each of 28 farms distributed across the three Eastern seaboard States of Australia.

2. The geometric mean for *Salmonella* was 44 Most Probable Number (MPN)/g for the 20 positive samples. Five samples were between 100 – 1000 MPN/g and one at 10^5 MPN/g, indicating a range of factors are contributing to these varying loads of this organism in litter.

3. The geometric mean for *Campylobacter* was 30 MPN/g for the 10 positive samples, with seven of these samples being <100 MPN/g. The low prevalence and levels of *Campylobacter* were possibly due to the rapid die-off of this organism.

4. *E. coli* levels were markedly higher than the two key pathogens (geometric mean 2.0 X 10^5 colony forming units (cfu)/g) with overall levels being more or less within the same range across all samples in the trial, suggesting a uniform contribution pattern of these organisms in litter.

5. *Listeria monocytogenes* was absent in all samples and this organism appears not to be an issue in litter.

6. The dominant (70% of the isolates) *Salmonella* serovar was *S.* Sofia (a common serovar isolated from chickens in Australia) and was isolated across all regions. Other major serovars were *S.* Virchow and *S.* Chester (at 10%) and *S.* Bovismorbificans and *S.* Infantis (at 8%) with these serovars demonstrating a spatial distribution across the major regions tested.

7. There is potential to re-use litter in the environment depending on end use and the support of relevant application practices and guidelines.

3.2 Introduction

It has been estimated that the Australian chicken meat industry uses around 0.95 million m^3 of bedding material per annum and generates around 1.67 million m^3 of litter per annum (Runge *et al.* 2007). Around 70% of the Australian chicken meat industry uses fresh bedding for each broiler cycle (Runge *et al.* 2007). In the other 30% of the industry, the brooder end of the shed always has fresh bedding while the grower end of the shed has the re-used litter – with re-use occurring for 2-5 broiler cycles (Runge *et al.* 2007). However in the USA unlike in Australia, several flocks are reared on a single batch of litter, which is kept in the houses during downtime between the flocks (Volkova *et al.* 2009).

While precise figures are difficult to locate, most chicken litter in Australia is utilised off the poultry farm (Runge *et al.*, 2007). Unlike countries such as the USA where a large portion of appropriately treated chicken litter is used as cattle feed ingredient (Martin *et al.*, 1998), the use of chicken litter as a feed ingredient for ruminants in Australia is illegal, regardless of treatment procedures (Runge *et al.*, 2007). Direct application of un-treated chicken litter to agricultural production (small crops, tree crops, grain crops and pastures) is the traditional and most popular method of utilising chicken litter in Australia (Runge, *et al.*, 2007). However composting of litter in terms of producing a value added and safe product (Dorahy 2007) can have a better potential for targeting pathogen issues in the environment.

The application of animal manures to agricultural land has been identified as route by which food-borne pathogens such as *Campylobacter, Listeria* and *Salmonella* can enter the human food chain (Nicholson *et al.* 2005b). Clearly, knowledge of the presence and levels of key food-borne pathogens in chicken litter is a vital first step in the establishment of guidelines for the safe and sustainable re-use of a valuable resource and with minimum impact to the environment and food chain.

The paucity of knowledge about the presence and levels of key food-borne pathogens limits the development of sound and effective guidelines to ensure that litter can be used for agricultural applications or across production cycles either directly or indirectly (following treatments such as composting) with minimum risk to human health.

The current study was designed to sample litter from farms representing different production operations and geographical locations within the production facilities across 3 Eastern Australian States. Litter was sourced from 28 different chicken meat operations across Queensland, New South Wales and Victoria and tested for the presence and level of three major food-borne pathogens – *Campylobacter coli/jejuni, Listeria monocytogenes* and *Salmonella* spp. As well, we have determined the levels of *Escherichia coli* present in the litter.

3.3 Materials and Methods

3.3.1 Sample Collection

Litter samples were collected from a single shed within a farm. Samples were collected prior to removal when the litter was still spread and within 24 hours of the final bird pick up. This gave the best opportunity to effectively sample the litter prior to being removed. A total of 28 sheds were sampled, these representing 10 broiler farms in Queensland and nine farms each in New South Wales and Victoria. The farms were selected in a structured manner. In each State, the farms were selected to represent the market share of the various processing companies. Samples were collected in a formal random method. Within each shed, sixty sites were sampled – with the sites being selected by a random number generator. Of the sixty samples, three sets of 20 were composited to form three samples for the entire shed. At each selected site within the shed, a 50 mm diameter sample was taken to the full depth of the litter. The three pooled sub-sets were then shipped at ~10^0C to the laboratory. On arrival at the laboratory, the core temperature of each bag was recorded to confirm that the transport had occurred under chilled conditions. The samples were tested within 24 h of collection. Information on the shed type, number of chickens, chickens placed per m^2, depth of bedding placed, depth of litter and general condition of the litter were collected as well.

3.3.2 Sample preparation

The litter was initially broken up by using a mallet, on a uniformly spread sample still within the polythene bag. Any clumps that could not be broken up with the mallet were broken up by hand. Any stones were manually removed from the sample. The sample was mixed again to form a uniform sample by shaking the polythene bag several times. The samples were then aseptically quartered several times until

an approximately 200 g sample was obtained. This sample was transferred aseptically to a fresh sterile bag. Lots of 25 g were then weighed into sterile bags, to which 225 ml of sterile diluent was added. The diluent varied according to the organism tested. The samples were allowed to soak for 30 min, after which they were aseptically blended using a homogeniser for 1 min. The blended samples were immediately used for the enumeration of *E. coli, Salmonella*, *Campylobacter* and *Listeria monocytogenes* as described below.

3.3.3 *Salmonella*

Serial dilutions were prepared in 0.1% buffered peptone water, after which 1 ml of appropriate dilution was inoculated into 10 ml buffered peptone water in triplicate and incubated at 37^{o}C overnight. Six 30 µl volumes of each incubated broth were inoculated on to a single MSRV (Oxoid) plate and incubated at 42^{o}C overnight. The plates were observed for motile zones and a sample of the zone was inoculated onto an XLD agar (Oxoid) which was then incubated at 37^{o}C overnight. Positives colonies from XLD were biochemically confirmed using O.B.I.S. *Salmonella* kit (Oxoid). Positives from the XLD were streaked onto Nutrient agar (Oxoid), incubated overnight at 37^{o}C and further confirmed using *Salmonella* O antiserum, Poly A – I and Vi (Difco). Most Probable Numbers (MPN) of *Salmonella* were obtained from MPN tables, with counts being expressed as MPN per g of litter. The minimum detection limit was 0.3 MPN/g of litter. A formal random method was used to select 30 isolates per farm (State A) or 10 isolates per farm (States B and C) which were sent to a central reference laboratory for serotyping.

3.3.4 *Campylobacter* spp.

Serial dilutions were prepared in Preston Broth without antibiotics i.e. Nutrient broth No 2 (Oxoid) and 5% lysed horse blood, after which 1 ml of appropriate dilutions were inoculated into 5.5 ml of Preston Broth with antibiotics - Nutrient Broth No 2 (Oxoid), *Campylobacter* growth supplement SR232 (Oxoid), *Campylobacter* selective supplement SR117 (Oxoid) with 5% horse blood - in triplicate. The broths were incubated under microaerobic conditions using Campygen (Oxoid) gas generating kits for 37^{o}C for 4 h followed by 42^{o}C for 44 h. The broths were then streaked on to CCDA (Oxoid) which were incubated under microaerobic conditions at 37^{o}C for 48 h. Typical colonies were streaked onto Abeyta-Hunt-Bark Agar plates (Food and Drug Administration, 2001), which were incubated under microaerobic

conditions at 37°C overnight. The isolates were tested for typical motility, cell morphology as well as oxidase and catalase reactions. The minimum detection limit was 3 MPN/g of litter. Most Probable Number (MPN) of *Campylobacter* was reported as MPN per g of litter.

3.3.5 *Listeria monocytogenes*

Serial dilutions were prepared in Demi-Fraser broth without ferric ammonium citrate (Oxoid), to which ferric ammonium citrate was added aseptically at a concentration of 500 mg per litre. One ml of appropriate serial dilutions were added to 10 ml Demi-Fraser broth (supplemented with ferric ammonium sulphate as above) in triplicate and incubated at 30°C for 24 h. Simultaneously, 25 g of litter was added to 225 ml of Demi-Fraser broth (supplemented with ferric ammonium citrate as described above) which was then incubated at 30°C for 24 h. The 10 ml Demi-Fraser Broths were then stored at 10°C and used only if the 225 ml master broth was positive. A 0.1 ml volume of the incubated 225 ml master broth was inoculated into 10 ml of *Listeria* Enrichment Broth (Oxoid – TM 0986), which was incubated at 30°C for 24 h. The presence of *Listeria* spp in this broth was determined by use of the Oxoid *Listeria* Rapid Test. If the master sample was positive, MPN levels were tested using the stored Demi-Fraser broths as previously described for the master broth. Broths yielding positives in the *Listeria* rapid test were streaked on to PALCAM (Oxoid) and Chromogenic Listeria Agar (oxoid) for further identification of *Listeria monocytogenes*. The minimum detection limit of the MPN was 3 MPN /g of litter.

3.3.6 *E. coli*

Serial dilutions were prepared in 0.1% peptone, after which 1 ml of appropriate dilution was added to *E. coli* Petrifilm (3M). The Petrifilm samples were incubated at 36°C for 24 h after which typical blue colonies were counted. Counts were reported as colony forming units (CFU) per g of litter.

3.3.7 Statistical analysis

Fisher's exact test was used to compare the *Campylobacter* and *Salmonella* positive rate for single use and re-used litter. The level of significance was $P \leq 0.05$. Statistix software by Analytical Software was used for this analysis.

3.4 Results

3.4.1 Farm information

Key information, i.e. litter practice, bedding used, ventilation system, floor type and number of chickens for the 28 sheds examined in this study is provided in Table 1. A range of bedding material such as shavings or saw dust sourced from either pine or hardwood as well as the use of rice hulls ranged across the sheds (farms) tested.

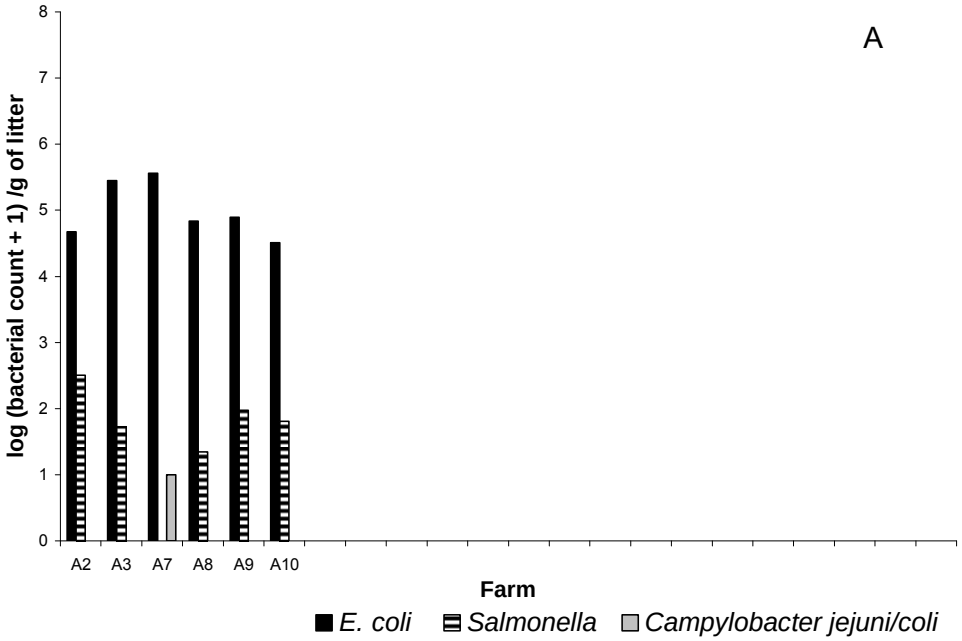
Table 1 Key characteristics of 28 sheds sampled in study

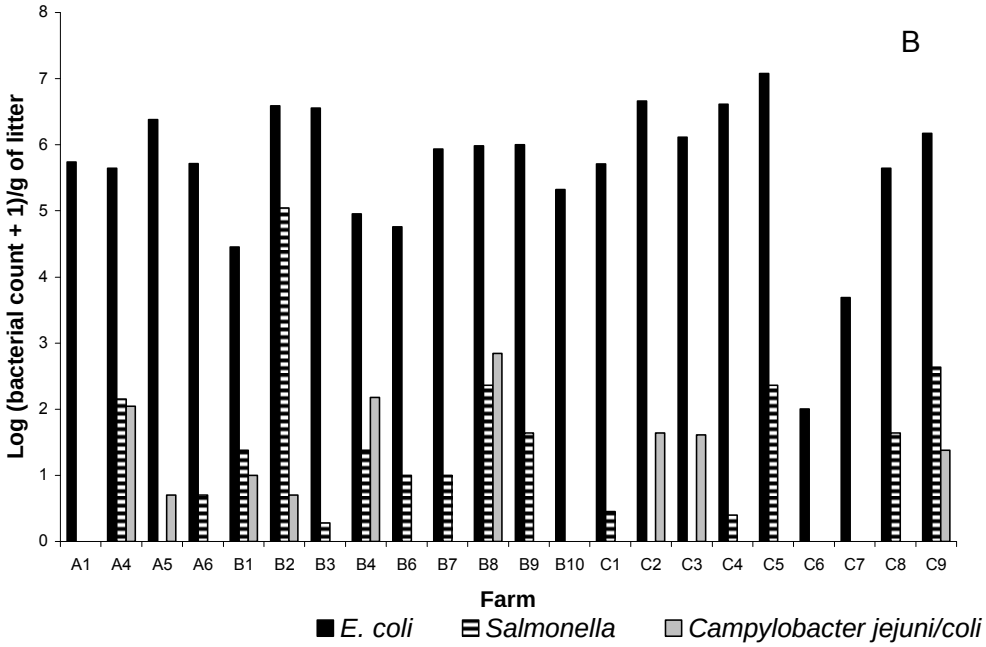
Characteristic	Number of Sheds
Litter	
Single Use	22
Partial Re-use	6
Bedding	
Pine Shavings	13
Pine Sawdust	5
Hardwood Sawdust	3
Rice Hulls	2
Shredded Paper	1
Rice Hulls/Pine Shavings (50/50)	1
Pine Sawdust/Pine Shavings (50/50)	1
Rice Hulls/Pine Sawdust (50/50)	1
Unknown	1
Ventilation System	
Tunnel	22
Cross-flow	5
Unknown	1
Floor type	
Compacted Clay (or Earth)	25
Asphalt	2
Unknown	1
Number of chickens placed in shed	17,400 – 50,000 (Average – 31,115)
Depth of bedding placed in shed	40 – 100 mm (Average 63 mm)

3.4.2 Microbiological results

A single shed was tested per farm and thus these sheds will be referred to as representing the different farms. No farms yielded any detectable *Listeria* spp. However *Salmonella*, *Campylobacter* and *E. coli* were detected at varying levels across the litter tested from the various farms. Figure 1 presents the results from all 28 sheds from 28 different farms for *Campylobacter jejuni/coli, E. coli* and *Salmonella* with the results of the single use and re-use litters presented separately. The geographical regions (or States) where the farms originated are coded as A, B and C.

Figure 1 Levels of *Campylobacter, E. coli* and *Salmonella* in both re-used (A) and single use (B) litter. The A4 *Campylobacter* count is a minimum estimation as the end point was not reached.





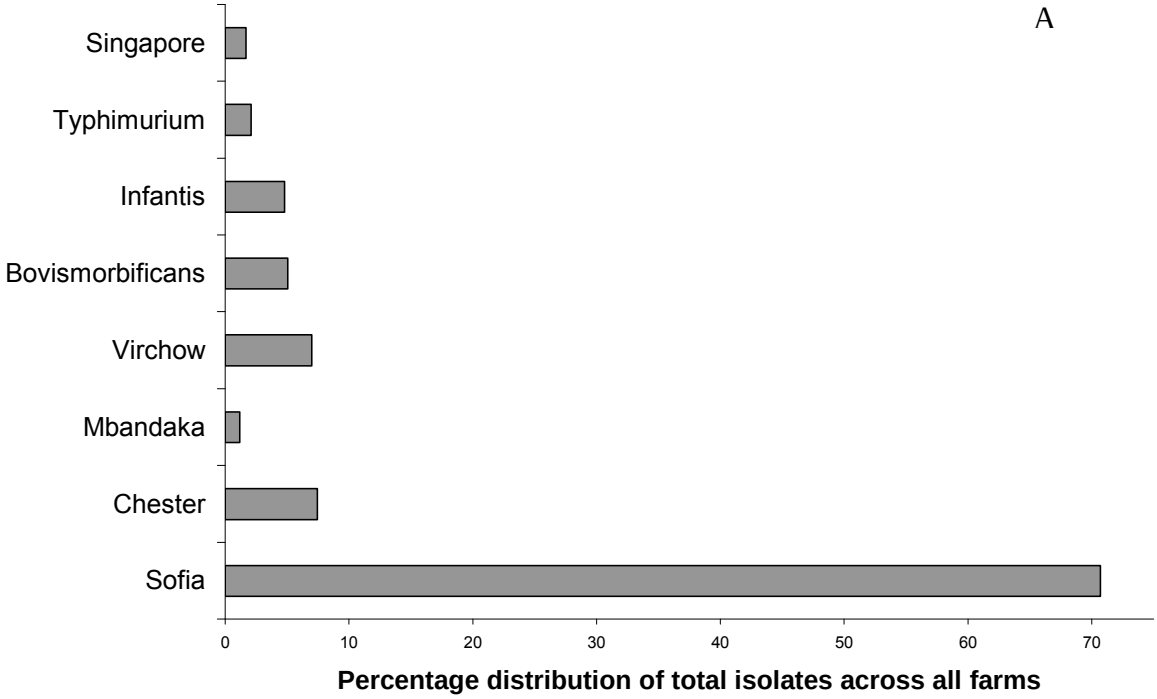
E. coli was detected on all 28 farms, with the range being from 3 X 10^4 to 3.6 X 10^5 CFU/g in re-used litter and 1 X 10^2 to 1.2 X 10^7 CFU/g in single use litter. The geometric mean for the farms re-using litter was 9.7 X 10^4 CFU/g while the geometric mean for single use litter was 4.2 X 10^5 CFU/g.

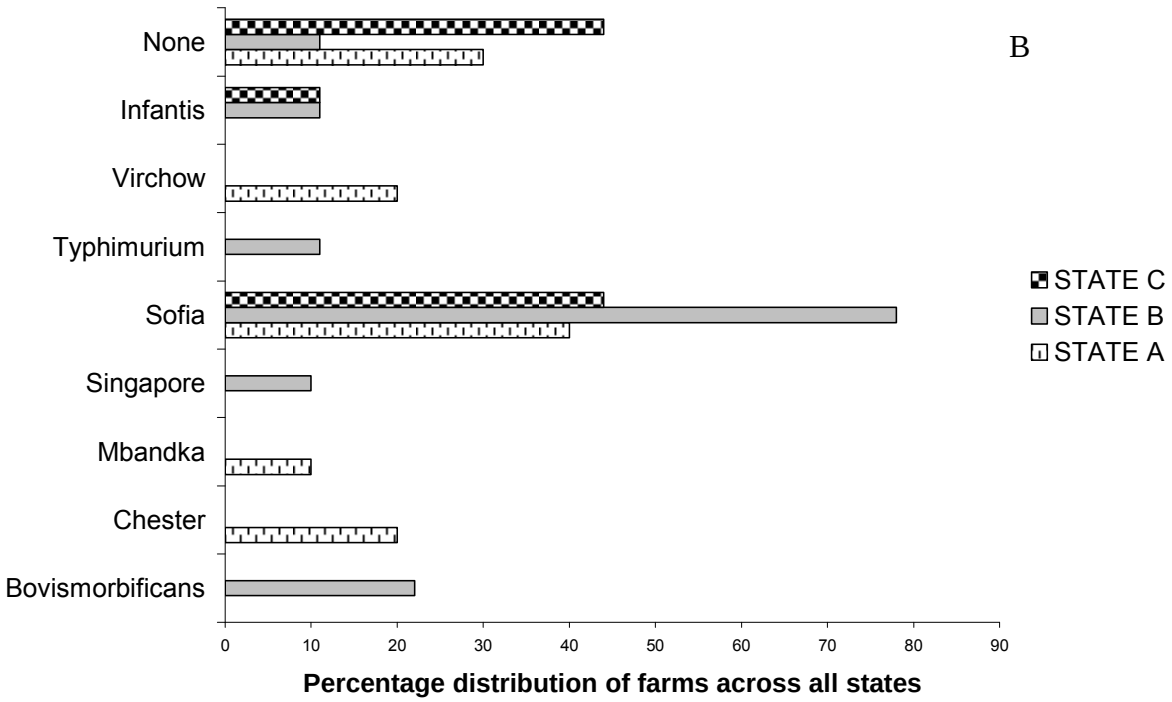
Salmonella was detected in five of the six (83.3%) re-use litter farms and in 15 of the 22 (68.2%) single use litters. In the five re-use farms that were positive for *Salmonella*, the level ranged from 4 to 930 MPN/g, with the geometric mean of these positive samples being 59 MPN/g. In the farms with single use litter that were positive for *Salmonella*, the level ranged from 4 to 1.1 X 10^5 MPN/g litter, with a geometric mean of 36 MPN/g litter. *Salmonella* levels were 100 MPN/g for 10 of 16 positive farms. Only one farm yielded a count of 1.1 X 10^5 MPN/g.

There was no significant difference in the rate of positive samples for either *Campylobacter* or *Salmonella* between single use and re-used litters ($P \leq 0.05$).

A total of 260 isolates of *Salmonella* were serotyped. Figure 2 presents both the percentage distribution of *Salmonella* isolates recovered across all farms (Figure 2A) and the percentage distribution of the serovars across the three States (Figure 2B). Among the total isolates 70% were *S*. Sofia, while *S.* Virchow and *S.* Chester were the next most common, contributing around 10% of the isolates, followed by *S.* Bovismorbificans and *S*. Infantis (around 8%). There was a major dominance of *S*. Sofia across the three States tested. When a comparison of the farms within States was made, *S*. Sofia dominated across the three States (Figure 2B). However with the exception of *S*. Infantis, the other six serovars recovered in this study were only present in a single State, showing a spatial effect on the appearance of these serovars. For example *S.* Chester, *S*. Mbandaka and *S.* Virchow were found only in State A, while State B was the only State to yield *S.* Bovismorbificans, *S.* Singapore and *S.* Typhimurium (Figure 2B). Other than *S*. Sofia, State C was only ever linked to a single serovar, *S*. Infantis, with *Salmonella* being absent on 50% of the farms in State C. State B, which had the highest number of serovars had only 10% of the farms negative for *Salmonella,* while State A had 30% farms negative for *Salmonella*.

Figure 2 Percentage distribution of *Salmonella* serovars across all isolates (A) and across States A, B and C (B).





Campylobacter was detected in only 1 of 6 (9 MPN/g) re-used litters tested. Of the 22 single use litters, nine were positive for *Campylobacter*. In one positive sample, the end point was missed and the level was recorded as being above 110 MPN/g. In the remaining eight samples, the level of *Campylobacter* varied from 4 to 700 MPN/g, with the geometric mean of these positive samples being 34 MPN/g. Only four litter samples, all being single use litters, had a level of greater than 100 MPN/g of *Campylobacter,* these being 110, 150, 700 and >110 MPN/g.

3.5 Discussion

The present study has demonstrated the presence and levels of the common indicator organism, *E. coli,* and two key pathogens (*Salmonella* and *Campylobacter)* in both single use and re-used litter across three States in Australia. An understanding of the levels is important in assessing the extent of contribution of these organisms to the food chain via litter during possible environmental use either with or without further treatment such as stockpiling. As all three organisms were indeed present, at varying levels, there is at least the potential for the organisms to remain present for some time. The survival period would be dependent upon a range of factors such as the robustness of the organism and the environment in which the used litter is placed.

It appears that pathogen survival was not influenced by the litter types, source material or physical characteristics given that detection was consistent across States. The litter was collected prior to any treatment such as windrowing. Thus, rather than the litter type it is perhaps the litter conditions such as temperature, pH and water activity (Macklin *et al.* 2006) that influence survival of the key pathogens.

The levels of *E. coli* (the common pathogen indicator organism) were much higher than the two key pathogens. *E. coli* is commonly present in the gut of chickens (Barnes *et al.* 2008), faeces and is a dominant organism in litter. With an established potential to survive in more challenging environments such as water and soil (Byappanahalli, *et al.*1998), it is not surprising to find the presence of *E. coli* in litter shortly after chicken removal.

Salmonella is recognised as a good survivor in the environment. For example *S.* Enteritidis persisted for at least 1 year in the dust of an empty broiler breeder house even after cleaning and disinfection (Davies & Wray 1996). In the current study, *Salmonella* was present in the majority of litter samples (20 of 28) but only at low levels across both single and re-use litter farms. There could be several factors contributing to the organism's presence in litter.

The presence of *Salmonella* in litter is typically a reflection of the presence of the organism in the chicken flock (Bhatia *et al.* 1979). Santos *et al.* (2005) have reported that there is a close relationship between *Salmonella* populations in both the chicken faeces and the litter. Transmission of *Salmonella* from the parent flock to the broiler flock is also well recognised, with a study of 10 French broiler farms concluding that the greatest contribution of *Salmonella* serovars in the broiler houses was the incoming chicks and not the surrounding environment (Lahellec *et al.* 1986). In contrast the overall dominance of the serovar *S*. Sofia (with respect to percentage distribution among total isolates) suggests a possible environmental link rather than flock to flock transmission of this serovar.

Other sources of *Salmonella*, such as feed, litter beetles, wild-life and rats are also well recognised (Gast 2003). For example litter beetles (a common feature in the poultry environment) can also play a role in the transmission of *Salmonella* (and *Campylobacter*) across successive broiler flocks (Hazeleger *et al.* 2008). Artificially contaminated litter beetles have shown 100% colonisation of the inoculated strain, Paratyphi B Variant Java (Hazeleger *et al.* 2008), and have been shown to act as a reservoir of *S*. Indiana between flocks (Skov *et al.* 2004). Similarly, poor quality feed (Barbour & Nabbut 1982) and water (Murray 1991) also has the potential to play a role in transmission. Thus, along with transmission by parent flock other external factors also have a contributory role in the transmission of *Salmonella* presence (or increase in levels) of this organisms depending on the prevailing conditions at the time.

The current study found a range of serovars in the litter and as these serovars (except for *S.* Sofia) showed a spatial distribution it is possible that these spatial patterns arose from links to the various parent flocks. As previously stated *S*. Sofia was by far the dominant serovar in all farms tested across these three states. This high prevalence of *S.* Sofia in the Australian poultry environment is well recognised

with *S.* Sofia representing over 50% of chicken-derived *Salmonella* submitted to the *Salmonella* Reference Laboratory at the Institute for Medical and Veterinary Science (Australian *Salmonella* Reference Centre Annual Report 2002). Despite this high prevalence in Australian chickens, the serovar is rarely isolated from humans (Australian *Salmonella* Reference Centre Annual Report 2002) The persistence of *S.* Sofia in Australian chickens as the predominant *Salmonella* isolate (over a 20 year period) is unique and not seen anywhere else in the world and there also appears to be no dominant clone (Heuzenroeder *et al.* 2004) The dominance of *S.* Sofia in Australian chickens has lead to speculation that possibly this serovar is acting as a "natural" competitive exclusion agent, reducing the prevalence of other serovars (Heuzenroeder *et al.* 2004). Definitive Australian-based pen trials have shown no evidence of such activity by *S.* Sofia (Heuzenroeder *et al.* 2001). Moreover, in the current study, four of the 28 litter samples examined, contained *S.* Sofia as well another serovar, indicating that *S.* Sofia does not appear to be acting as an exclusion agent. Based on the current observations *S.* Sofia appears to be of environmental origin due to its non-spatial distribution (i.e. not being confined to different geographic locations) as well as the dominance of this serovar across these regions tested, which are separated across thousands of kilometres. *S.* Sofia has shown the potential to be well adjusted to the chicken production environment under Australian conditions although the exact mechanism for such dominance is not clear.

The *Campylobacter* levels detected in the current study were low – the geometric mean in the single use litter was 34 MPN/g while the sole positive re-use positive litter had a level of 9 MPN/g. There is evidence that *Campylobacter* has a poor survival capacity in litter in the absence of the chicken. Shanker *et al.* (1990) reported that the litter level of *Campylobacter* dropped from 2.9 X 10^6 CFU/g to levels of 2.8 x 10^3 CFU/g at 24 h post chicken removal, to 5.3 X 10^2 CFU/g at 48 h post chicken removal and finally to <5 CFU/g at 72 h post chicken removal. While the current study did collect samples within 24 h of the last chicken pick up, it would be likely that the levels of *Campylobacter* in the litter were dropping from the time the last chickens left the shed.

Though the current results were not designed to include the comparison of the major litter practices used in Australia, the current study suggested that there may be a difference in the survival potential of the two pathogens studied between the two

major litter practices. The re-used litter samples showed a lower percentage positive for *Campylobacter* (17%), than the single use litter (41%). Interestingly, the relative percentages were reversed for *Salmonella* – with more re-used litters being positive for *Salmonella* (83%) than the single use litters (54%). There is considerable evidence that, in other countries such as the USA, re-used litter has a lowered level of *Salmonella* colonisation (Olesiuk, *et al.* 1971). The re-use practice on the Australian farms, described in the introduction, in the current study was markedly different from that typically used in the USA where chicks are placed onto re-used litter. This difference in litter re-use practices may have an impact on the intrinsic parameters of the different types of used litter to inhibit *Salmonella*, which is also reflected on the initial levels present in such litter.

Listeria is a common environmental organism (Ojeniyi *et al.* 1996). The absence of *Listeria* from the litter samples in the current study supports the earlier findings of others that this organism is not common in broiler chickens. Ojeniyi *et al.* (1996) reported that caecal samples from 2078 broilers in 90 random broiler flocks, 40 bedding samples and 640 dead broilers were negative for *Listeria.* In contrast, Ojeniyi *et al.* (1996) reported a higher prevalence in parent flocks (11 of 236 caecal samples being positive). This suggests that birds which live longer than 8 weeks may have greater chances of acquiring the organism. The findings of Ojeniyi *et al.* (1996) confirm that *Listeria* is likely to be in the general poultry production environment.

An important consideration when considering the implications of the current study for use of the litter in agricultural settings is the impact of any treatment of the litter prior to the agricultural use. Treatment processes such as deep stacking have the potential to reduce *Salmonella* levels markedly. Jeffrey *et al.* (2001) have shown that deep stacked litter that is spiked with *Salmonella* has no detectable *Salmonella* within 28 h. Any treatment process must, of course, be managed with appropriate guidelines. Indeed, even composting which is widely regarded as an effective pathogen reduction process (Sidhu *et al.* 2001), can suffer *Salmonella* re-growth problems if the composted material is not handled appropriately (Sidhu *et al.* 2001).

Overall, the current study has provided a baseline of information on the microbiological characteristics of Australian broiler litter. This information can now

be used to develop appropriate guidelines that ensure that this material can be safely and effectively used in the environment.

3.6 Acknowledgements

I acknowledge my co-authors M. Redding[1], G. Runge and P. J. Blackall

This research was supported by the Australian Rural Industries and Research Corporation. The technical assistance of Kate Russel and Adam Gale is acknowledged. The support of the companies and farms concerned is also acknowledged.

Chapter 4

Impact of the Australian litter re-use practice on *Salmonella* in the broiler farming environment

This Chapter is published in *Food Research International* (Special Issue, *Salmonella* in foods: Evolution, Stratergies and Challenges)

Citation:

H.N. Chinivasagam, T.Tran, P. J. Blackall (2010) Impact of the Australian litter re-use practice on *Salmonella* in the broiler farming environment, *Food Research International,* **45**: 891 - 896.

4.1 Summary

This study has examined the dynamics (in terms of levels and serovar diversity) of *Salmonella* in the “dual litter environment” that occurs within a single shed as a result of a management practice common in Australia. The study also looked at the physical parameters of the litter (pH, moisture content, water activity and litter temperature) as a means of understanding the *Salmonella* dynamics in these litter environments. The Australian practice results in the brooder end of the shed having new litter each cycle while the grow-out end has re-used litter (a “dual litter environment”). Two farms that adopted this partial litter re-use practice were studied over one full broiler cycle each. Litter was sampled weekly for the levels (and serovars) of *Salmonella* during a farming cycle. There was a trend for lower levels of *Salmonella* (and a lower *Salmonella* serovar) diversity in the re-used litter environment as compared with the new litter environment. Of the physical parameters examined, it would appear that the lower water activity associated with the re-used litter may contribute to the *Salmonella* dynamics in the dual environment.

4.1 Introduction

Salmonella was the second most common cause of notified cases of food-borne outbreaks in Australia in 2008 (8,310 cases with a rate of 39 cases per 100,000 populations) (OzFoodNet Working Group 2009). Figures from the United States suggest that *Salmonella* causes 1.4 million cases, 15,000 hospitalizations and 400 deaths each year (Voetsch *et al.* 2004). There is a general acceptance that poultry and poultry products are an important source of human food borne salmonellosis (Gast 2008) and thus emphasis needs to be placed on the contribution of the farming environment to reducing both *Salmonella* levels (and serovar diversity) along the food chain.

In Australia, it has been estimated that around 70% of broiler chickens are grown on new bedding (sawdust or pine shavings) (Runge *et al.* 2007). In the remaining 30% of production, litter re-use occurs with the chickens grown under a partial litter re-use practice (Runge *et al.* 2007). Due to constraints in sourcing suitable bedding material this re-use practice is set to grow in the future, despite increasing concerns about possible pathogen transfer across cycles via used litter. Under this partial re-use regime, the litter at the end of the first broiler cycle is windrowed, the shed cleaned and the windrowed litter spread out over the grow-out end (back) of the shed. The brooder end (front) of the shed always receives fresh bedding. The young chickens are kept on this fresh bedding until around 14 days of age (and separated from the rest of the shed via a curtain) and are then allowed full access to the back of the shed. This cycle will repeat for another 3-5 broiler cycles (with some litter often being removed to reduce the accumulation of used litter at the end of each cycle) until a full removal of all litter occurs and the process re-starts (Runge *et al.* 2007).

The present study was designed to take into consideration the “dual environments” that occur as a result of the Australian re-use practice and the impact of these environments on *Salmonella* levels and serovar diversity during the broiler production cycle. The study was performed on farms with different cycle ages to allow an understanding of the overall picture of the Australian litter re-use practice.

Materials and methods

4.2.1 Broiler farms and litter management

Two farms, coded A and B, were examined in this study. On both farms, a single shed was selected for study with both study sheds being tunnel-ventilated controlled environment sheds. The two farms operated on an all in all out basis with chicks being placed as day olds (32,000 – 35,000 chicks per shed). Both farms used the partial litter re-use practice as described in the Introduction, with both farms using pine shavings. On Farm A, a full clean-out occurred after three broiler cycles while on Farm B, the full clean out occurred after the sixth broiler cycle. On Farm A, the study was performed during broiler cycle 2 while on Farm B, the study was performed on broiler cycle 6. The young chicks were released from the brooder area at day 14 on the two farms. On both farms, multiple harvesting (a standard Australian practice) of chickens occurred. Under this practice, the bird density (expressed in kilograms of chicken meat per square metre of shed area) reaches a maximum around Day 35 of the cycle. A partial thin-out then occurs, reducing the numbers of birds in the shed. Depending upon market demands, further partial thin-outs may occur. The final collection of chickens occurred at Day 56 on Farm A and Day 53 on Farm B.

4.2.2 Litter and surface dust sampling

Litter samples were collected from both the brooder and grow out ends of the shed at weekly intervals over the full broiler cycle. The shed was divided into five different zones to represent the different litter types.

N1 – New litter – the half of the brooder end near the shed entrance that received new litter

N2 – New litter - the half of the brooder end near the centre of the shed that received new litter

Buffer - this area was in the centre of the shed and was not tested due to the possible mixing of both litter types and remained as a buffer zone where no samples were collected

R1 – Re-use litter - the half of the grow-out end near the centre of the shed that received re-used litter after the windrowing process

R2 – Re-use litter - the half of the grow-out end at the end of the shed that received re-used litter after the windrowing process

Within each sampling zone (N1, N2, R1 and R2), three segments or bays (defined by the structural supports of the building) were randomly selected on each sampling date. Within each selected bay, three litter samples were collected. The samples were collected using a specially designed stainless steel sampler that collected litter to a depth of 40 cm over an area of 400 cm^2. Hence a total of nine samples were collected per sampling zone on each sampling date. Once collected the samples were stored chilled and transported to the laboratory. At the laboratory, the nine samples per sample zone were composited and a representative subsample obtained from which both physical and microbiological sampling was carried out. The preparation of the representative 25 g samples was as previously described (Chinivasagam *et al.* 2010a)

Surface dust sampling was carried out on Farm A. The sampling was done using a moistened cotton-tipped swab. The swab was used to sample a surface (25 cm^2) in a zig-zag motion (both vertically and horizontally). The swab was then placed in transport media and transported on ice to the laboratory. The surfaces sampled were as follows:- drinker line, feeder line, drinker and feeder suspension ropes (1 m length sampled), bottom ledge of side wall, top ledge of side wall, surface of mini-vents in side wall, surface of ceiling curtain, surface of heater (brooder end only) and surface of feeder pipe (grow out end only). The sampling was done in two sheds. In Shed 1, sampling occurred at Days 39 and 47 in one cycle and Days 0, 12, 26, 33, 47 and 54 in the next cycle. In Shed 6, the sampling occurred at Day 47 in one cycle and then Days 1 and 15 in the next cycle. At each sampling date, the above sites were sampled in four bays each in the N and R regions. As this sampling was not performed on the same cycles as the full litter study, litter (1 gm per bay) was also aseptically collected.

4.2.3 Sample preparation and microbiological analysis

The representative 25 g samples were placed into 225 ml of 0.1% buffered peptone water, soaked and then homogenised as previously described (Chinivasagam *et al.* 2010a). For the enumeration of *Salmonella,* a three tube Most Probable Number (MPN) method using 0.1% buffered peptone water and MSRV and XLD was used as previously described (Chinivasagam *et al.* 2010a). Confirmation by the O.B.I.S.

Salmonella kit (Oxoid) and polyvalent *Salmonella* O antisera was performed as previously described (Chinivasagam *et al.* 2010a). As well as the MPN methodology, a presence absence test was performed on the initial dilution (25 g litter in 225 ml). A typical *Salmonella* colony from one positive XLD plate at all positive dilutions, including the presence absence test was randomly picked. All selected isolates were sent to a central reference laboratory for serotyping.

The environmental swabs were all handled individually. All swabs were subjected to a presence/absence test by placing the swab head in 10 ml of buffered peptone water and processing as described above. The 1 gm of litter was placed in 10 ml of buffered peptone water and processed in a presence/absence test as described above.

4.2.4 Physical measurements

The temperature of the spread litter, both brooder end (new bedding) and grow-out end (re-used litter) were measured using a data logger (DataTaker DT80). Four probes per litter type (new and re-used) were used. The temperature measurements were made over a 24 hour period prior to chicken placement and at days 18, 25, 38, 42 and 52.

The water activity of the samples was measured (Decagon Safe Storage Quick Check) as soon as the samples were collected. The probe was inserted into the sample bag, as much air as possible eliminated and the measurement taken after a minute.

Both pH and moisture content were measured on sub-samples taken from the same quarter allocated to microbiological sampling. The sample for moisture analysis was around 30 g and was placed in a zip lock bag without head space to minimise moisture transfer and held at 4^0C till analysed. Moisture content was determined in a standard laboratory assay. The moisture content was defined as the difference in weight of the litter sample before and after drying at 100^0C for 18 hours. The moisture content was calculated using the following formula from Opara *et al.* (1992):-

Moisture Content = $(W_O - W_D)/ W_O \times 100$

where W_O = original weight of sample and W_D = dry weight of sample.

Litter pH was measured by mixing a 6 g litter sample in 24 ml of distilled water with the pH being taken by pH meter after this suspension was left standing for 15 min.

4.2.5 Statistical analysis

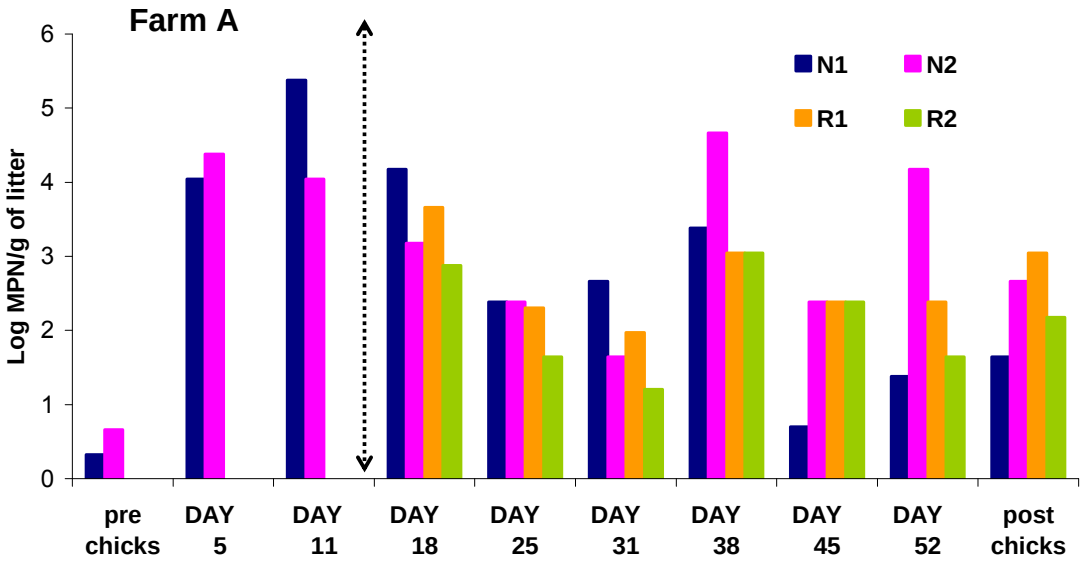
Chi-square analysis (using Statistix software) was used to test for a statistical difference ($p < 0.05$) in the number of litter samples above 10^2 MPN/g, the number of *Salmonella* serovars detected in the different litter types, the number of litter samples with a_w above 0.9 and the number of litter samples above a pH of 8.0.

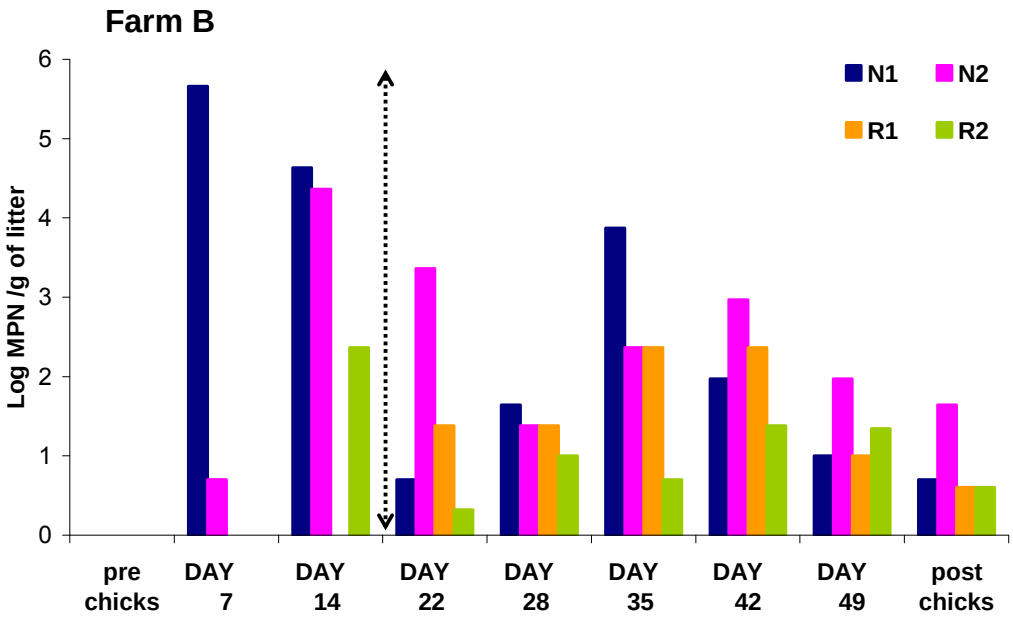
4.3 Results

4.3.1 *Salmonella* levels

The levels of *Salmonella* detected in the litter on Farms A and B are presented in Figure 1 A and B (respectively). On Farm A, no *Salmonella* was detected in the re-used litter prior to chicken placement but was present at low levels in the new litter. Following placement of the chickens, the *Salmonella* levels in the new litter (brooder) reached levels of between 10^4 to 10^5 cfu/g on days 5 and 11. In contrast, on days 5 and 11, the re-used litter did not yield *Salmonella* except in R2 at day 5 (present in 25g). At this stage, chickens were not present in the grow-out end where the re-used litter was placed. At day 18, when chickens were present in all parts of the shed, the two litter types had similar levels of *Salmonella*. From then onwards the levels tended to follow the same pattern, falling at days 25 and 31, rising at day 38 and stable on days 45 and 52, irrespective of litter type.

Figure 1. Levels of *Salmonella* detected in the litter over a full broiler cycle at Farms A (Fig. 1A) and Farm B (Fig. 1B). For Farm A, the R2 litter was positive in the presence / absence test only at day 5. For Farm B, the N1 litter was positive in the presence – absence test only prior to chick placement. The dotted line marks the date at which the chickens were allowed to move out from the brooder end of the shed.





On Farm B, a very similar picture of *Salmonella* levels were seen, i.e higher levels (10^5 to 10^6 cfu/g) in the new litter during the early stages (days 7 – 14) of the cycle, a fall during the mid cycle (day 35). As with the previous farm there was a drop in levels towards the end of the cycle (Fig 1B).

To assess differences in *Salmonella* levels in the two litter types a marker point of 10^2 MPN/g was selected. On both Farms, in the presence of chickens, there was no significant difference in the number of samples positive above and below this marker point in the two litter types.

The environmental (surface dust sampling) resulted in *Salmonella* Sofia being found in Shed 1, in both litter types from Day 26 onwards. All other samples (both litter and surface dust in the prior cycle, and surface dust in the current cycle) did not yield *Salmonella*. In Shed 6, the sampling (surface dust and litter) confirmed the presence of *Salmonella* in the litter at Day 47 in both litter types. At Day 1 in the next cycle, no litter was positive, while at Day 15 all litter samples were positive. Of the surface dust samples, only one drinker line (at Day 47) was positive. All other surfaces tested were negative for *Salmonella,* as in Shed 1.

4.3.2 *Salmonella* serovars

A total of 250 *Salmonella i*solates were serotyped. The detailed results are presented in Figures 2 and 3 (Farms A and B, respectively). On Farm A, *Salmonella* Chester and *Salmonella* Virchow were detected in the new litter prior to placement of the chickens. Both serovars were commonly detected in the new litter for the rest of the cycle. *Salmonella* Senftenberg was detected only early in the cycle in the new litter. *S.* Sofia was a common serovar in the new and re-used litter from days 31 (new) and 25 (re-used). The re-used litter yielded three serovars over the cycle (one occurring only once – *Salmonella* Zanzibar) while the new litter yielded five serovars with all serovars being detected on at least two occasions. Several serovars were present in the new litter but not the re-used litter (*S.* Senftenberg, *Salmonella* Singapore and *S.* Virchow) and only in the re-used litter but not the new litter (*S.* Zanzibar).

Figure 2 Change in *Salmonella* serovars across New and Re-used litter zones of a single shed on Farm A. At all sampling times, each litter type was represented by 18 samples across the relevant zone of the shed, which was composited into a single laboratory sample for each zone (i.e. N1, N2, RI, R2). Each bar represents the total number of colonies taken from the positive MPN dilutions (and the presence absence test).

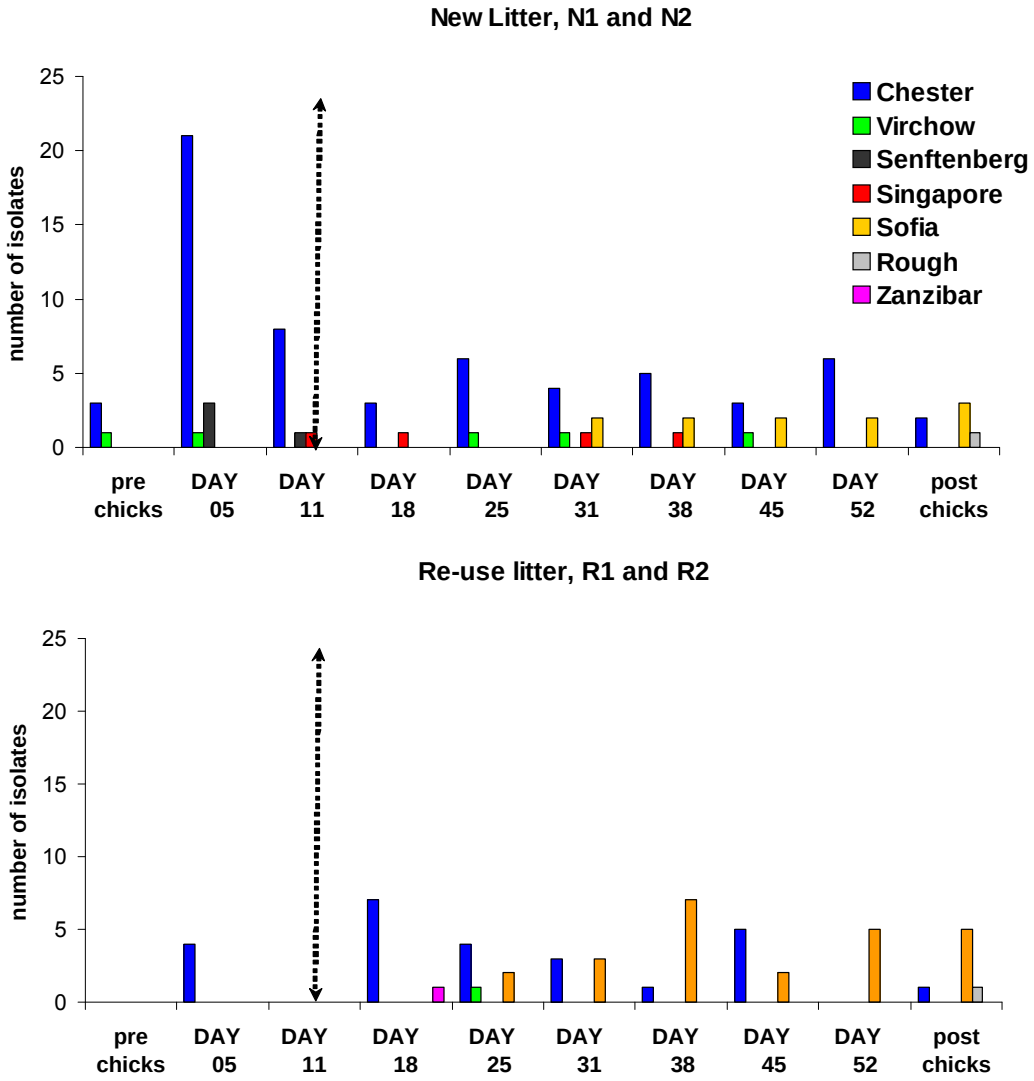
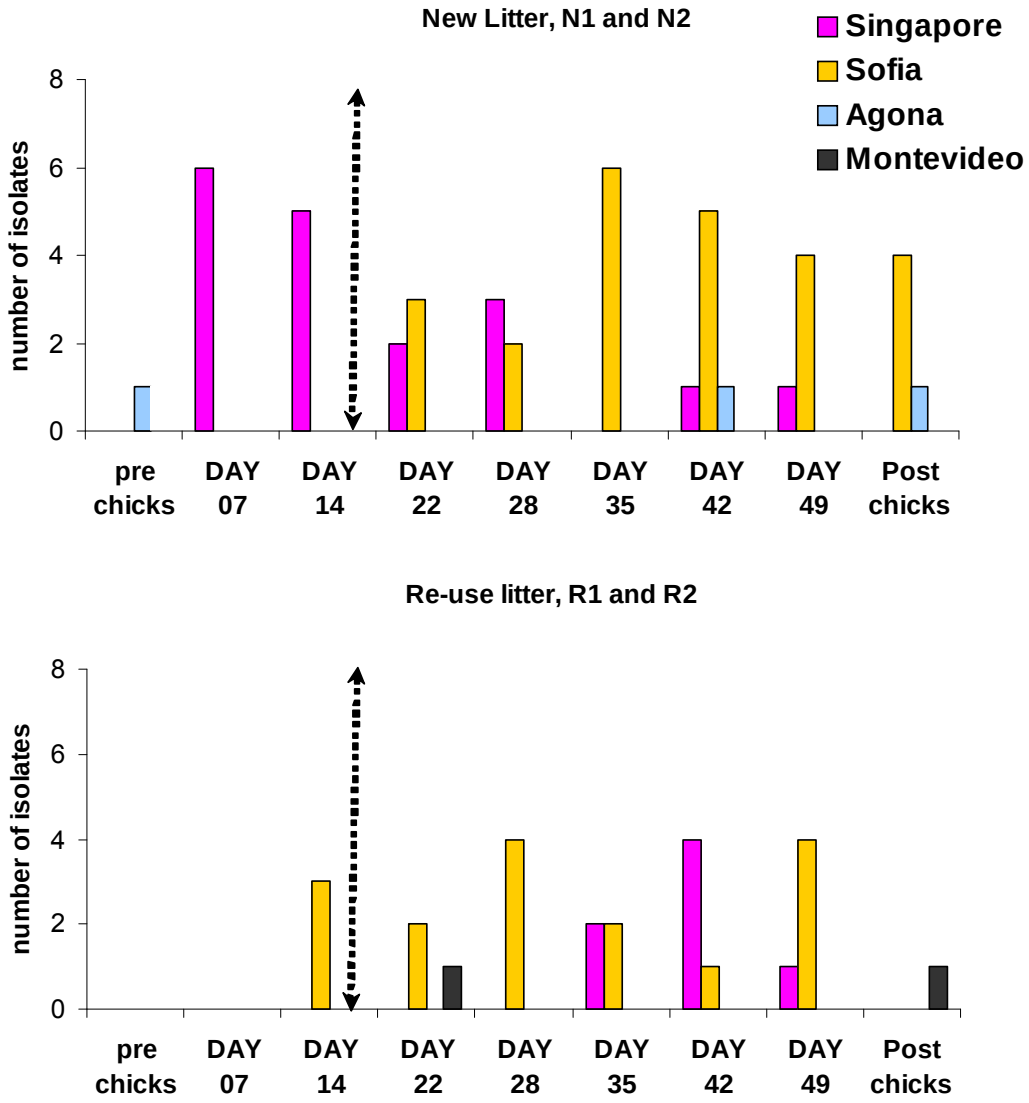


Figure. 3 Change in *Salmonella* serovars across New and Re-used litter zones of a single shed on Farm B. At all sampling times, each litter type was represented by 18 samples across the relevant zone of the shed, which was composited into a single laboratory sample for each zone (i.e. N1, N2, RI, R2). Each bar represents the total number of colonies taken from the positive MPN dilutions (and the presence absence test).



On Farm B (Figure 3), *Salmonella* Agona was detected prior to chicken placement in the new litter and then again several times later in the cycle. Two other serovars were consistently detected in the new litter after days 7 (*S.* Singapore) and 14 (*S.* Sofia). In the re-used litter, serovars *S.* Singapore and *S.* Sofia were present at most samplings from day 14 onwards. Two serovars were detected on single dates – *S.* Zanzibar at day 22 and *Salmonella* Montevideo at day 53 (post harvest).

On both farms, *S.* Sofia while a dominant serovar in the latter parts of the cycle (in both litter types) was not detected in the early days of the cycle. In looking at the number of serovars that were detected at least two times in a cycle, the new litter supported more serovars (5 and 3) than the re-used litter (2 and 2) on Farms A and B respectively (a difference that was not statistically significant).

4.3.3 Water activity and moisture levels

The a_w and moisture levels in both litter types across the production cycle on Farms A and B are presented in Table 1. As a_w levels of above 0.9 are seen as supportive of *Salmonella* growth, the results were analysed in terms of this marker point. On Farm A, the new litter recorded levels of above 0.9 on 17 of 20 testing occasions, which was significantly more than the re-used litter (8 of 20 occasions) (p = 0.008). On Farm B there was a tendency for the new litter to have more occasions of levels above 0.9 (10 of 18 sampling occasions) as compared with the re-used litter (5 out of 18 occasions), although the difference was not statistically significant (p = 0.17). The percent moisture in both litter types on both farms tended to be around the 25% level once the chickens were present on the litter. On Farm B the peaks in water activity observed through the shed on day 35 coincided with the time at which there was a maximum of bird density which is around the time that the first thin-out of birds occurred (Day 35).

Table 1 Changes in pH, Moisture and Water Activity across zones of New (N1, N2) and litter, Re-used litter (R1, R2) on Farms A and B

Farm A												
Time	pH				Moisture				Water activity			
	NI	N2	R1	R2	NI	N2	R1	R2	NI	N2	R1	R2
pre	7.45	7.89	8.23	8.5	43.4	39.4	23.4	26.3	0.95	0.99	0.94	0.91
day 5	8.14	7.88	8.67	8.76	25.8	26.0		24.5	1.02	0.98	0.93	0.92
day 11	7.89	7.74	8.65	8.64	24.7	23.5	20.9	21.5	0.96	0.92	0.81	0.83
Chicken movement across the shed												
day 18	7.17	8.10	8.21	8.30	23.9	22.2	23.3	25.7	0.90	0.87	0.82	0.83
day 25	8.08	8.27	8.41	8.47	28.2	26.8	31.1	29.5	0.98	0.99	0.95	0.99
day 31	8.32	8.56	8.27	8.29	26.7	26.5	24.9	27.7	1.00	0.92	0.87	0.89
day 38	8.57	8.59	8.16	8.31	25.3	27.1	29.4	26.4	1.00	0.92	0.87	0.90
day 45	8.27	8.18	8.12	8.15	22.8	22.6	25.5	27.0	1.00	0.93	0.88	0.88
day 52	7.87	8.36	8.38	8.24	27.5	24.8	27.3	26.1	0.99	0.97	0.94	0.93
post	8.06	8.52	8.63	8.06	27.5	23.8	24.9	37.4	0.83	0.80	0.79	0.83
Farm B												
Time	pH				Moisture				Water activity			
	NI	N2	R1	R2	NI	N2	R1	R2	NI	N2	R1	R2
pre	6.55	6.9	7.88	8.13	13.1	11.6	26.5	27	0.59	0.60	0.80	0.77
day 7	7.26	6.28	8.3	8.14	22.4	28.3	19.8	20.1	0.93	0.87	0.77	0.82
day 14	8.25	8.34	7.9	7.84	20.8	23.4	23.8	20.9	0.88	0.86	0.78	0.78
Chicken movement across the shed												
day 22	8.19	8.36	7.72	7.34	25.6	22.9	26	24.3	0.85	0.88	0.85	0.86
day 28	8.34	8.96	8.21	8.2	25.3	28.5	27.8	26	0.87	0.93	0.83	0.81
day 35	8.46	8.58	8.3	8.3	28.6	28.4	28.6	29.4	1.03	1.04	0.99	1.03
day 42	8.45	8.53	8.52	8.52	26	25.8	24.7	27.2	0.96	0.97	0.91	0.95
day 49	8.48	8.37	8.44	8.32	24.3	22	22.8	25	0.91	0.90	0.88	0.86
Post	8.25	8.61	8.7	8.38	24.5	24.3	24.3	24.7	0.91	0.90	0.92	0.87

4.3.4 pH levels

The pH results are presented in Table 1. On both farms, the re-use litter had a higher pH than the new litter prior to chicken placement. The new litter at Farm B showed a lower pH than the new litter at Farm A. After the chickens were present in all areas of the shed (day 18 for Farm A and day 14 for Farm B), the two litter types tended to have similar pH levels, with the levels being between 8 to 8.5 from around day 22 onwards. To look for differences in pH levels associated with litter types, the marker point of a pH level above 8.0 was used. On both farms there was no statistical difference in the number of re-use litter samples above pH 8.0 as compared with new litter samples.

No statistical analysis of litter type and pH was attempted prior to chicken placement as the differences are marked, due absence of chicken waste in one litter type.

3.5 *Temperatures*

On both farms the temperature of the re-used litter was higher than the new litter prior to chicken placement. As an example on Farm A on the day of placement, the new litter temperature was between 23 – 25^{o}C. In contrast the re-used litter ranged between 26 – 34^{o}C. A similar difference was seen on Farm B. On all the remaining sampling days, the temperature of the two litter types did not show any marked difference on either Farm.

4.4 Discussion

The litter management practice used on the farms in this study (new litter in the brooder end and re-used litter in the grow-out end) means there is a “dual litter environment” within the shed and perhaps a “dual set of conditions” impacting on pathogen survival across a chicken cycle. This study set out to assess *Salmonella* dynamics under such conditions, which also can be impacted by re-use practices adopted.

The outcomes from this study seem to suggest that this “dual environment” does result in variation in the *Salmonella* dynamics in the litter types. There was a tendency for a higher level of *Salmonella* in the new litter across both farms. Overall the older litter had lower *Salmonella* levels (in 25 out of 28 sampling events where both litter types were sampled in the presence of chickens). There was also tendency for a greater variety of serovars in the litter originating from the brooder end compared to the grow-out end with the possibility that this re-use litter was inhibitory for *Salmonella*.

There is evidence that used litter can have considerable anti-*Salmonella* activity. In pen trials, used litter contaminated with *S.* Typhimurium did not cause transfer to newly placed chickens and the *Salmonella* in the litter could not be detected in 3-5 days (Olesiuk *et al.* 1971). It has been shown that *Salmonella* does not persist as long in old poultry litter as in fresh litter (Fanelli *et al.* 1970). In addition *Salmonella* levels in the caeca of chickens raised on used litter were significantly lower than those in chickens raised on new litter (Corrier *et al.* 1992). Hence, the finding in the current study that the re-used litter tended to be associated with lower levels of *Salmonella* and lower serovar diversity matches with the existing literature. It is possible that the windrowing process used on these farms had some influence on the anti-*Salmonella* capacity of the re-used litter. It is certainly clear that the re-used litter is no worse or possibly even better (in food-safety terms) than the new litter.

Used litter following a chicken cycle can be a source of residual *Salmonella,* raising concerns of the possible transfer of these organisms through to the next chicken cycle (post treatment). The litter re-use procedures vary across countries and there are universal concerns with regards to possible pathogen transfer across flocks. Multiple sampling of chicken litter during the growing period across three consecutive flocks monitored on four farms in Nova Scotia resulted in 16 % of the

used litter from two farms using wood shavings being positive for *Salmonella* (with 13 different serovars) (Long *et al.* 1980) with concerns of transfer across broiler cycles. Similarly, the present study has demonstrated that *Salmonella* can persist in litter through various stages of the chicken cycle. However, in the current study, when litter was tested at the grow-out end of the shed prior to the movement of the young chickens to the area, all samples taken by day 11 or earlier (five sampling dates across the two farms) were negative for *Salmonella*. On one sampling occasion the presence of *S*. Chester (a serovar that was dominant with the placed flock) was detected in the presence absence test and could have occurred as result of chickens escaping into the re-use end from the brooder end. All the evidence gained in this work points out that *Salmonella* levels only increased in re-used litter after the movement of chickens to this area of the shed. This is a positive food-safety outcome in terms reducing concerns of the possible transfer and an increase in levels due to the previous cycle.

Studies carried out under laboratory conditions have shown *Salmonella* to survive in poultry feed and litter at 25^{o}C for 16-18 months and at 38^{o}C for 40 days in feed and 13 days in litter (Williams & Benson 1978). Hence, while the re-used litter on both study farms had a temperature above that of the new litter prior to chick placement, the difference is not likely to have any impact on *Salmonella* dynamics.

On both study farms, the two litter types generally showed a pH range of 7.5 to 9.0. Under experimental conditions, *Salmonella* populations have been shown to exhibit an initial growth (of around 2 logs) and then a stable period of up to 42 days under conditions of pH 7 and 9 (with a suitable a_w of 0.96) (Payne *et al.* 2007). Hence, while necessary to consider the interaction with a_w (see discussion below), the two litter types had a pH range which, in isolation, would seem unlikely to have any major impact on *Salmonella* dynamics.

Studies on broiler litter have shown the following to be a general indicator for the presence of *Salmonella* - an a_w of 0.75 – 0.83, 0.83 – 0.90 and 0.90 – 0.96 being negative, a transition zone and positive, respectively (Carr *et al.* 1994). As well, a water activity of < 0.84 is regarded as effective for reducing *Salmonella* populations (Payne *et al.* 2007). It has been suggested that holding chicken manure at a water activity of 0.89 can reduce *Salmonella* levels by a million fold and hence adjustment

of suitable ventilation rates for a few hours to achieve appropriate water activity can help to control *Salmonella* in poultry houses (Himathongkham *et al.* 1999a).

In the current study, one farm (Farm A) showed a statistically significant number of samples with an a_w of <0.9 in the re-used litter as compared with the new litter while the second farm (Farm B) had a similar trend, although not significant. Overall, the results in the current study suggest that a_w differences in the new and re-used litter may be part of the reason for the trend to lower numbers and lower serovar diversity in the re-used litter. It is important to consider the interaction of pH and a_w in this context. (Payne *et al.* 2007) have shown that litter at pH 9 and an a_w of 0.96 will support initial growth and a steady *Salmonella* population while litter at pH 9 and an a_w of 0.91 will support only a slight growth and then a steady decline, with a_w levels of less than 0.84 being associated with even more marked declines.

The presence of *Salmonella* within the shed environment even after a shed clean out can impact *Salmonella* dynamics in the litter and thus the chicken. As an example *Salmonella* was isolated from settled dust within a shed, in the absence of chickens, possibly as a result of a residual effect of a previous broiler cycle (Chinivasagam *et al.* 2009). In the present study, the possible presence of *Salmonella* was examined on shed surfaces such as drinkers, feeders, ropes, ledges, air vents, curtains, heaters and feeder pipes through the cycle. *Salmonella* was absent from the surfaces tested and though such surfaces could be a source of cross contamination they appear not to be a major source of contamination of the litter.

4.4 Conclusions

This study has examined *Salmonella* levels and serovar diversity in the "dual environment" that occurs under the litter re-use practices used on the two study farms. There was a trend for lower levels (and a lower serovar diversity) in the re-used litter environment as compared with the new litter environment. Of the physical parameters examined (pH, temperature, moisture content and a_w), it would appear that the lower a_w associated with the re-used litter may play a role in the *Salmonella* dynamics in the litter environment.

4.5 Acknowledgements

I acknowledge my co-authors T. Tran and P.J. Blackall

The financial support of the Australian Poultry CRC is gratefully acknowledged. The co-operation of the broiler integrator company, the farm management teams and Mr Geoff Runge (Queensland Department of Primary Industries and Fisheries) was an essential component on this work. We also acknowledge the support of the *Salmonella* Reference Laboratory at the Institute of Medical and Veterinary Science.

Chapter 5

Investigation and application of methods for enumerating heterotrophs and *Escherichia coli* in the air within piggery sheds

This Chapter is published in the *Journal of Applied Microbiology*

Citation:

H.N. Chinivasagam, P. J. Blackall (2005) Investigation and application of methods for enumerating heterotrophs and *Escherichia coli* in the air within piggery sheds*, Journal of Applied Microbiology*, **98**(5): 1137-1145.

5.1 Summary

Aims: To investigate methods for the recovery of airborne bacteria within pig sheds and to then use the appropriate methods to determine the levels of heterotrophs and *Escherichia coli* in the air within sheds.

Methods and Results: AGI-30 impingers and a six stage Andersen multi-stage sampler (AMS) were used for the collection of aerosols. Betaine and catalase were added to impinger collection fluid and the agar plates used in the AMS. Suitable media for enumerating *E. coli* with the Andersen sampler were also evaluated. The addition of betaine and catalase gave no marked increase in the recovery of heterotrophs or *E. coli*. No marked differences were found in the media used for enumeration of *E. coli*. The levels of heterotrophs and *E. coli* in three piggeries, during normal pig activities, were 2.2 X 10^5 CFU m^{-3} and 21 CFU m^{-3} respectively.

Conclusions: The failure of the additives to improve the recovery of either heterotrophs or *E. coli* suggests that these organisms are not stressed in the piggery environment. The levels of heterotrophs in the air inside the three Queensland piggeries investigated are consistent with those previously reported in other studies. Flushing with ponded effluent had no marked or consistent effect on the heterotroph or *E. coli* levels.

Significance and impact of the study: Our work suggests that levels of airborne heterotrophs and *E. coli* inside pig sheds have no strong link with effluent flushing. It would seem unlikely that any single management activity within a pig shed has a dominant influence on levels of airborne heterotrophs and *E. coli*.

5.2 Introduction

The piggery environment is a source of airborne contamination that consists of organic matter (dust) as well as various microbes (Mackiewicz 1998). The nature of these aerial contaminants is likely to vary with the ages of the pigs as well as the types of pig sheds. For example, pig activity tend to be minimal in the nursery area, while the growing and finishing areas involve much more pig activity and hence higher levels of airborne contaminants could be expected. Chang *et al*. (2001) found that the finishing units contained the highest level of both culturable and gram negative bacteria. High stocking rates as well as the body size of the pig, the accumulation of waste material and the moist air were all suggested as reasons for the high level of airborne bacteria in finishing units (Chang *et al.* 2001). In contrast, another study has reported that farrowing buildings had 50 –150% higher amounts of airborne microbes than finishing buildings (Donham 1991). Overall, inefficient effluent removal and lack of general hygiene are regarded as major contributing factors to the elevated airborne bacterial levels in piggeries (Chang *et al.* 2001). It has been suggested that control of airborne bacterial levels could be achieved by reducing the stocking rate and improving the ventilation as well as general hygiene (Banhazi & Cargill 1997).

Management of airborne bacterial levels is important for the health of piggery workers as well as neighbours and also helps reduce the overall environmental impact of piggeries. Clearly, the management of airborne bacterial levels in a piggery requires an ability to measure these levels. Any management strategies should be based on the use of optimised bacterial sampling methods. The six stage Andersen multi-stage sampler (Andersen 1958) and the AGI – 30 impinger (May & Harper 1957) both have a long history of use for the capture of airborne microbes in different environments, e.g. sewage treatment plants (Pillai *et al.* 1996) and wastewater spray irrigation sites (Bausum *et al.* 1983). These samplers are able to capture microbes by impaction (Andersen sampler) or impingement (AGI-30) with the bacteria then being enumerated by conventional methods.

Bacteria in aerosols are subjected to stresses associated with exposure to the atmosphere, with these stresses being typically associated with temperature and relative humidity (Marthi *et al.* 1990). The stress of collection due to impingement (into liquid collection media) or impaction (on to agar surfaces) (Stewart *et al.* 1995,

Terzieva *et al.* 1996) further compounds the difficulties associated with the recovery of viable airborne organisms for enumeration. To overcome this problem of stress and hence low viability, several studies in environments other than pig sheds have shown that the use of additives, such as betaine and catalase, can aid in the recovery of captured airborne bacteria (Marthi & Lighthart 1990, Marthi *et al.* 1991).

The present study looked at (a) the use of betaine and catalase for optimum recovery of airborne bacteria in piggery sheds and (b) the typical levels of heterotrophs and *Escherichia coli* in aerosols inside Queensland piggery sheds and (c) the effect of flushing (washing the gutters that collect faecal material with water from the effluent pond) on airborne bacterial levels in piggery sheds. In an earlier study of effluent in 13 Queensland piggeries, we found that key pathogens – specifically *Salmonella* spp and *Campylobacter* spp – were present at much lower levels (maximum of 0.5 MPN ml $^{-1}$, 930 MPN ml $^{-1}$ respectively) than the typical faecal indicator organisms – *E. coli* and thermotolerant coliforms (maximum of $1.96X10^5$ CFU 100 ml^{-1}, $2.1X\ 10^5$ CFU 100 ml^{-1} respectively) (Chinivasagam *et al.* 2004). Hence, in this work, we have concentrated on enumerating heterotrophs and *E. coli*.

5.3 Materials and Methods

5.3.1 Sampling plan

Three piggeries located in South-East Queensland, Australia were studied. Table 1 describes the sampling strategy adopted for the present study. Trials 1-3 assessed methodology for the optimum recovery of airborne heterotrophs and *E. coli*. In these trials, sampling was done in the morning before any effluent flushing was performed. Trials 4-8 evaluated levels of both airborne heterotrophs and *E. coli* during normal pig activity as well during flushing of the shed with re-cycled water from the effluent pond. In Trial 4, two sampling periods were used – one before effluent flushing and one during effluent flushing. In Trials 5, 7 and 8, sampling was done before effluent flushing, during effluent flushing and after effluent flushing. Trials 7 and 8 also continued the evaluation of media for the recovery of *E. coli*.

Table 1 Sampling strategy for piggeries used in study

Trial	Piggery (date)*	Media used	Aim of Trial (Instruments Used)
1	Piggery G (29/02)	R2A MLSA	Use of catalase and betaine for improving recovery (AMS and AGI-30)
2	Piggery G (21/03)	R2A MLSA	Use of catalase and betaine improving recovery (AMS and AGI-30)
3	Piggery W (19/10)	EMB MMGM	Assess use of different media for the recovery of *E. coli* (AMS)
4	Piggery G (20/06)	R2A EMB	Establish bacterial levels in shed, with flushing (AMS and AGI-30)
5	Piggery G (28/08)	R2A	Establish bacterial levels in shed, with flushing (AGI-30)
6	Piggery W (19/10)	EMB	Establish bacterial levels in shed, with flushing (AMS)
7	Piggery B (02/11)	R2A EMB Chromocult	Establish bacterial levels in shed, with flushing Assess use of different media for the recovery of *E. coli* (AMS and AGI-30)
8	Piggery W (16/11)	R2A EMB Chromocult	Establish bacterial levels in shed, with flushing Assess use of different media for the recovery of *E. coli* (AMS and AGI-30)

* Date given in day/month format

5.3.2 Nature of pig sheds

On Piggery G, the shed studied housed pigs from around 25 days of age to around 50 days of age. The building was naturally ventilated and had automatic self-feeders and nipple waterers. The pens were concrete floored with the rear area covered with slats. Beneath the slats was a gutter for the collection of urine and manure. Once daily, the gutter was flushed with water from the main piggery

effluent pond. The building was 50 m long, 10 m wide and 2.5 m high. The pigs were housed in pens containing around 22 pigs.

On Piggery W, the shed studied housed pigs from around 28 days of age to around 52 days of age. The building was naturally ventilated and had automatic self-feeders and nipple waterers. The pens were raised on a platform with a solid floor except for the rear area which was covered with slats. There was a gutter for the collection of urine and manure beneath the slats. Once daily, the gutter was flushed with water from the main piggery effluent pond. The building was 50 m long, 20 m wide and 3 m high. The pigs were housed in pens containing around 20 pigs.

On Piggery B, the shed studied housed pigs from around 28 days of age to around 55 days of age. The building was naturally ventilated and had automatic self-feeders and nipple waterers. The pens were raised on a platform with a slated floor. Once daily, the entire area under the pen was flushed with water from the main piggery effluent pond. The building was 50 m long, 10 m wide and 3 m high. The pigs were housed in pens containing around 20 pigs.

All three piggeries operated on a “continuous flow” system. In this system, pigs enter the shed at one end and are progressively moved along the shed until they reach the end of the shed. The pigs then move to the next shed. Hence, on these three farms, no shed was ever empty as pigs keep moving into and then out of the sheds.

5.3.3 Aerosol collection methods

AGI-30 impingers (Ace Glass, Inc., Vineland, N.J) with a 30 mm jet-to-bottom spacing and a six stage AMS viable particle sizing sampler (Graseby-Andersen Inc. Atlanta, Ga.) were used for collection of aerosols from the sheds. The flow rate of the impinger was maintained at a flow rate of 12.5 l min^{-1} while the flow rate of the AMS was 28.3 l min^{-1}. The flow rate for the AGI-30 impingers was established by use of a rotameter (Ace Glass, Inc., Vineland, N.J) inserted between the pump and the impingers. Glass petri dishes as supplied with the AMS plus additional glass dishes made to the AMS specifications were used with the AMS. Sampling height was set at 1.2 metres from the ground and sampling was performed in the centre of the shed. Two impingers were used at a point per sampling time, facing the same direction. They represented duplicate samples. Sampling time for all impingers was

20 min. All plates used with the AMS contained 27 ml of agar. The sampling time for the AMS varied and is set out in the following section.

5.3.4 Bacteriological methods

Media and sampling with AGI-30 impinger: The medium in the AGI-30 impingers was 19 ml of 0.1% peptone and 1 ml of 0.3% Antifoam A (Dow Corning). The antifoam was added prior to sterilisation.

For the evaluation of additives, the 16 ml of liquid remaining in each impinger after the 20 min collection time was split into two equal halves immediately after the collection period. Betaine (final concentration of 2 mmol l^{-1}), catalase (final concentration of 5 U ml^{-1}) and a combination of both betaine and catalase (with final concentrations as indicated for the single treatments) were added to one of the 8 ml volumes. The other 8 ml volume was retained as the untreated control.

On arrival at the laboratory, the impinger samples were stored at 4^{o}C overnight. All microbiological culturing was commenced within 24 h of arrival at the laboratory. A 2 ml volume of 0.1% peptone was used to wash the neck of each impinger. A similar washing of the neck was performed on those impingers involved in the additive evaluation trials. In the additive trials, the washing was performed immediately after the sampling. The washing was performed as it is a recommended procedure when using AGI-30s to determine the total airborne micro-organism count (Jensen *et al.* 1994).

For enumeration of airborne heterotrophs, 0.1 ml volumes of appropriate serial dilutions were spread on R2A agar (Oxoid). The inoculated plates were incubated at 30^{o}C for 48 h. Colonies were counted after 48 h and re-incubated for a further 48 h to observe any further growth of stressed organisms.

For enumeration of *E. coli,* a 5 ml volume of impinger fluid was filtered (0.45 μm pore size, Millipore) and the filter was then placed on Membrane Lauryl Sulphate agar (Oxoid). The plates were incubated at 30^{o}C for 4 h and 44.5^{o}C overnight. Presumptive colonies were confirmed as *E. coli* by observing gas production in EC broth (Oxoid) and indole production in tryptone water at 44.5^{o}C overnight.

Media and sampling with the AMS. For the enumeration of airborne heterotrophs, R2A Agar was used in the AMS with a sampling time of 40 – 45 s. After sampling, the plates were incubated at 30^{o}C for 48 h. In the trials, evaluating the role of additives, betaine and/or catalase were added to the R2A agar plates immediately before pouring. The final concentration of betaine and catalase was the same in the agar as in the impinger fluids.

The following were used in comparison of media for the recovery of *E. coli*; Eosin Methylene Blue (EMB) agar (Oxoid), Mineral Modified Glutamate Medium (MMGM) agar (Oxoid) and Chromocult (Merck), all with a sampling time of 12 min. The plates were held in a cool environment while being transported to the laboratory (arrival within 4 hr). The EMB and MMGM plates were incubated at 37^{o}C for 24 h while the Chromocult plates were incubated at 35^{o}C for 24 h. Typical *E. coli* colonies on MGMM and EMB were subcultured onto EMB and then confirmed as *E. coli* by testing for the presence of β-galacturonidase in an overnight tryptone broth containing 4 methylumbelliferyl- β –D- glucuronide (50 mg l^{-1}) (Oxoid BR071E). Suspect *E. coli* colonies on Chromocult (based on colony morphology and colour) were confirmed with a spot indole test as recommended by the manufacturer. All counts were calculated as CFU per cubic meter of air, (CFU m^{-3}).

5.3 Results

Effect of betaine and catalase on the recovery of heterotrophs and E. coli. Table 2 shows the effect of additives in the impinger fluid on the recovery of heterotrophs and *E. coli*. Table 3 shows the effect of adding additives to the agar plates used in the AMS. The results indicate no marked difference between the presence or absence of the additives, either singly or as a combination, for either heterotrophs or *E. coli*.

Table 2 Use of the additives, betaine and catalase, with impinger recovery fluid in assessing the recovery of heterotrophs on R_2A agar and *E. coli* via membrane filtration and MLS agar from piggery aerosols. Betaine was included at 2 mmol l^{-1} and catalase at 5 U ml^{-1} (final concentration). Three sets of AGI-30s were used in each Trial with repeats also used in Trial 1. The results are expressed as colony forming units (CFU) m^{-3}

Trial	Organism	Set 1		Set 2		Set 3	
		Betaine (CFU m^{-3})	Control (CFU m^{-3})	Catalase (CFU m^{-3})	Control (CFU m^{-3})	Betaine & Catalase (CFU m^{-3})	Control (CFU m^{-3})
1	Heterotrophs	$8.4 X10^4$	$1.9 X 10^5$	$9.0 X10^4$	$1.1 X 10^5$	$8.4 X10^4$	$1.1 X 10^5$
		$3.3 X 10^5$	$2.3 X 10^5$	$1.0 X 10^5$	$1.0 X 10^5$	$2.8 X 10^4$	$2.5 X 10^4$
2	Heterotrophs	$3.2 X10^5$	$8.4 X10^5$	$4.5 X10^5$	$9.0 X10^5$	$2.0X10^5$	$2.2X10^5$
1	*E. coli*	Not recovered	Not recovered	22	22	11	Not recovered
		Not recovered	Not recovered	11	Not recovered	Not recovered	Not recovered

Use of AGI-30 and AMS for heterotroph counts. Trials 1 and 2 involved the use of both AMS and AGI-30 to recover airborne heterotrophs. In Trial 1, the heterotrophic count using AGI-30s, ignoring the presence or absence of additives, varied from 8.4 X 10^4 to 2.3 X 10^5 CFU m^{-3} (Table 2) while the single AMS heterotroph count was 2.8 X 10^4 CFU m^{-3} (Table 3). Further work, again ignoring the presence or absence of additives, was performed in Trial 2 – with the AGI-30 count varying from 2 – 9 X 10^5 CFU m^{-3} and the AMS count varying from 1.8 – 4.1 X 10^4 CFU m^{-3}

Recovery levels in the different stages of the AMS. When the AMS was used for heterotroph counts, and ignoring the presence or absence of additives, the highest level of recovery of CFUs was obtained on stage 1 ($\geq$ 7.0 μm), ranging from 40-52% of the total count, with stages 2 (4.7 – 7.0 μm) and 3 (3.3 – 4.7 μm) showing the next levels with 12 – 24% for stage 2 and 13 – 17% for stage 3 (Table 3).

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Table 3 Use of Andersen multi-stage sampler AMS for assessing the recovery of heterotrophs with or without the presence of additives on R2A agar from piggery aerosols. Betaine was included at 2 mmol l^{-1} and catalase at 5 U ml^{-1} (final concentration). The counts are expressed as CFU m^{-3} and as an overall percentage of the total count of the sample

AMS Stage	Size Range (μm)	Trial 1	Trial 2			
		Control (CFU m^{-3}) (% count)	Control (CFU m^{-3}) (% count)	Betaine (CFU m^{-3}) (% count)	Catalase (CFU m^{-3}) (% count)	Betaine & Catalase (CFU m^{-3}) (% count)
1	≥ 7.0	$1.1X10^4$ (39.6)	$1.9X10^4$ (52.8)	$7.0X10^3$ -	$1.7X10^4$ (42.5)	$7.7X10^3$ (43.0)
2	4.7 – 7.0	$6.6X10^3$ (23.7)	$4.4X10^3$ (12.3)	$7.7X10^3$ -	$9.2X10^3$ (22.2)	$4.1X10^3$ (22.8)
3	3.3 – 4.7	$4.3X10^3$ (15.5)	$4.6X10^3$ (13.0)	$8.0X10^3$ -	$7.0X10^3$ (16.8)	$3.7X10^3$ (21.0)
4	2.1 – 3.3	$2.1X10^3$ (7.6)	$5.3X10^3$ (15.0)	Spreading colonies, not possible to count	$2.0X10^3$ (4.8)	$1.4X10^3$ (8.1)
5	1.1 – 2.1	$2.1X10^3$ (7.6)	$1.5X10^3$ (4.3)	$1.1X10^3$ -	$5.2X10^3$ (12.6)	$7.0X10^2$ (3.9)
6	0.65 – 1.1	$1.7X10^3$ (6.1)	$9.9X10^2$ (2.7)	$5.1X10^3$ -	$4.2X10^2$ (1.0)	$2.3X10^2$ (1.3)
Total	Total	$2.8X10^4$	$3.6X10^4$	not calculated	$4.1X10^4$	$1.8X10^4$

Table 4: *Escherichia coli* counts (CFU^{-3}) using AMS (including stage recovered) using EMB, MMMA and Chromocult during aerosol sampling in Trials 3, 6 and 7.

Trial	*E. coli* (CFU^{-3}) count					
	EMB	Stages recovered (AMS)	MMMA	Stages recovered (AMS)	Chromocult	Stages recovered (AMS)
3	38	1	38	1, 3, 4	Not done	-
	27	1, 2, 3				-
6	59	1	Not done	-	35	1, 2
	24	1	Not done	-	15	1
	9	1, 2	Not done	-	9	1
7	32	1, 2, 5, 6	Not done	-	32	1, 2
	47	1, 2, 5	Not done	-	9	1, 3, 4
	35	1, 3	Not done	-	18	1, 2

Recovery of E. coli. We found no marked difference in any of the three media used for the recovery of *E. coli* in the AMS (Table 4). Similarly, we could find no great difference in the *E. coli* count using either impingement (the AGI-30) or impaction (AMS) (see Table 5, data for Trial 4). As shown in Table 5, the AGI detected 10 CFU m^{-3} *E. coli* while the AMS detected 3 CFU m^{-3} *E. coli* in the first sampling in Trial 4. A second sampling, done 30 minutes later, resulted in counts of 21 CFU m^{-3} *E. coli* in the AGI and 42 CFU m^{-3} *E. coli* in the AMS (Table 5).

Table 5 Results of air sampling, within a piggery environment, for heterotrophs (CFU m^{-3}) and *E. coli* (CFU m^{-3}), using AGI-30 impingers and AMS respectively

Trial (Piggery)	Time of Day	Activity in Shed	Heterotrophic count (CFU m^{-3}) AGI-30	*E. coli* count (CFU m^{-3}) AMS
4	8:30 am	Normal pig activity	4.3×10^5	3 (10)*
	9:00 am	Effluent flushing	6.0×10^5	42 (21)*
5	8:45 am	Normal pig activity	3.5×10^4	Not Done
	9:15 am	Effluent flushing	4.5×10^4	"
	10:20 am	Normal pig activity	1.0×10^5	"
	11:20 am	Normal pig activity	6.0×10^4	"
	12:20 pm	Normal pig activity	6.0×10^4	"
	13:20 pm	Normal pig activity	8.7×10^4	"
7	10:35 am	Normal pig activity	7.8×10^5	59
	11:35 am	Effluent flushing	2.6×10^5	24
	11:55am	Normal pig activity	1.7×10^5	9
8	11:00am	Normal pig activity	2.0×10^5	32
	11:50am	Effluent flushing	3.2×10^5	47
	13:30 pm	Normal pig activity	2.7×10^5	35

* Results in brackets are the corresponding count obtained using AGI-30

The levels of airborne bacteria in pig sheds. The levels of airborne heterotrophs and *E. coli* were determined before effluent flushing, during effluent flushing and one hour after effluent flushing for all three piggeries in this study (Trials 5, 7 and 8 on Piggeries G, B and W) (Table 5). Additional sampling was done at Piggery G and is also presented in Table 5. The additional sampling consisted of sampling on a separate occasion (Trial 4) and prolonged sampling during Trial 5. In these trials, AGI-30 impingers were used to sample heterotrophs and the AMS with EMB agar was used to sample *E. coli*.

The counts of the heterotrophs across the piggeries and in the presence or absence of effluent flushing showed no great variation. In Trial 5 on Piggery G, samples were taken every hour, again with no great change in the heterotroph count which varied only from a minimum of 3.5×10^4 to a maximum of 1×10^5 CFU m^{-3}.

Similarly, there was no marked change in the counts for *E. coli* – either between piggeries or within a piggery in the presence or absence of effluent flushing. The highest count for *E. coli* was 59 CFU m^{-3} in Trial 7 (Piggery B) before effluent flushing. The lowest *E. coli* count was 3 CFU m^{-3} in Trial 4 (Piggery G), again before effluent flushing.

As there was little variation in the counts of either the heterotrophs or *E. coli*, we pooled the counts for the respective organisms for all trials performed in this study to calculate geometric means. The means were calculated for all heterotroph counts performed using AGI-30 impingers and R2A either in presence or absence of effluent flushing. A similar pooling and calculation was done for all *E. coli* counts performed with the AMS, regardless of the actual agar used in the AMS. The resultant geometric means are shown in Table 6.

Table 6 Geometric means (and geometric standard deviations)* of all heterotrophs (CFU m^{-3}) and *E. coli* (CFU m^{-3}) counts across the trials performed in this study. The heterotroph counts were performed using AGI-30 impinger and R2A agar while the *E. coli* counts were done using the AMS and with several different media

Activity in piggeries	Heterotrophic Count (10^5CFU m^{-3})	*E. coli* (CFU m^{-3})
Flushing	2.2 (X $2.8^{\pm 1}$)	23 (X $2.1^{\pm 1}$)
No Flushing, normal pig activity	2.2 (X $2.8^{\pm 1}$)	21 (X $2.1^{\pm 1}$)

* The geometric standard deviation (GSD) (Kirkwood 1979) is the anti-logarithm of the SD on the log scale, and is a multiplicative factor such that a range of mean ± SD on the log scale is equivalent to a range of $e^{\text{mean} \pm \text{SD}}$ on the original counts scale, and is obtained by dividing and multiplying the geometric mean by GSD.

5.4 Discussion

The aerosols within a piggery are unique to the environment in which they are created. Bacteria and fungi as well as endotoxins form a major biological component of piggery aerosols (Donham 1991). An understanding of the levels of bacteria (as well as pathogens) in the air inside piggeries is necessary to guide efforts to manage the levels of these organisms. Management of the levels of airborne bacteria inside pig sheds will have a positive influence on the health of the workers in the sheds as well as reducing any pollution of the surrounding environment.

It is well recognised that enumeration of airborne bacteria by culture can underestimate the actual bacterial concentration due to problems with the ability of available media to culture these bacteria (Shahamat *et al.* 1997). As an example, Heidelberg *et al.* (1997) found that less than 10% of artificially aerosolised bacteria were capable of forming colonies on recovery media. However, it is not clear whether the laboratory based artificial aerosolisation process used by Heidelberg *et al.* (1997) to generate airborne bacteria (with the bacteria themselves being laboratory cultures) reflects the reality of a pig shed where environmental and pig associated bacteria are being aerosolised by natural processes. Hence, it is not clear if the low efficacy of culture methods reported by Heidelberg *et al.* (1997) apply to airborne bacteria in pig sheds, which are in their natural environment.

The enumeration of airborne bacteria is further complicated by the problem of stress (Marthi *et al.* 1990). Airborne bacteria are thought to be stressed by a number of factors with the aerosolisation process and a drop in osmotic pressure caused by the loss of cellular water regarded as being or prime importance (Marthi & Lighthart 1990). However culture methods still remain one of the popular methods used in bioaerosol studies as it allows the estimation of both composition and concentration simultaneously (Chang *et al.* 2001). In an attempt to assess bacterial levels in the pig shed environment, the present study investigated potential improvements in collection and enumeration methods for airborne heterotrophs and *E. coli*.

The addition of betaine, an osmoprotectant, has been shown to improve both the heterotrophic and Gram-negative bacterial count by 21-61% (Marthi & Lighthart 1990). In the present study, the use of betaine in the impinger fluid as well as in the

collection plates used in the AMS, did not result in any marked increase in the heterotrophic count. This finding implies that the heterotrophs were probably not suffering osmotic shock. It is possible that the nutrient-rich and moist environment that is typical of a pig shed may have reduced any problems associated with osmotic stress in the airborne heterotrophs. The prior studies reporting the beneficial results of adding betaine have all been performed in environments other than pig sheds e.g. home and outdoor environments in Mexico (Rosas *et al.* 1997) and an activated sludge tank, a farmland and off a roof environment (Marthi & Lighthart 1990). Possibly, airborne bacteria in pig sheds are not stressed by dehydration.

Catalase mediates the breakdown of toxic hydrogen peroxide and other peroxides generated by bacterial metabolism. Exogenous catalase, when added to injured cells, also mitigates the effect of toxic peroxides (Marthi 1994). The incorporation of catalase into enumeration media has been reported to result in a 63% increase in colony counts for airborne bacteria, although the beneficial effect of the catalase disappeared at high relative humidities of 80 -90% (Marthi *et al.* 1991). The use of catalase in the present study did not result in a marked increased recovery of either heterotrophs or *E. coli*. This would suggest that airborne bacteria inside pig sheds are not injured or susceptible to toxic peroxides.

Another possible reason for our failure to detect any response to the addition of catalase, or indeed betaine, on heterotrophic counts is the fact that we used R2A agar. R2A was specifically formulated to grow injured bacteria from potable water (Reasoner & Geldreich 1985). R2A is a minimal medium that contains sodium pyruvate, which has the capacity to degrade accumulated peroxides. Repair of dehydration stress in many species of bacteria occurs in minimal media (Marthi 1994). It is possible that the combination of the media and incubation temperature ($30^{o}C$) we used meant that additional improvement in recovery rates due to the addition of betaine or catalase was not possible. It is worth noting that other studies on the bacterial load in the air inside pig sheds have not used any protective additives such as catalase or betaine e.g. Elliot *et al.* (1976), Cormier *et al.* (1990), Thorne *et al.* (1992), Zucker *et al.* (2000) and Chang *et al.* (2001).

It is also possible that the approaches we have taken may not have been adequate enough to overcome any stress or injury present. As the methods we have tested

have been shown to be adequate in a number of different studies (Marthi 1994), this does not seem likely.

We investigated several different options for the enumeration of *E. coli* – membrane filtration of impinger fluid and a range of different agar in the AMS. The low numbers that we found with all methods meant that we found it difficult to detect any marked difference between any of the methods. On the basis of practical convenience, we elected to use the AMS with EMB agar for *E. coli* enumeration. Studies performed in piggeries in both Taiwan (Chang *et al.* 2001) and the United States (Thorne *et al.* 1992) have concluded that the AGI-30 is the best instrument for performing a total heterotrophic count while the AMS is the best instrument for performing a count of enteric bacteria.

We consistently found that the AMS results indicated that approximately only 40% of the heterotrophic bacteria present in the air were in the respirable range (< 5 μm). Few of the previous studies on the bioaersols present in pig sheds have separated the total count from the respirable size count. Cormier *et al.* (1990) did report this division – with the respirable component comprising from 25 – 35% for the heterotrophic count and 12 – 15 % for the Gram negative count. Similarly, Crook *et al.* (1991) found that most airborne bacteria were detected in stage 1 of a six-stage AMS.

Our finding that between 40-50% of heterotrophs colonies were recovered from the first stage of the AMS, which was a >7 μm pore size, means that either single or multiple cells of heterotrophic bacteria were part of large particle aggregates. It is possible that these particle aggregates may also have protected the attached bacterial cells from environmental stress, explaining our failure to detect any increased recovery in the presence of such compounds as catalase and betaine. It is probable that sampling by the AGI-30 resulted in a break-up of these aggregates. This particle break-up would be part of the reason why we have found that AGI-30 counts were higher than the matching AMS counts.

Table 7 presents a comparison of the results of the heterotroph and *E. coli* counts of this study and previous studies. Overall, a notable feature is that the results for the heterotrophic bacterial counts are broadly similar, despite the studies being performed under different conditions in terms of pig facilities and geographical location.

Table 7 Levels of heterotrophic bacteria and *E. coli* reported in various studies

***E. coli* or faecal coliforms*or Gam-negatives**, (number / m^3)**	**Heterotrophic Bacteria (number/m^3) (Range in brackets)**	**Conditions**	**Study**
21 (3 – 59)	220,000 (28,000 – 900,000)	Growers; naturally ventilated sheds with flushing; normal pig activity	Current Study
140** 71-495	492,000 (199,000 – 1,248,000)	Fattening units shed B	Canada (Cormier *et al.* 1990)
180** (18 – 371)	544,000 (292,000 – 996.000)	Fattening units shed D	Canada (Cormier *et al.* 1990)
		Fattening unit with ventilation	
	419,000	2 air changes h^{-1}	Canada (Butera *et al.* 1991)
	394,000	5 air changes h^{-1}	
	460,000	8 air changes h^{-1}	
25.3**	105, 972	Fan forced / fattening unit	Germany (Zucker *et al.* 2000)
7,000** (7,600 – 145,000)	86,000 (7,600 – 1,589,000)	Fattening unit with mechanical ventilation	Netherlands (Attwood *et al.* 1987)
	930,600 (613,700 – 1,246,700)	Breeding units	Poland (Mackiewicz 1998)
		Grower –	Scotland

	(300,000 – 8,000,000)	finishing unit	(Crook *et al.* 1991)
75** (4 – 450)	127,213 (214,813 – 717,421)	Growers with mechanical ventilation	Taiwan (Chang *et al.* 2001)
1,900* (8.8 – 4,100)	130,000 (420 – 1,500,000)	Growing finishing unit: 3 air changes h^{-1}	USA (Elliot 1976)
	210,000		USA (Thorne *et al.* 1992)
	58,000	Natural ventilation	USA (Predicala *et al.* 2002)
	65,000	mechanical ventilation	

* Indicates that the results are for Gram-negatives

** Indicates that results are for faecal coliforms

Our study is the only study of piggery shed aerosols to determine the level of *E. coli*. All of the other studies have used a broader group/category – either Gram-negative bacteria or faecal coliforms. Hence, it is not strictly possible to directly compare our *E. coli* results, which are for a single species, with the other studies that report results for a broad class of bacteria (that include *E. coli).*

The initial aims of our study were to provide base-line information, using optimised methods, on the levels of bacteria present in the air in Queensland pig sheds and to then investigate whether the re-use of effluent to flush away manure caused any marked increase in air-borne bacteria, particularly *E. coli*. As we could find no marked increase in either total air-borne bacteria or in air-borne *E. coli* during effluent flushing, we conclude that the use of effluent as a flushing liquid in a piggery shed is not a major factor in the bioaerosol load within the shed.

An important aspect of the study piggery and other animal derived aerosols is the challenge faced in recovering the maximum levels of organisms. Our results indicate that heterotrophs can be enumerated with the standard method of AGI-30 impingers and a minimal medium such as R2A agar, with no need for additives such as catalase and betaine. The challenge remains with Gram negative bacteria. Gram negative bacteria are a very small component of the typical total air-borne

bacterial count - being estimated at 0.04% (Chang *et al.* 2001) and 0.02 – 5.1% (Kiekhaefer *et al.* 1995). We found *E. coli,* a specific subsection of the overall Gram negative population, to be present at around 0.001% of the total airborne population. Hence, the enumeration of air-borne Gram negative pathogens that are common in animal environments, and which are of public health significance, such as *Salmonella*, *Campylobacter*, remains a significant challenge.

5.5 Acknowledgements

I acknowledge my co-author P. J. Blackall

This work was made possible by funding from Australian Pork Limited. The co-operation of the management of the piggeries examined in this study is gratefully acknowledged.

Chapter 6

Detection of *Arcobacter* spp. in piggery effluent and effluent irrigated soils in South East Queensland

This Chapter is published in the *Journal of Applied Microbiology*

Citation:

H.N. Chinivasagam, B.G. Corney, L.L. Wright, I.S. Diallo and P.J. Blackall (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland, *Journal of Applied Microbiology* **103**(2): 418-426.

6.1 Summary

Aims: To investigate the occurrence and levels of *Arcobacter* spp. in pig effluent ponds and effluent treated soil.

Methods and Results: A Most Probable Number (MPN) method was developed to assess the levels of *Arcobacter* spp. in seven pig effluent ponds and six effluent-treated soils, immediately after effluent irrigation. *Arcobacter* spp. levels in the effluent ponds varied from 6.5 X 10^5 to 1.1 X 10^8 MPN 100 ml^{-1} and in freshly irrigated soils from 9.5 X 10^2 to 2.8 X 10^4 MPN g^{-1} in all piggery environments tested. Eighty three *Arcobacter* isolates were subjected to an abbreviated phenotypic test scheme and examined using a multiplex PCR. The PCR identified 35% of these isolates as *A. butzleri,* 49% as *A. cryaerophilus* while 16% gave no band. All 13 non-reactive isolates were subjected to partial 16S rDNA sequencing and showed a high similarity (>99%) to *A. cibarius*.

Conclusions: *A. butzleri, A. cryaerophilus* and *A. cibarius* were isolated from both piggery effluent and effluent-irrigated soil, at levels suggestive of good survival in the effluent pond.

Significance and impact of the study: This is the first study to provide quantitative information on *Arcobacter* spp. levels in piggery effluent and to associate *A. cibarius* with pigs and piggery effluent environments.

6.2 Introduction

The genus *Arcobacter* was originally created to house organisms that were initially regarded as aerotolerant *Campylobacter* species (Vandamme *et al.* 1991). The genus currently consists of five species – *A. butzleri, A. cibarius, A. cryaerophilus, A. nitrofrigilis* and *A. skirrowii* (Houf *et al.* 2005). *A. butzleri* has been found in human extra intestinal diseases but little is known about the organism's pathogenicity and virulence (Lehner *et al.* 2005). Even though *A. butzleri* has not been directly linked to food-borne illness, the fact that the organism is found on meats and causes diarrhoeal illness in humans suggests it is a possible food-borne pathogen (Mansfield & Forsythe 2000).

Arcobacter spp. are found to survive in a wide range of environments such as the gut and faeces of pigs (Van Driessche *et al.* 2004, Wesley *et al.* 1996), poultry meat/carcass (Corry & Atabay 2001, Houf *et al.* 2002), poultry litter (Eifert *et al.* 2003), cattle (Kabeya *et al.* 2003), lamb meat (Rivas *et al.* 2004), drinking water (Jacob *et al.* 1998) and river water (Morita *et al.* 2004). Experimental infections of caesarean-derived, colostrum-deprived piglets showed that *A. butzleri, A. cryaerophilus* and *A. skirrowii* could all colonise the piglets but that severe gross pathology was absent (Wesley *et al.* 1996).

Arcobacter has been isolated from water treatment plants in Germany (Jacob *et al.* 1993), well- water in USA (Rice *et al.* 1999), river and canal waters in Japan and Thailand respectively (Morita *et al.* 2004) and sewage plants in Italy (Stampi *et al.* 1999). A laboratory strain of *A. butzleri* (NCTC 12481) was able to maintain membrane integrity after 35 d of starvation in non-chlorinated drinking water (Moreno *et al.* 2004). Water and effluent clearly have the potential to play a role in the transmission of *Arcobacter* spp.

Pigs are a source of *Arcobacter* with the prevalence in faeces (16-85%) increasing with age and the dominant species being *Arcobacter butzleri* (Van Driessche *et al.* 2004). *Arcobacter* spp. have been isolated from nursing sows, grower pigs and market-age pigs at slaughter (Hume *et al.* 2001). *A. butzleri* is a routine contaminant of pork, with plants in the USA showing a prevalence that varied between 0-90% (Collins *et al.* 1996). Thus, pigs have been found to be a reservoir

for *Arcobacter* spp. with the potential for the organism to transfer into the environment as result of effluent management practices. However, limited data are available on the role of piggery effluent in the survival and transfer of *Arcobacter* to the environment.

Being an organism of recent interest, no standard, widely-accepted methodologies for the isolation and enumeration of levels of *Arcobacter* exist. Several studies have compared various media formulations as well as enrichment procedures for the recovery and isolation of *Arcobacter* spp. (Corry & Atabay 1997, Atabay & Corry 1998, Johnson & Murano 1999a, b, Houf *et al.* 2001). There is a need for suitable, optimal recovery media and conditions that can detect the levels of *Arcobacter* spp. in a range of different sources such as faeces, carcasses and the environment.

The present study was carried out to understand the presence, levels and species-distribution of *Arcobacter* spp. in piggery effluent and soil using a Most Probable Number (MPN) technique. The study also evaluated the possibility of an abbreviated phenotypic testing scheme in comparison with a multiplex PCR for species identification.

6.3 Materials and Methods

6.3.1 Samples for initial evaluation of direct plating and the development of a MPN method

Fresh pig faeces and pig effluent were collected and held on ice until arrival at the laboratory (4 hours from collection), and processed on the day of collection. In addition, soil was placed in a plastic pot (pot volume of 200 ml) and the soil then saturated with pig effluent.

6.3.2 Direct plating method

Direct plating involved the use of CAT agar which consisted of CCDA agar (Oxoid CM 739) with added C.A.T. supplement (Oxoid SR 174). *Arcobacter* agar was also used and consisted of *Arcobacter* broth (Oxoid CM 965) with added C.A.T. supplement (Oxoid SR 174) and bacteriological agar (Oxoid L11) (12 g l^{-1}). All

media were prepared and supplemented as per the manufacturer's instructions. Direct plating was performed by mixing 10 g of faeces or 10 ml of effluent in 90 ml of 0.1% peptone. The mixture was shaken for 15 min. Serial 10-fold dilutions were prepared in 0.1% peptone by mixing 1 ml in 9 ml. Direct plating was performed by spreading 0.1 ml of the relevant dilution across the surface of the relevant medium. All inoculated plates were incubated at 28^0C for 24-48 h.

6.3.3 MPN Methods A, B and C

All MPN methods were three tube methods and 0.1% peptone was used as diluent. All dilutions were prepared as an initial 10 g (faeces or soil) or 10 ml (effluent) sample in 90 ml of 0.1% peptone. All subsequent serial dilutions were performed as 1 ml in 9 ml of 0.1% peptone. If an undiluted sample was tested, then 1 ml of effluent or 1 g of soil was added directly to the enrichment broth.

Method A The enrichment broth used was *Arcobacter* broth (Oxoid CM 965) with added C.A.T. supplement (Oxoid SR 174) (Atabay and Corry, 1998). The inoculated broths were incubated at 25^0C in tightly capped bottles under aerobic conditions for 48 h. The broths were then inoculated onto 5% sheep blood agar which was incubated for 48 h under aerobic conditions.

Method B This method used the same basic broth for the enrichment stage, *Arcobacter* broth (Oxoid CM 965), as used in method A with the supplements used by (Houf *et al.* 2001), ie. 5% lysed horse blood, amphotericin B (10 mg l^{-1}), cefoperazone (16 mg l^{-1}), 5-fluorouracil (100 mg l^{-1}), novobiocin (32 mg l^{-1}) and trimethoprim (64 mg l^{-1}). The broths were incubated under microaerobic conditions, at 28^oC, for 48 h. The plating medium consisted of *Arcobacter* broth (Oxoid CM 965), bacteriological agar (Oxoid L11) (12 g l^{-1}) and the same additives as the broth. The supplements were aseptically added just before the agar was poured. The inoculated plates were incubated at 28^oC, under aerobic conditions, for 48 h.

Method C The enrichment broth, termed JM broth (Johnson & Murano 1999a), contained special peptone (Oxoid L72) (10 g l^{-1}), yeast extract (5 g l^{-1}), beef extract (5 g l^{-1}), NaCl (4 g l^{-1}), potassium phosphate (monobasic) (1.5 g l^{-1}), sodium

phosphate (dibasic) (3.5 g l^{-1}), sodium pyruvate (0.5 g l^{-1}), sodium thioglycolate (0.5 g l^{-1}), charcoal (0.5 g l^{-1}), bile salts No 3 (Oxoid L56) (2 g l^{-1}) and bacteriological agar (Oxoid L11) (2 g l^{-1}). After sterilisation by autoclaving, the following supplements were added:- 5-fluorouracil (200 mg l^{-1}) and cefoperazone (32 mg l^{-1}). The inoculated broths were incubated at 30^0C for 48 h. After incubation, the JM broths were plated onto JM agar (Johnson and Murano, 1999b). JM agar contained special peptone (Oxoid L72) (10 g l^{-1}), yeast extract (5 g l^{-1}), beef extract (5 g l^{-1}), NaCl (4 g l^{-1}), potassium phosphate (monobasic) (1.5 g l^{-1}), sodium phosphate (dibasic) (3.5 g l^{-1}), sodium pyruvate (0.5 g l^{-1}), sodium thioglycolate and bacteriological agar (Oxoid L11) (12 g l^{-1}). Immediately before pouring, JM agar was supplemented with cefoperazone (32 mg l^{-1}) and defibrinated sheep blood (50 ml l^{-1}). The inoculated JM agar plates were incubated at 30^0C, under aerobic conditions, for 48 h.

6.3.4 Assessment of levels of *Arcobacter* spp. in effluent and soil

Effluent was collected from seven piggeries across south east Queensland over a period of 3 years. Around 1 l of effluent was collected from the final pond of each piggery and transported to the laboratory as previously mentioned. At five piggeries, effluent from the same pond was irrigated onto pasture near the piggery. Following this irrigation samples of soil were collected aseptically using a stainless steel core and to a depth of 4 cm within an hour after effluent application. The soil samples were composited and a 10 g sample aseptically weighed. The sample was then shaken for 30 min in 90 ml 0.1% peptone diluent. Appropriate 1 ml serial dilutions from both soil and effluent (in 0.1% peptone) were then used in the Method C MPN. Selected typical isolates were picked for further identification. The results were expressed as MPN / 100 ml of effluent.

6.3.5 Confirmatory identification of presumptive isolates

Typical colonies (greyish yellow to grey moist) were subcultured, as a single colony pick, onto Abeyta-Hunt-Bark agar without antibiotics (AHB) (Hunt *et al.* 2001) which consists of heart infusion agar (Difco Cat # 244400) (40 g l^{-1}) and yeast extract (2 g l^{-1}). After overnight incubation at 30^0C, subcultures on AHB agar were examined, under dark ground microscopy, for typical *Arcobacter* cell shape (slender, curved rods) and typical spiral motility. If the cell shape and motility were correct, the

following tests were performed – catalase (using 3% H_20_2), and oxidase (using MVD strips – Cat # BS210). The catalase reactions were termed as "weak" catalase positive meaning visible bubbles within10 -15 s or "rapid" catalase positive, meaning instantaneous bubbling.

Cadmium chloride sensitivity (Kazmi *et al.* 1985) was carried out using sterile blank discs that were impregnated with 20 μl of a solution that contained 2.5 μg of cadmium chloride per 20 μl. The cadmium chloride sensitivity test was performed by placing the disc on the AHB subculture plate in the primary inoculum area. After 24 h incubation, any zone of inhibition around the cadmium chloride disc was regarded as indicating a sensitive isolate. The indoxyl acetate reaction (On & Holmes 1992) was performed by preparing a 10% indoxyl acetate solution in ether and impregnating sterile blank discs with 25 μl of this solution. Dried indoxyl acetate discs were inoculated with a heavy smear of an overnight AHB agar culture and observed for 5 min. A dark blue colour under and around the growth was recorded as positive. To be regarded as *Arcobacter* spp, an isolate had to have the typical cell shape and motility, typical colony morphology on both JM agar and AHB agar and be oxidase and indoxyl acetate positive. $CdCl_2$ and catalase reactions were used to assign isolates to a presumptive *Arcobacter* species.

Arcobacter isolates were selected from effluent and soil over the period of the study to represent the dominant colonial morphologies (large and small - on JM agar) and biochemical variations based on catalase reactions ("weak" and "rapid") and $CdCl_2$ sensitivity.

6.3.6 *Arcobacter* Multiplex PCR

The type strains of *A. butzleri* (CCUG 30485 ^{T}), *A. cryaerophilus* (CCUG 17801 ^{T}) and *A. skirrowii* (CCUG 10374^T) were obtained from the Culture Collection of the University of Göteborg, Sweden.

The multiplex PCR for the identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* described by Houf *et al*. (2000) was used with some modifications. Template was prepared as follows. An overnight culture on JM agar was harvested

into 100 μl of sterile water using a standardised 1 μl loop. The suspension was heated for 10 min at 98^{0}C and then centrifuged for 45 seconds at 14,000g. A 2 μl volume of this preparation was used as the template for the PCR. The PCR was performed using a PCR High Fidelity Master Mix (Roche Cat # 2 140 314) and consisted of 25 μl of Master Mix, 2 μl of template, 50 pmol of primers ARCO, BUTZ, CRY1 and CRY2 and 25 pmol of primer SKIR and sterile water sufficient to make a final volume of 50 μl. PCR consisted of an initial denaturation at 94^{0}C for 2 min and 32 cycles of denaturation at 94^{0}C for 45 s, primer annealing at 61^{0}C for 45 s and chain extension at 72^{0}C for 30 s on a Hybaid Omnigene thermocycler (Thermo Hybaid Ltd, Middlesex, UK). A 10 μl sample of the assay was electrophoresed through a 1.5% agarose gel containing Tris-Acetate-EDTA (40 m mol l^{-1} Tris-acetate, 2mmol l^{-1} EDTA, pH 7.5) and ethidium bromide (0.5 μg ml^{-1}) in TAE buffer at 5.5 V cm^{-1} for 1h. The gel was viewed by ultraviolet illumination.

6.3.7 Amplification and sequencing of 16S rRNA

Arcobacter isolates that did not react in the multiplex PCR were subjected to partial 16S rDNA sequencing. DNA from two-day-old cultures was extracted using the QIAamp DNA Mini Kit (QIAGEN Cat # 51306) as per manufacturer's instructions. The DNA concentration was estimated using a spectrophotometer (Biophotometer, Eppendorf) and 16 to 160 ng of DNA was used in each PCR reaction. The 100 μl PCR reaction mixture contained 10μl of 10 x PCR buffer (Roche Cat 11146173001), 200 mmol l^{-1} of each dNTP (Roche Cat #11814362001), 0.4 μmol l^{-1} of each of the forward (27f) and reverse (1525r) primers (Lane, 1991) and 1.6 units *Taq* DNA polymerase (Roche Cat # 11146173001). The PCR was performed using a Hybaid Express Thermal Cycler (Thermo Hybaid Ltd, Middlesex, UK). Cycling consisted of an initial denaturation at 98^{0}C for 2.5 min followed by 29 cycles of denaturation at 93^{0}C for 1 min, annealing at 52^{o}C for 45 s and extension at 72^{0}C for 2 min. This was followed by a final cycle of denaturation at 93^{0}C for 1 min, annealing at 52^{o}C for 45 s and extension at 72^{0}C for 10 min. Amplicons were purified from the PCR reaction using a Montage PCR column (Millipore, Cat # UFC7PCR50) as per manufacturer's instructions. The resulting DNA was sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Cat # US81050) on an ABI Prism 377 DNA sequencer (PE Applied Biosystems Inc., Foster City, CA, USA). Both strands of the 16S rDNA were sequenced.

The resulting sequences were analysed using a FASTA search on the European Bioinformatics Institute website (http://www.ebi.ac.uk).

6.4 Results

6.4.1 Evaluation of direct plating media

When inoculated directly with dilutions of either pig faeces or pig effluent, both CAT agar and *Arcobacter* agar were overgrown with *Pseudomonas*-like organisms. There was evidence of colonies typical of *Arcobacter* present on some plates; however, the *Pseudomonas*-like organisms overwhelmed the slower growing *Arcobacter*-like organisms.

6.4.2 Selection of a suitable MPN method for *Arcobacter* spp.

The MPN methods, A B and C, were evaluated using the same sample of fresh piggery effluent. Method A did not yield typical *Arcobacter* spp. colonies at any of the three dilutions tested (zero, 10^{-1} and 10^{-2}) meaning a count of < 30 *Arcobacter* spp. MPN 100 ml^{-1}. Little or no growth following enrichment and plating was observed with method B (again yielding a count of < 30 *Arcobacter* spp. MPN 100 ml^{-1}). However, method C yielded positives for *Arcobacter* spp. for all three dilutions tested (zero, 10^{-1} and 10^{-2}) – a count of > 11,000 *Arcobacter* spp. MPN 100 ml^{-1} of effluent. When tested using soil freshly irrigated with effluent, method C again yielded positives for *Arcobacter* spp. for all three dilutions tested (zero, 10^{-1} and 10^{-2}) – a count of > 110 *Arcobacter* spp. MPN gm^{-1} for treated soil. Thus, method C was selected as the MPN method for the enumeration of *Arcobacter* spp. in both piggery effluent and soil, in all further work in this study.

6.4.3 Levels of *Arcobacter* spp. in piggery effluent and effluent treated soil

The levels of *Arcobacter* spp. in pig effluent and freshly irrigated soil are shown in Table 1. The effluent levels ranged from a minimum of 6.5 X 10^5 to a maximum of 1.1 X 10^8 MPN 100 ml^{-1} and did not vary much between winter (15^{o}C – 25^{o}C) and summer (20^{o}C-35^{o}C). The mean level of *Arcobacter* spp. in the ponds was 2.7 X 10^7 MPN 100 ml^{-1}. The levels in soil varied from 9.5 X 10^2 to 2.8 X 10^4 MPN g^{-1}.

Table 1. *Arcobacter* spp. levels in pond effluent and soil freshly irrigated with effluent at six piggeries

Piggery	Season	MPN (expressed as 100 ml^{-1} of effluent or gm^{-1} of soil)	
		Effluent	Soil
C	Summer 2004	4.3×10^7	ND*
	Winter 2003	2.3×10^6;1.1×10^8	ND
D	Summer 2004	1.1×10^8	ND
G	Summer 2002	4.3×10^6	5.4×10^3
	Winter 2002	2.5×10^7	1.4×10^4
K	Summer 2003	4.3×10^6	4.1×10^3
	Winter 2003	4.3×10^6	2.8×10^4
R	Summer 2003	1.4×10^6	1.4×10^4
	Winter 2003	6.5×10^5	9.5×10^2
T	Summer 2004	$>1.1 \times 10^7$	2.4×10^4
	Winter 2004	4.3×10^7	4.3×10^3
W	Summer 2002	9.3×10^5	3.3×10^3
	Winter 2002	4.6×10^6	2.5×10^4

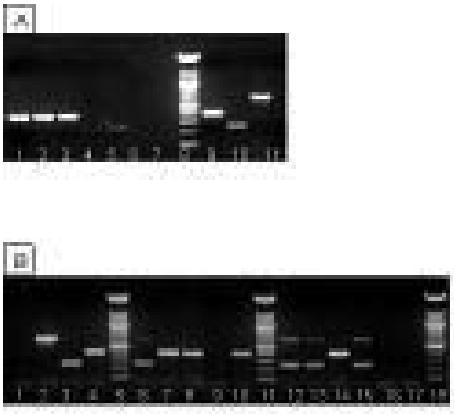
* ND = not done

6.4.4 Validation of multiplex PCR

When used on the reference strains, the multiplex PCR of Houf *et al*. (2000) gave the expected bands of 641 bp for *A. skirrowii*, 401 bp for *A. butzleri* and 257 bp for *A. cryaerophilus* (see Fig. 1 A and B). We intermittently observed non-specific bands for both the *A. cryaerophilus* reference and field isolates at around 640 bp

(Fig 1B, lanes 6,12,13 and 15). These non-specific bands were considerably fainter than the specific band and did not interfere with the ability of the PCR to correctly identify *A. cryaerophilus*.

Figure 1. Example of multiplex *Arcobacter* PCR results



A Lanes 1, 2, 3 field isolates of *A. butzleri*; Lanes 4, 5 field isolates of *A. cryaerophilus*; Lanes 6, 7 Negative control; Lane 8 Molecular weight marker; Lane 9 *A. butzleri* CCUG 30485^{T}; Lane 10 *A. cryaerophilus* CCUG 17801^{T}; Lane 11 *A. skirrowii* CCUG 10374^{T}.

B Lane 1 negative control; Lane 2 *A. skirrowii* CCUG 10374^{T}; Lane 3 *A. cryaerophilus* CCUG 17801^{T}; Lane 4 *A. butzleri* CCUG 30485^{T}; Lanes 5, 11 and 18 Molecular weight markers; Lanes 6, 12, 13 and 15 field isolates of *A. cryaerophilus*; Lanes 7, 8, 10 14 field isolates of *A. butzleri*; Lanes 9, 16 and 17 field isolates that did not react in the multiplex PCR.

6.4.5 Diversity of *Arcobacter* spp.

Over the 3 year course of the study, 83 isolates, 38 from effluent and 45 from soil treated with effluent, were selected to represent the phenotypic variance (colony morphology, catalase reaction and $CdCl_2$ sensitivity) seen during the confirmation of *Arcobacter* spp. All these isolates were examined by the multiplex PCR. The PCR confirmed 29 isolates (18 from soil, 11 from effluent) as *A. butzleri* and 41 isolates (17 from soil, 24 from effluent) as *A. cryaerophilus.* A further 13 isolates (10 from soil, 3 from effluent) did not give a band in the multiplex PCR. However, when subjected to partial 16S rDNA sequencing, all 13 multiplex PCR negative *Arcobacter* isolates showed a high similarity (>99%) to *A. cibarius* as determined by the FASTA searchs.

The distribution of three *Arcobacter* spp. detected in this work is shown in Table 2. *A. butzleri* and *A. cryaerophilus* were well distributed in both soil and effluent. *A. butzleri* was present in 5 of the 6 soil samples and 4 of 6 effluent samples. *A. cryaerophilus* was present in 5 of 6 effluent and soil samples. In contrast *A. cibarius* was present in only 1 effluent sample and 3 soil samples.

Table 2. Distribution of *Arcobacter* species across different piggeries in effluent and soil samples*

Piggery	Substrate	Season	*A. butzleri*	*A. cryaerophilus*	*A. cibarius*
D	Effluent	Winter	4/12	8/12	0/12
G	Effluent	Winter	0/4	4/4	0/4
	Soil	Winter	0/5	5/5	0/5
K	Effluent	Winter	2/3	1/3	0/3
	Soil	Winter	6/12	2/12	4/12
R	Effluent	Winter	1/2	1/2	0/2
	Soil	Winter	2/6	4/6	0/6
T	Effluent	Summer	4/4	0/4	0/4
	Soil	Summer	7/12	4/12	1/12
	Soil	Winter	2/2	0/2	0/2
W	Effluent	Winter	0/13	10/13	3/13
	Soil	Winter	1/8	2/8	5/8

* Results are presented as number positive over number tested

6.4.6 Correlation between key phenotypic characteristics and PCR / sequencing results

Table 3 shows the results of the comparison between the key phenotypic characteristics, catalase reaction and $CdCl_2$ sensitivity and the species identification as confirmed by PCR or 16S rDNA sequencing. All 29 isolates that were "weak" catalase positive and $CdCl_2$ resistant were confirmed as *A. butzleri*. All 33 isolates that were "rapid" catalase positive and $CdCl_2$ sensitive were confirmed as *A. cryaerophilus*. Of the 17 isolates that had a "weak" catalase reaction and were sensitive to $CdCl_2$ 13 were identified as *A. cibarius* and four as *A. cryaerophilus*. A further four confirmed *A. cryaerophilus* isolates had the unique combination of being "rapid" catalase positive and resistant to $CdCl_2$.

Table 3. Comparison of PCR results with phenotypic characteristics

PCR identification	Number of isolates	Sensitivity to $CdCl_2$*	Catalase Reaction†
A. butzleri	29	R	W
A. cibarius	13	S	W
A. cryaerophilus	33	S	P
	4	S	W
	4	R	P

* Any zone of inhibition recorded as sensitive (S); growth continuous to the disc was recorded as resistant (R)

† P = positive – frank bubbling immediately on mixing; W = weak (bubbling detected 10-15 s after mixing cells with reagent

6.5 Discussion

Arcobacter species are present in the faeces of healthy pigs (Van Driessche *et al.* 2004) and thus can be expected to be present in stored effluent. Thus, effluent could be a source of transfer of this organism via the food process chain if the effluent is used within a food production context e.g. for irrigation. The present study deals with the isolation, enumeration and species distribution of *Arcobacter* in Australian piggery effluent.

Our work has shown the presence of *A. butzleri*, *A. cryaerophilus* and *A. cibarius* in piggery effluent. The effluent ponds examined in this study differed in terms of their retention times and other physical parameters. Despite these variations, *Arcobacter* spp. were present in all seven ponds examined (across seasonal variations) and at levels of at least 10^6 MPN 100 ml^{-1}.

As indicated in Table 2, we established the simultaneous presence of two species of *Arcobacter* in four of the six effluent ponds. (Van Driessche *et al.* 2004) have reported that healthy pigs can simultaneously be shedding two or three species of *Arcobacter*. We did not detect the presence of *A. skirrowii* – a species that has been found in the internal organs of aborted piglets (On *et al.*, 2002) and the faeces of healthy pigs (Van Driessche *et al.* 2004). Van Driessche *et al.* (2004) reported that *A. skirrowii* was the least common of the three species present (*A. butzleri* 67%, *A. cryaerophilus* 23% and *A. skirrowii* 7%).

A. cibarius has only been recently recognised and only in association with broiler carcasses (Houf *et al.* 2005). Our study appears to be the first to associate this organism with pigs and the pig environment. The pathogenicity of *A. cibarius* is unknown and the significance of *A. cibarius* in piggery effluent is also unknown. It should be noted that On *et al.* (2002) reported that six of 27 *Arcobacter* isolates associated porcine abortions could not be assigned to a recognised species. It is possible that a range of currently unrecognised species of *Arcobacter* may be present in pigs.

The multiplex PCR of Houf *et al.* (2000) gave some occasional non-specific bands with both field isolates and the reference strain of *A. cryaerophilus*. This non-

specific reaction has not been reported by others. These non-specific bands occurred at around the same molecular weight as the specific band for *A. skirrowii*. As we used the multiplex PCR only on pure cultures, this non-specific band was not a problem. However, if this multiplex PCR was used on direct samples or on enrichment broths, it would be difficult to confidently conclude whether *A. cryaerophilus* or *A. cryaerophilus* and *A. skirrowii* were present.

We have shown that the enumeration of *Arcobacter* spp. from piggery effluent and soil treated with piggery effluent can be performed by an MPN method, the first such report of an MPN method. We developed the MPN approach by adopting an existing method for the selective isolation of *Arcobacter* spp. (Johnson & Murano 1999b, a). This MPN method resulted in a low level of competing bacteria under aerobic incubation thus allowing recognition of the typical *Arcobacter* spp. We found that the colony morphology of *Arcobacter* spp. was distinct, as originally reported (Johnson & Murano 1999b, a). Using this MPN method, we were able to isolate the faster growing *A. butzleri,* as well as the slower growing *A. cryaerophilus* and *A. cibarius,* after 48 h at 30^{o}C under aerobic conditions. The distinct colony size difference between *A. butzleri* and *A. cibarius/A. cryaerophilus* makes it possible to recognise the presence of multiple species within the one sample.

Of the three methods initially trialled to enumerate *Arcobacter* levels, our work demonstrated that two alternative MPN methods, one based on CAT supplements and the other on the antimicrobials of (Houf *et al.* 2001) were not suitable for piggery effluent. We found that the alternative method based on the use of antimicrobials described by (Houf *et al.* 2001) was too selective when used with piggery effluent. The difficulty of overgrowth on isolation media containing CAT supplements that was observed in the present study has also been reported by others (Atabay & Corry 1997, Rivas *et al.* 2004, Atabay *et al*. 1997) were able to overcome this problem by using either the (Steele & McDermott 1984) or the Lammerding *et al.* (1996) filter methods on the enrichment before plating onto agar. In our view, the use of filtration after enrichment and before plating is neither a convenient nor a suitable process within a context of an MPN method.

We used an abbreviated phenotypic testing scheme to screen the isolates of *Arcobacter* spp. The scheme proved useful and effective. By comparing the results of the testing with the PCR results, we have shown that all *A. butzleri* isolates are resistant to $CdCl_2$ and show weak catalase activity (Table 3). The catalase test for *A. butzleri* has been ambiguously described in the literature, been reported as positive in 33% of isolates (On *et al.* 1996, On *et al.* 2002) or in 100% of isolates weak positive (Vandamme *et al.* 1992, Schroeder-Tucker *et al.* 1996, Harrass *et al.* 1998 , Atabay *et al.* 2006). Our results suggest that *A. butzleri* could be regarded as being uniformly weakly catalase positive. It is possible that those studies that reported variable catalase activity were not aware of the need for a careful examination of the catalase reaction. Resistance to $CdCl_2$ has been consistently found in *A. butzleri* (Schroeder-Tucker *et al.* 1996).

We found that most, but not all, *A. cryaerophilus* isolates are strongly catalase positive and are sensitive to $CdCl_2$. While a number of other studies have reported that *A. cryaerophilus* are catalase positive and sensitive to $CdCl_2$, the occurrence of $CdCl_2$ resistance and weak catalase reaction has been reported by (Kiehlbauch *et al.* 1991).

We found that our abbreviated phenotypic system resulted in a clear distinction of *A. butzleri* and *A. cryaerophilus* (Table 3). However, *A. cibarius* isolates produced a pattern that was not distinguishable from a small percentage of *A. cryaerophilus* isolates – being sensitive to $CdCl_2$ and showing a weak catalase reaction. Based on the work of (Houf *et al.* 2005), *A. cibarius* is uniformly unable to reduce nitrates. As well, *A. skirrowii* has the ability to grow in 4% NaCl (Atabay & Corry 1998). Hence, the addition of two tests would allow a quick phenotypic screening of suspect *Arcobacter* isolates and a presumptive allocation to the four species now recognised as being present in pigs (see Table 4). While the original description of *A. cibarius,* which was based on 20 isolates, noted that the catalase reaction was variable (Houf *et al.*, 2005), we found that all 13 of our isolates showed weak catalase activity. If isolates of *A. cibarius* do have strong catalase activity, the scheme in Table 4 should recognise the isolates provided that they also fail to reduce nitrates.

Table4. Suggested extended phenotypic scheme for the presumptive differentiation of *Arcobacter* species known to be associated with pigs*

Organism	Sensitivity to $CdCl_2$†	Catalase Reaction‡	NO_3 Reduction	Growth in 4% NaCl
A. butzleri	R	W	+	-
A. cryaerophilus				
(80%)	S	P	+	-
(10%)	S	W	+	-
(10%)	R	P	+	-
A. cibarius	S	W	-	-
A. skirrowii	?	P	+	+

*Data for NO_3 reduction and growth in 4% NaCl and all data for *A. skirrowii* from Houf *et al.* (Houf *et al.*, 2005). All other data from current study.

† Any zone of inhibition recorded as sensitive (S); growth continuous to the disc was recorded as resistant (R)

‡ P = positive – frank bubbling immediately on mixing; W = weak (bubbling detected 10-15 s after mixing cells with reagent

An understanding and appreciation of suitable isolation media by clinical microbiologists would increase the frequency of isolation of *Arcobacter* spp. in general and in particular *A. butzleri*, an emerging pathogen of concern, from clinical stool specimens. *Arcobacter* isolates are often obtained using *Campylobacter* selective media – media which are recognised not to be optimal for *Arcobacter* (Prouzet-Mauleon *et al.* 2006). In France, *A. butzleri* formed 1% of the total *Campylobacter* like isolations from a surveillance network using a specialised *Campylobacter* selective medium - Campylosel (bioMérieux, Marcy l'Etoile, France) (Prouzet-Mauleon *et al.*, (2006). The adoption of more appropriate isolation media such as that trialled in the present study may aid in better understanding the contribution of *A. butzleri* to human enteric infections as well as animal reservoirs.

There appear to be no previous reports on levels of *Arcobacter* spp. in effluent. However, levels of up to 10^4 CFU g^1 have been reported in pig faeces, with the dominant species being *Arcobacter butzleri* (Van Driessche *et al.* 2004). We have previously found that the mean level of *E. coli* in 13 piggery effluent ponds in South-East Queensland is 1 X 10^5 MPN 100 ml^{-1} (Chinivasagam *et al.* 2004). Hence, it would appear that *Arcobacter* levels are around 100 times higher than *E. coli* levels in effluent ponds (10^7 compared to 10^5 MPN 100 ml^{-1}). However, the relative levels of the two organisms appear to be reversed in pig faeces – *Arcobacter* spp. being present at levels up to 10^4 CFU g^{-1} (Van Driessche *et al.* 2004) while *E. coli* has been reported to be typically present at levels of up to 10^8 CFU g^{-1} (Shuval 1991). This difference between *E. coli* and *Arcobacter* levels raises the possibility of *Arcobacter* spp. having the potential to grow in these anaerobic, nutrient rich piggery effluent ponds. There is clearly then a potential for the transfer and survival of these organisms within environments receiving effluent. We found *Arcobacter* levels in freshly irrigated soil were around 10^4 MPN g^{-1}. There is a need to understand the survival of *Arcobacter* in soils receiving piggery effluent.

In conclusion, *Arcobacter* species were isolated from piggery effluent in high levels and can be enumerated using an MPN technique. Three species were identified – *A. butzleri, A. cryaerophilus* and *A. cibarius* – with the latter species being associated with pigs for the first time.

6.6 Acknowledgements

I acknowledge my co-authors B.G. Corney, L.L. Wright, I.S. Diallo and P.J. Blackall

Australian Pork Limited provided funding for this study. The co-operation of the owners of the seven piggeries involved in this study is gratefully acknowledge

Chapter 7

The aerobiology of the environment around mechanically ventilated broiler sheds

This Chapter is published in the *Journal of Applied Microbiology*

Citation:

H.N. Chinivasagam, T. Tran, L. Maddock, A. Gale and P.J. Blackall (2010) The aerobiology of the environment around mechanically ventilated broiler sheds, *Journal of Applied Microbiology* **108**: 1657-1667.

7.1 Summary

Aim: To investigate the aerobiology of the environment around mechanically ventilated broiler sheds with the aim of understanding dispersion in the surrounding environment.

Methods and Results: Aerosol samples were collected weekly on four different commercial broiler farms through the cycle of 55 days from 2005 to 2007. Samples were collected inside the shed and at distances from the sheds. Litter and dust from within the shed were also examined. Members of the genera *Staphylococcus* (and to a lesser extent *Corynebacterium*) dominated (10^6 cfu m^{-3}) in the outside air at 20 m from the fan and were shown to decrease with distance. At distances of around 400 m, the levels of staphylococci/coryneforms returned to levels typical of those present before the placement of chickens. *Escherichia coli* levels were low (maximum 100 cfu m^{-3}) at 20 m. Fungi were present at uniform levels across the broiler cycle.

Conclusions: Staphylococci are the dominant organisms present in the air around mechanically ventilated broiler sheds and have the potential to act as an airborne "marker organism".

Significant Impact of the study: The outcomes of this study suggest that the impact of aerosols emitted from broiler sheds could be monitored and managed by examining the levels of staphylococci/coryneforms.

7.2 Introduction

Australian poultry production systems are currently dominated by mechanically operated tunnel ventilated sheds that are designed to improve production as well as create an optimum environment for the bird (Runge *et al.* 2007). However, concerns are raised with all animal production systems and their wastes where there is an increased potential for microbial pathogens to enter the aerosol environment (Pillai 2007). Thus an understanding of relative risks can only be put into perspective with a clear knowledge of the actual levels of the various organisms at realistic distances from sheds.

Few studies have dealt with quantifying the levels of microbes in aerosols in and around mechanically ventilated poultry environments. Birds grown on litter (sawdust or straw with a litter thickness of 8 to10 cm) under intensive broiler production in mechanically ventilated sheds yielded total bacterial counts in the air that depended on bird age (Baykov & Stoyanov 1999). These bacterial counts ranged from 1.25 X 10^5 m^{-3} at the beginning of the trial to 1.68 X 10^7 m^{-3} at the end of the trial (day 56) (Baykov & Stoyanov 1999). Another study (with more or less similar conditions to the previous study) also showed an increase in levels with the increase in bird age and body weight recording concentrations of bacteria within the similar range in air inside the shed (1.7 × 10^4 to 2.2 × 10^5 cfu m^{-3}) (Vučemilo *et al.* 2007). Additionally fungi were present at lower levels than bacteria (9.8 × 10^3 to 8.5 × 10^4 cfu m^{-3}) (Vučemilo *et al.* 2007). However little work has been done on the levels (and thus the survival potential) of these organisms once they enter the external environment, a key to understanding the external impacts of mechanical ventilation and the dispersion of organisms.

Bacteria that enter the aerosol environment can be stressed and not all organisms survive well either, during the mechanical transfer via the fans or subsequent transport. For example, biological death rates for organisms such as *Escherichia coli* in aerosols have been shown to be linked to particle size and prevailing temperatures (Ehrlich *et al.* 1970b). The prevailing weather can also impact on survival patterns with better survival shown to occur on sunny days compared to cloudy days (Tong & Lighthart 1998). Laboratory based studies have shown that various Gram negative organisms once in an aerosol state show a good survival at higher relative humidity (70 – 80%) (Marthi *et al.* 1990) while other species are not

significantly affected by a wide range of humidities (25 to 99%) (Ehrlich *et al.* 1970a).

The present study was carried out to address the issue of transfer and survival of key organisms in the surrounding natural farm aerosol environment under normal farm operating conditions to assess an overall pattern of transmission. Organisms were tested at extended distances from the front (exhaust end) of the fan as well as the back and sides of the shed. The focus of this study was the estimation of the levels of commonly occurring organisms such as total bacteria, staphylococci, *E. coli* and fungi. The basis of the study was to capture organisms under prevailing environmental conditions, wind speed, direction, temperature and relative humidity as well as take into account site specific dilution effects that can help to understand the aerobiology surrounding the broiler farm over typical broiler cycles.

7.3 Materials and methods

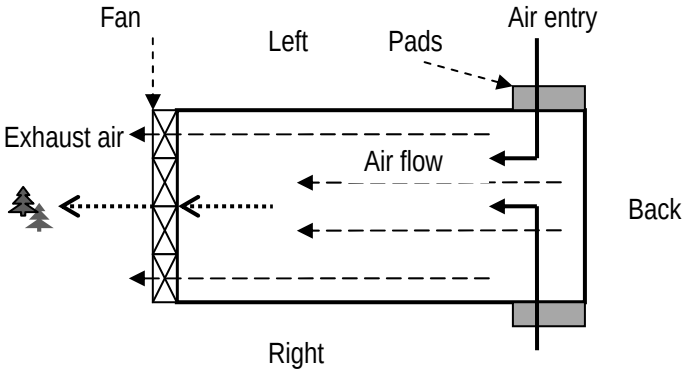
The major focus of the present study was assessing microorganisms at varying distances from the fan. Additional samples collected in a parallel study on *Salmonella* and *Campylobacter* (Chinivasagam *et al.* 2009) were also used to quantify the presence of bacteria relevant to the current study.

7.3.1 Farms, topography and distance sampling design

The selection of the four study farms was mainly influenced by the surrounding topography of the farm to help assess bacterial dispersion trends. The trials were performed under prevailing environmental conditions including the presence of open fields surrounding the study farm or barriers that were natural (trees) or constructed (embankments or Hessian barriers). The farms each consisted of a cluster of four to six sheds with a random selection of a single shed for the trial. Farm S (three trials carried out on June to August 2005, September to October 2005 and April to June 2006) had a flat expanse of land in the front (exhaust end) and back of the shed. Farm X (single trial from August to October 2006) had an embankment and surrounding treed boundaries. Farm D (single trial from January to March 2007) had treed surroundings with Hessian barriers at the back of some sheds. Farm L (single trial from August to October 2007) had a mix of treed surrounding and open areas.

Fig. 1 illustrates the sampling set up. Sampling commenced in the morning taking about 3 h and was carried out weekly prior to chicken placement and over a normal broiler cycle of approximately 55 days. Actual sampling at each sampling point was done in triplicate with the time varying according to the expected concentration. Samples were collected at distances at the exhaust end of the fan ranging from 2, 10, 20, 30, 50, 75 and 100 m (which slightly varied on each farm). As well, distances of 300 and 400 m were tested in a single trial on Farm S. Additionally samples were collected at distances of 2 and 20 m at the left and back of the test shed as well as the right of the overall shed complex.

Figure 1 Aerosol Sampling plan. Samples of exhaust air (EXH) were collected at distances of 2, 10, 20, 30, 50, 75, 100, 200, 300 and 400 m. Air samples were also collected at 2 and 20 m at the Right (R), Left (L) Back (B) positions. Air was pulled into these sheds via cool pads (indicated) and was exhausted via the fans (indicated).



7.3.2 Microbiological sampling for aerosols

The MAS 100 (Microbial Air Monitoring System www.merck.de) was used to test levels of total bacteria, staphylococci/coryneforms (as black pigmented colonies on Baird-Parker agar), *E. coli* and fungi. The MD8 airscan (www.sartorius.com) was used (Chinivasagam *et al.* 2009) to test total bacterial levels and staphylococci/coryneform levels both inside the shed and outside the shed at 10 m distances from the fans.

The MAS 100 aspirates air at the rate of 100 l of air min^{-1} and the sampling times used ranged from 10 sec to 10 min and depended on the organisms tested and the expected concentrations in the air. The MD8 was set to sample air at the rate of 133.33 l min^{-1} with sampling times of 45 min. The relevant agar plates were directly

loaded onto the MAS-100, whereas for the MD8 the filter was directly dissolved in 0.1% peptone on the completion of sampling. Both the plates and the filters were transported within 3 h to the laboratory for further processing or direct incubation.

The organisms tested were *E. coli* (Chromocult – Merck, incubated overnight 36^0C), staphylococci (Baird Parker Agar – Oxoid, incubated at 37^0C for 48 h) and total bacteria (R2A agar incubated at 30^oC for up to 5 d).

The number of colonies from the MAS-100 plates was corrected based on the Feller's statistical correction table (provided by the manufacturer) and then converted to colony forming units per cubic meter of air. The counts for MD8 were obtained following serial dilution and counting of countable plates and illustrated as cfu m^{-3} of air.

Additionally, Farm S (July to August 2006) was sampled (MAS 100 and MD8) for *E. coli* over different times of the day (9:00 am, 10.00 am, 12:00 pm and 3:00 pm) on days 19, 26 and 47 of the broiler cycle to assess an impact on the time of the day on the levels of the organisms tested.

7.3.3 Microbiological analysis of litter and dust

Testing of litter (Farms D, L, S, and X) and dust (Farms D and L) was used to estimate levels of staphylococci and total bacteria. A uniform and representative sample of litter was assembled from 20 sub-samples. The dust was randomly collected aseptically from around the mini vents in the shed. From the composite sample, 25 g litter was placed in 225 ml of 0.1% peptone water and 1 g dust in 18 ml of 0.1% peptone water. Appropriate serial dilutions were then prepared and plated onto Baird-Parker agar (staphylococci) and R2A agar (total bacteria). Baird-Parker agar was incubated at 37^oC for 2 d and R2A was incubated at 30^oC for 5 d.

7.3.4 Identification of isolates from Baird – Parker Agar

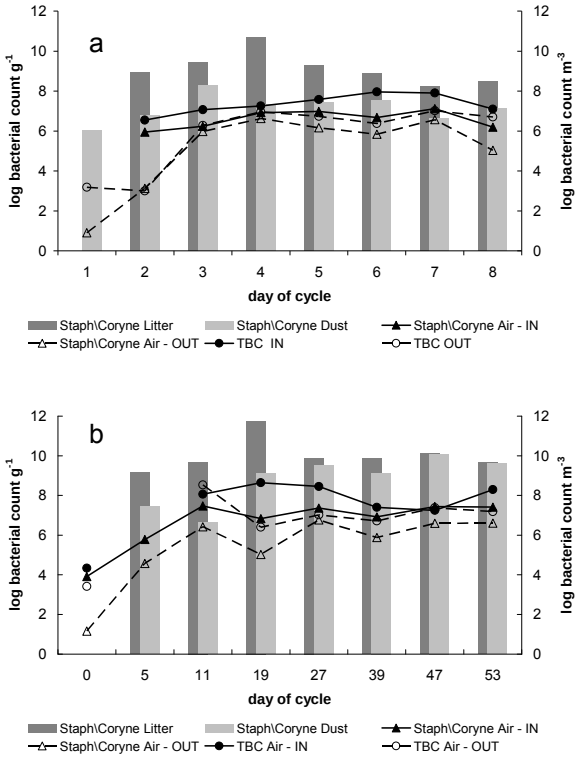
Two dominant colonial morphologies, grey or black colonies (both associated with tellurite reduction), were present on Baird- Parker. These were counted as staphylococci\coryneforms and were further identified. All isolates were picked from a segment of the plate (which was divided into 8 segments). A total of 180 colonies were isolated from both aerosols (in and out) and litter on Farms S and X. These isolates were categorised as either cocci or irregular rods with the cocci being identified using the Microbact 12 S kit (Oxoid) and the rods using an "API Coryne kit (BioMerieux).

7.4 Results

7.4.1 Staphylococci/coryneforms and total bacteria in litter, dust and internal and external aerosols

Fig. 2 illustrates staphylococci/coryneform and total bacteria levels in litter, dust (from shed surfaces) and aerosols (exhaust air at 10 m and inside air at 10 m from the fan) on Farms D (January to March 2007 trial) and L. The levels of staphylococci/coryneforms in litter on Farms D (January to March 2007), L (August to October 2007) and S (March to June 2006 – data not presented) ranged from 10^9 to 10^{12} cfu g^{-1} with lower levels (10^8 to 10^9 cfu g^{-1}) observed on Farm S (April to June 2007 – data not presented). The levels of these organisms in dust were typically lower (10^6 to 10^9 cfu g^{-1}) than the litter levels. After an increase in levels of staphylococci/coryneforms until around days 27/28, the levels in both dust and litter then remained almost in the same range until the end of the cycle (Fig. 2).

Figure 2 The levels of total bacteria (TBC) and staphylococci/coryneforms in litter, dust and aerosols in the exhaust air (out) and inside air (in) at 10 m from the fans on Farms L (August to October 2007, Fig. 1a) and D (March to May 2007, Fig. 1b) through a production cycle.



Corresponding to the high levels in either litter or dust the initial levels of staphylococci/coryneforms in the air inside the shed in the presence of chickens ranged from 10^6 to 10^7 cfu m^{-3} and remained almost steady at 10^7 cfu m^{-3} towards the latter stages of the cycles in both Farm L (August to October 2007) and Farm D (March to May 2007) (Fig. 2). In a second trial on Farm D (May to July 2007) the levels inside started at 10^4 cfu m^{-3} rose to 10^6 by day 25 and after that dropped to 10^4 cfu m^{-3} by the end of the cycle (data not presented).

The levels of staphylococci/coryneforms in the air outside the shed were lower by almost a single log cycle compared with the air inside the shed in all trials. These levels ranged from a minimum of 10^3 cfu m^{-3} to a maximum of around 10^6 cfu m^{-3} through most of the cycle on Farms D and L (Fig. 2). However, there was an exception on Farm S (April to June 2006 - data not presented) where the levels outside the shed were at least two log cycles lower than inside the shed levels for four of the seven sampling dates.

Fig. 2 also shows the levels of total bacteria in air inside the shed and in air outside the shed. These levels were generally slightly higher than the staphylococci/ coryneform levels. The total bacteria in air both inside and outside were more or less in the similar range to the staphylococci/coryneforms indicating the major contributor to the population was the latter group of organisms.

7.4.2 Staphylococci and coryneforms - species diversity

The identification of the selected isolates which produced the characteristic black pigmentation as a result of tellurite reduction on Baird-Parker Agar is illustrated in Table 1. These isolates were picked from both litter and aerosols on Farms S and X. None of these colonies showed the additional features characteristic of *Staph. aureus* (lecithinase and lipase activity).

Table 1 Percentage (numbers[#]) of *Staphylococcus* spp and coryneforms* distributed in litter and air on Farms S and X**

Organism	Present on Farm S (air or litter) at indicated section of the cycle					Present on Farm X (air or litter) at indicated section of the cycle				
	Before Chicks	Days 2 to 23		Days 29 to 57		Before Chicks	Days 4 to 26		Days 32 and 39	
	Litter	Air	Litter	Air	Litter	Air	Air	Litter	Air	Litter
Staphylococci	38 (3)	88 (21)	22 (7)	76 (22)	30 (9)	50 (2)	92 (12)	91 (20)	20 (1)	67 (6)
Staph. cohnii	0	4	3	4	3.5	0	23	38	20	22
Staph. hominus	0	33	0	21	0	0	0	0	0	0
Staph. intermedius	0	0	0	0	0	0	23	18	0	22
Staph. saprophyticus	13	15	3	31	7	0	0	4.5	0	0
Staph. xylosus	0	15	6	0	13	25	46	4.5	0	22
other species[#*]	25	15	9	21	7	25	0	27	0	0
Coryneforms	63 (5)	13 (3)	78 (25)	24 (7)	70 (21)	50 (2)	8 (1)	9 (2)	80 (4)	33 (3)
Corynebacterium[##]	50	4	62.5	14	70	50	0	9	80	33
other genera	13	8	16	10	0	0	8	0	0	0

[#] number of dominant colonies isolated from a segment of a well distributed plate

* identified as *Corynebacterium, Microbacterium* or *Propionibacterium*

** Among a total of 123 isolates from litter and aerosols, 50% staphylococci and 50% coryneforms

[##] In litter *Corynebacterium propinquun* formed 94% and 7% of the isolates on Farms S and X respectively

[#*] Other species were *Staph. capitis, Staph. choromogenes, Staph. lentus*, *Staph. simulans* (both Farms), *Staph. warnerii* (present on Farm S only) and *Staph. epidermidis* and *Staph. hyicus* (present only on Farm X).

On Farm S, the coryneforms (consisting of the genera *Corynebacterium, Microbacterium* and *Propionibacterium*) were more common than the staphylococci in litter, while the staphylococci were more common than the coryneforms in the aerosols. Among the total of 123 isolates obtained on Farm S from both litter and aerosols, 50% were identified as coryneforms and 50% identified as staphylococci. On Farm S, the dominant coryneform was *Corynebacterium propinquun.* A total of 94% of *Coryne. propinquun* isolates were from the litter, while only 6% were isolated from aerosols. This organism is clearly linked with the litter source material – pine wood. The dominant staphylococcal species identified in both litter and air were *Staph. saprophyticus*, *Staph. choromogenes* and *Staph. hominis* with *Staph. xylosus*, *Staph. simulans* and *Staph. lentus* occurring to a lesser extent.

On Farm X, smaller numbers of isolates were identified. Of the total of 57 isolates identified, 24% were coryneforms and 76% were staphylococci. While the percentage of coryneforms on this farm was lower than that of Farm S, the dominant coryneform was still *Coryne. propinquun* (85% of total coryneforms). On Farm X, the litter did not yield any coryneforms until day 26. The staphylococci were the dominant organism in both aerosols and litter.

On the basis of these identifications, Fig. 3 shows the relative proportions of the total of the identified staphylococci and coryneforms in litter and in air on Farm S and X over the production cycle. On Farm S, there is a clear dominance of coryneforms over staphylococci in the litter while the reverse applies in the aerosols where the staphylococci are dominant. At times, the staphylococci represented 80 to 90% of the aerosol population. On Farm S, coryneforms were present on day 0 in the new litter material prior to the placement of chickens (Fig 3a). Coryneforms continued to be a dominant organism in the litter through to day 52 on this farm. Staphylococci were also present on day 0 in the litter, albeit at low levels.

Figure 3 The percentage distribution of staphylococci and coryneforms in the litter and aerosols (exhaust air and inside air at 10 m from the fans) on Farms S (April to June 2006, Fig. 2a) and X (August to October 2006, Fig. 2b). The numbers on the top of the bars represent the day of the cycle when the sample was obtained.

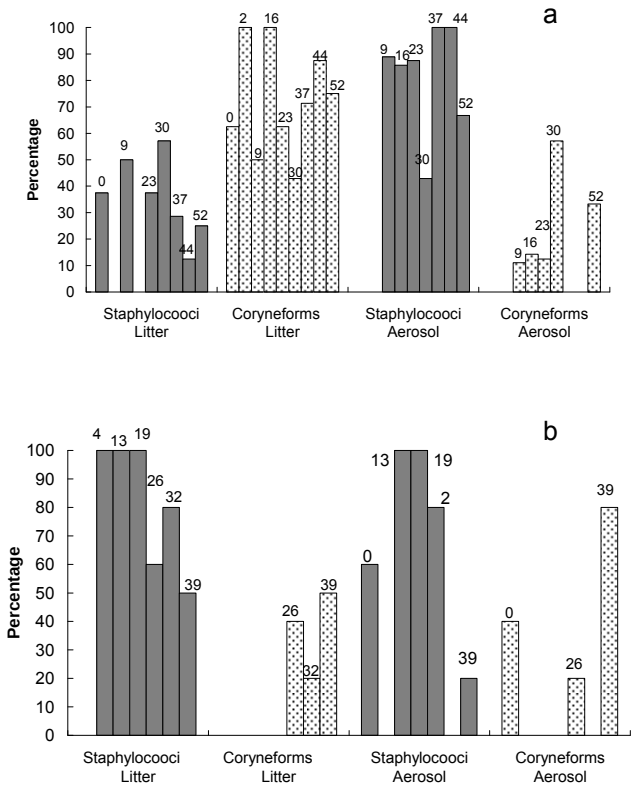


Fig. 3b clearly shows that the coryneforms were not detected in the litter on Farm X until day 26 and were generally lower than the staphylococci in both air and litter samples over the production cycle. On Farm X coryneforms did not dominate in the litter, as in Farm S, from the early stages. Staphylococci were the dominant flora in the litter from day 4 to day 39 (the sampling did not proceed beyond this date). The staphylococci remained at fairly high levels in both litter and aerosols except on day 39 where coryneforms were dominant in aerosols (Fig. 3b). This change in staphylococci/coryneform ratio may have been caused by the fact that Day 39 followed an initial thinning of the flock with the considerable potential generation of dust and litter matter in air.

7.4.3 Staphylococci/coryneforms in external aerosols

A total of five trials looking at the levels of airborne bacteria at external distances from the fans were performed – three on Farm S and one each on Farms D, and L. Table 2 shows the meteorological data collected at 10 m from the fan for the trials

on Farm D (January to March 2007), L and S (September to November 2005). Generally lower wind speeds (0.6 to 1.3 m s^{-1}) were recorded on Farm L than on Farm D and S (1.1 to 3.2 m s^{-1}). Day temperatures ranged from 20 to 33°C. Farm S had a lower relative humidity (22 to 47%) than Farm D and L (43.7 to 68.6%).

Table 2 Ambient outdoor Wind Speed (WS - m s^{-1}), Temperature (TP - °C) and Relative Humidity (RH%) during weekly testing for staphylococci/coryneforms, *E. coli*, fungi and total bacteria on Farms L, D and S

Farm L				Farm D				Farm S			
Cycle Day	WS m s^{-1}	TP °C	RH %	Cycle Day	WS m s^{-1}	TP °C	RH %	Cycle Day	WS m s^{-1}	TP °C	RH %
10	0.7	24.0	50.8	0	3.4	31.2	56.6	0	1.1	25.5	22.4
17	0.6	23.6	57.9	3	2.7	29.4	54.3	7	2.7	27.5	23.5
24	0.6	20.9	83.8	10	2.5	33.0	57.9	15	1.6	27.4	38.6
31	0.7	28.3	43.7	17	2.2	30.9	57.1	21	2.4	33.5	30.6
37	0.9	22.7	69.4	25	2.5	30.0	52.7	28	2.5	31.2	32.1
45	1.3	20.1	61.3	30	1.7	27.7	63.1	36	3.2	28.0	37.7
52	0.8	27.3	55.1	37	2.5	27.9	64.6	44	1.2	33.5	46.9
				44	1.8	29.0	68.6	53	1.9	24.6	37.2
				51	2.6	28.5	67.1				

For three of the trials, aerosol samples were collected before placement of the chickens and the mean count of airborne staphylococci/coryneforms varied from 18 to 39 cfu m^{-3} with an overall mean of 25 cfu m^{-3} (Table 3). This figure was calculated from all sampling positions, with Table 3 showing that only a small number of points (i.e. only 3 to 5 of the 13 sampling points) were actually positive for staphylococci.

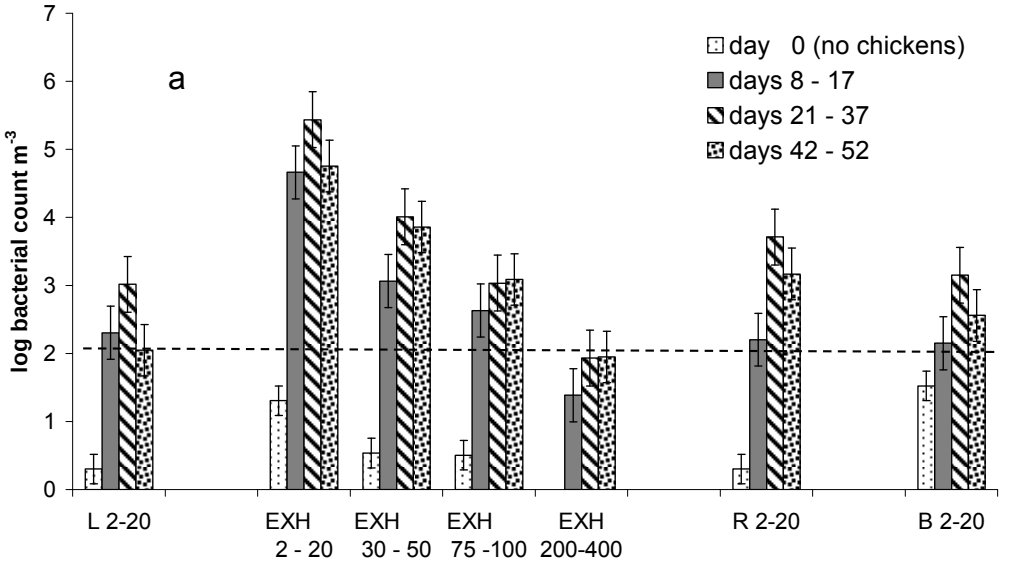
Table 3 Levels of staphylococci/coryneforms, total bacteria and fungi (expressed as cfu m^{-3}) in three trials prior to chicken placement (background). The data for each trial/organism combination are a summary of the levels detected at all sampling positions (with the geometric mean being shown).

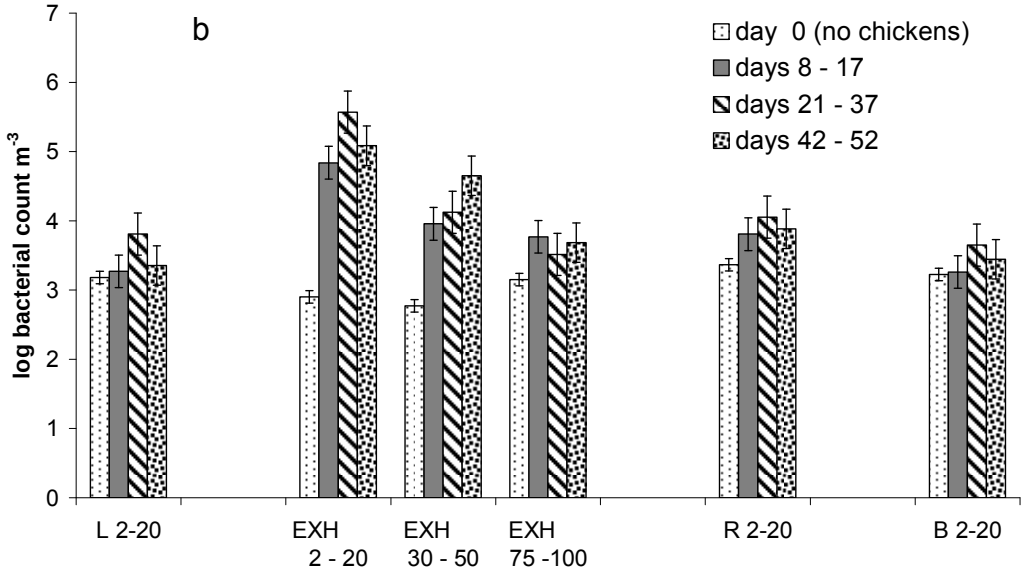
Trial	Staphylococci/coryneforms		Total bacteria		Fungi	
	Mean	Samples[a]	Mean	Samples	Mean	Samples
Farm S	18	3/13	1,497	12/12	997	12/12
Farm S	39	4/13	2,122	13/13	1,859	13/13
Farm D	25	5/12	278	8/8	507	8/8
Total	25	12/38	1,143	33/33	1,082	33/33

[a] Shows the number of samples positive/total number of samples tested.

Fig. 4a presents the assembled data on the levels of staphylococci/coryneforms from the trials carried out on Farms S (3 trials), D and L, at distances from the exhaust end of the fan. The sampling distances ranged from 2 to 100 m from the exhaust end of the fan (on all trials) with the sampling points at 200, 300 and 400 m from the fan coming from the third trial on Farm S. As previously discussed, the dominant organisms captured in aerosols were staphylococci while a smaller fraction were coryneforms (sourced from the original bedding material). Hence these two species, irrespective of source, are able to indicate shed-contributed aerosols at distances from the shed.

Figure 4 The levels of staphylococci/coryneforms (Fig 4a) and total bacterial (Fig 4b) in aerosols at distances from the fan (exhaust EXH) and (left L), (right R) and back (B) of the shed through a cycle on Farms S (June to August 2005, September to October 2005,), D (January to March 2007) and L (August to October 2007), across the production cycle. Additionally, staphylococci were tested at 200, 300 and 400 m (March to June 2006) at Farm S.





The mean background count of staphylococci/coryneforms on the farms varied from 18 to 39 cfu m^{-3} (Table 2). Hence, as a conservative estimate, a pre-chicken, background level of staphylococci/coryneforms of 100 cfu m^{-3} is marked (the dotted line in Fig. 4a). As shown in Fig. 4a, the staphylococci/coryneform count showed a decline over the sampling distance. These results indicate that aerosols contributed by the shed dropped off to background levels at 400 m. The data for the 200, 300 and 400 m sampling points all came from Farm S, a farm that featured no barriers or trees. Hence it would seem that dilution and dispersion caused the aerosols to reach background levels at 400 m.

The introduction of the chickens resulted in a marked increase in the levels of airborne staphylococci/coryneforms outside the shed at the 2-20 m sampling points. At these points, the levels rose to around 10^4 -10^5 cfu m^{-3} for days 8-17, with a further rise to 10^5 -10^6 cfu m^{-3} at days 31-37. The levels then dropped back to around 10^4 -10^5 at days 42-52. At 30-50 m, a similar pattern was seen, although the levels were lower overall. The levels continued to fall at the greater distances. On all farms, the highest counts recorded for staphylococci/coryneforms were typically at the 2 m distance. The counts were often at or near the maximum detection limit of the sampling unit (10^6 cfu m^{-3} and thus could be even higher than recorded. There is a clear reduction in levels of these organisms over distance.

The levels of staphylococci/coryneforms in the left, right and back sampling points clearly rose in response to the presence of the chickens (Fig. 4a). However, the levels at these sites were markedly lower compared to the levels in the exhaust air (Fig. 4a). As well, the levels recorded to the left and right sides were also lower than the exhaust end of the shed. On some farms, the presence of small fans (mini vents) on the side of the sheds did result in elevated counts of staphylococci/coryneforms.

While external factors such as topography and general farm activities on the day as well as meteorological factors such as temperature, relative humidity and wind speed are clearly contributory factors, the overall occurrence of airborne staphylococci/coryneforms on the trial farms did not vary to a great extent. Overall, Fig. 4a shows the potential of the use of staphylococci/coryneforms as “marker” organisms to assess the distance travelled by shed contributed aerosols.

7.4.4 Total bacteria, fungi and *E. coli*

The levels of total bacteria and fungi detected in air samples prior to the placement of chickens are shown in Table 3. No *E. coli* were detected at any sampling point on any farm prior to chicken placement. The total bacterial count was a mean of 1,143 cfu m^{-3} for all three studies. Importantly, all sampling sites were always positive for these organisms – confirming that the organisms are normal air-borne flora. The fungal levels were broadly similar to the total bacterial levels (a mean of 1,082 cfu m^{-3}) and all samples in all three trials were positive. Again this indicates that the fungi are part of the normal airborne flora.

Fig. 4b shows the assembled data on the levels of total bacteria on all study farms. Like the staphylococci/coryneforms, the total bacteria count did show a marked rise in response to the presence of chickens in the exhaust air at the 2-20 m sampling points. A similar marked response was seen in the exhaust air at 30-50 m sampling points. At all other sampling positions (exhaust air at 75-100 m, left, right and back) there was no marked response to chickens. In general, the total bacteria, with levels at distances from the exhaust are only marginally above background levels (Fig. 4b), are not as sensitive a marker of the presence of chickens as the staphylococci/coryneforms (Fig. 4a).

The results for the fungal levels (Fig. 5) are markedly different from the total bacteria. The only sampling points showing any response to the presence of chickens were the exhaust air 2-20 m where a small rise was seen. Overall, there is little evidence that the chicken shed contributes much to the airborne levels of fungi outside the shed.

Figure 5 The levels of fungi in aerosols at distances from the fan (exhaust EXH) and (left L), (right R) and back (B) of the shed through a cycle on Farms S (June to August 2005, September to October 2005), D (January to March 2007) and L (August to October 2007).

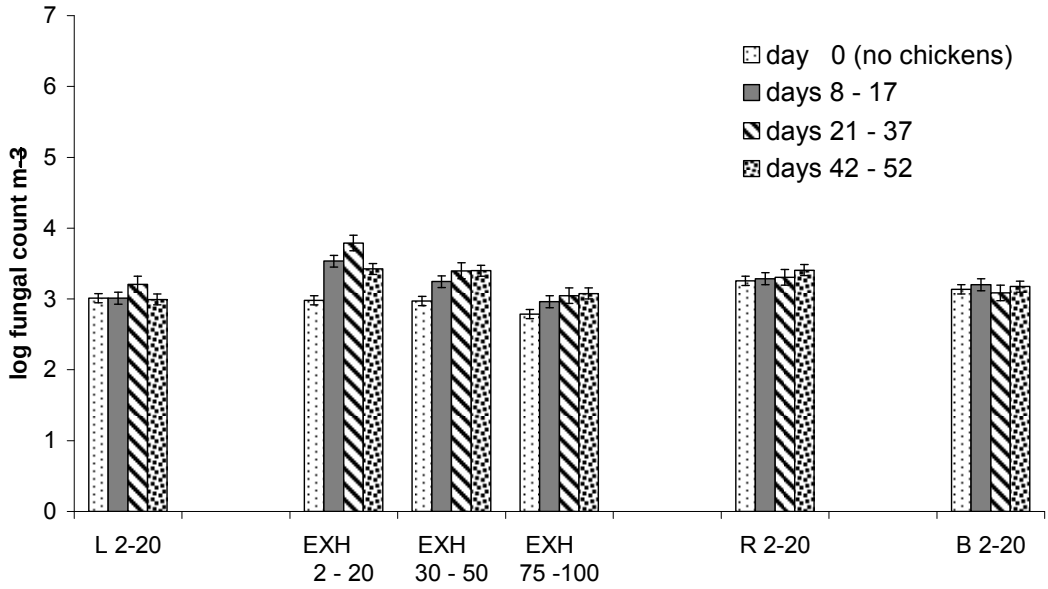
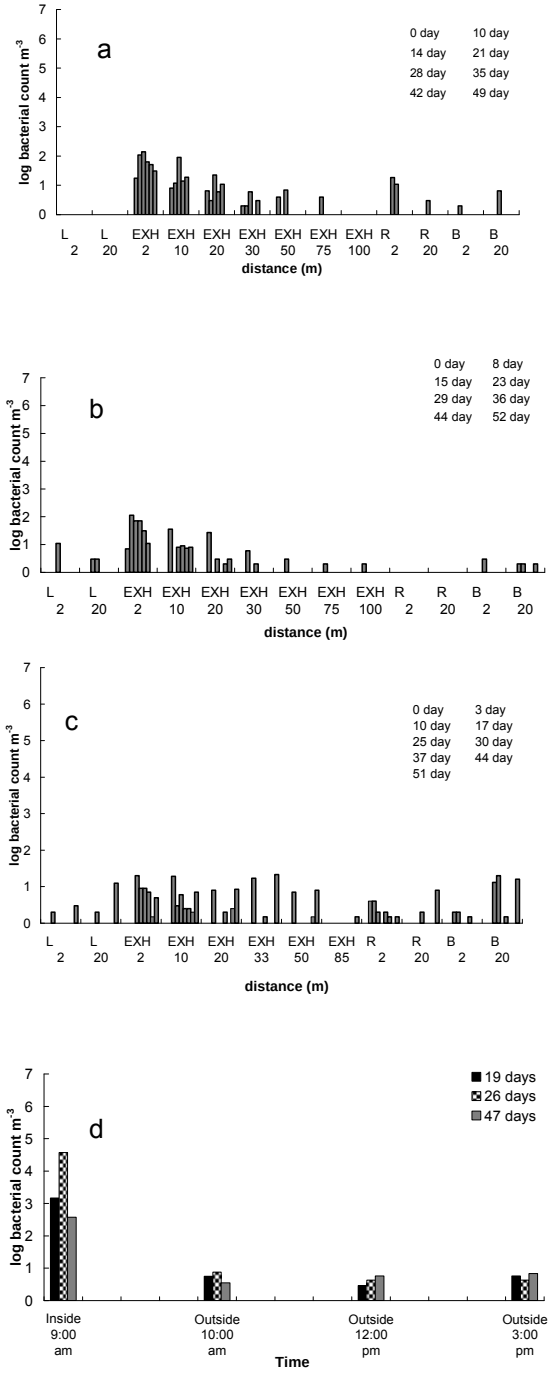


Fig. 6 (a, b and c) shows the levels of *E. coli* detected at weekly samplings and all distances for three trials (two on Farm S and one on Farm D) while Fig. 6 (d) shows the levels of *E. coli* at different times of the day (from Farm S on days 19, 26 and 47 of a single cycle). Only low levels of *E. coli* were detected at distances from the fan, with the majority of these detections occurring at the close distances of 2, 10 and 20 m at the exhaust end of the fan. The highest level of *E. coli* detected was around 100 cfu m^{-3}. Very similar results were found on the other trials on Farms S and L (data not presented).

Figure 6 The levels of *E. coli* in aerosols. (i) At distances from the fan (exhaust end) and left, right and back of shed through a cycle on Farms S (June to August 2005, Fig. 6a; September to October 2005, Fig. 6b) and D (January to March 2007, Fig. 6c) (ii) at different times of the day at three points in the weekly cycle of Farm S (July to August 2006, Fig 6d)



E. coli was used to determine the impact of time of the day (from 10:00 am to 3:00 pm) had on the levels of detectable aerosolised organisms. Collectively the levels of *E. coli* inside the shed were well over 10^4 cfu m^{-3} of air at 9:00 am. The levels outside the shed at 10 m from the fan (100 cfu m^{-3}) remained unchanged when measured at 10:00 am and 12:00 pm and 3:00 pm on days of 19, 26 and 47 of the broiler cycle. This outcome shows no impact of the time of day on the levels of *E. coli* outside the shed

7.5 Discussion

The impact of tunnel ventilated sheds on the aerobiology of the surrounding environment is determined by the survival potential of the organisms as well as other site related factors. These situations have been studied in a number of agricultural and waste management applications, where the organisms are able to transfer to the air environment via droplets or organic particulates, the latter situation likely to occur in tunnel ventilated poultry environments.

Pillai and Ricke (2002) state that there is a lack of clear understanding of the fate and transport of bioaerosols in intensive animal and livestock production facilities, especially within the open environment, and thus there is an inability to accurately predict the health risks associated with bio-aerosolised pathogens. Studies have been carried out with an attempt to develop sampling strategies in commercial poultry houses for the routine monitoring of bacteria (Woodward *et al.* 2004). The present study has addressed these issues.

An increase in the concentration of air-borne bacteria does occur as a result of particulate matter transferring into the environment via feathers, faeces or litter material exiting the sheds during fan activity as demonstrated by the high levels of organisms captured at 10 m from the fan in the present study. Dust from poultry housing can be high and originate from feed, litter, animal surfaces and faeces and a lesser amount from friction from floors and walls (Hartung 1994 cited in Pillai and Ricke (2002) The viable airborne particle concentration inside the shed can be influenced by the ventilation type of the shed, amongst other factors (Banhazi *et al.* 2008a). In the current study, only sheds of a single ventilation type have been studied. While ventilation rates undoubtedly varied across sheds, the current study

found that the overall dispersion pattern of all of the organisms studied did not markedly differ between the study farms.

The organisms isolated in the current study are common and are also a feature of other aerosol generating sites such as sewage treatment plants (via aerosol droplets) (Adams & Spendlove 1970, Fannin *et al.* 1985). A long term waste water plant operation showed high bacterial (coliforms, *E. coli* and staphylococci) and fungal concentrations in almost all of the sites around the plant including downwind positions (Brandi *et al.* 2000) as in the present study.

In the current study, total bacteria were captured at distances from the fan at levels higher than *E. coli*, staphylococci and fungi. These organisms showed a general pattern of dispersion – with little difference across the different farms studied and thus formed an overall pattern linked to the types of environments studied. Importantly, agricultural settings could provide a range of other sources for elevated total bacteria counts in air. This is a confounding factor as the distance from the poultry shed increases. At extended distances (for example beyond 500 m) and outside the close vicinity of the farm higher levels of total bacteria can be attributed collectively to various other sources. Baykov and Stoganov (1999) attributed total bacterial levels at distances as far as up to 3000 m from the intensive poultry production environment. It is possible that other sources could have been a contributory factor. However, in the present study, the use of a more specific "marker organism" - staphylococci (and coryneforms) – provides a better connection to the source, especially at extended distances such as over 1,000 m. While local topography will clearly influence the levels of these marker organisms, the current study has shown that on Farm S (with a very flat and open topography), the contribution of the marker organisms associated with the shed reduced to background levels by 400 m.

While high levels of *E. coli* were captured through the cycle in aerosols within the shed (Chinivasagam *et al.* 2009), the current study has demonstrated only low levels at 20 m outside the shed. Similarly the activity of biosolid loading is known to be a source of *E. coli* (Brooks *et al.* 2005b) and coliforms, which although found at high levels (10^6 g^{-1} dry weight) in the biosolids were not routinely detected in aerosols (Brooks *et al.* 2004).

One of the key findings of the present study is that *E. coli,* the indicator, was almost always absent at distances beyond 20 m and that the levels captured at 20 m were low. This outcome suggests that there is little possibility of pathogens such as *Salmonella* and *Campylobacter* being commonly present beyond 20 m from the shed. This is further supported by our parallel study that dealt with *Salmonella* and *Campylobacter* where these organisms were only rarely detected outside the shed (Chinivasagam *et al.* 2009). The low levels of *E. coli* seen in the current study could be either due to die off or dilution. Experimental studies carried out to understand the die-off of *E. coli* in an atmospheric environment have found that the impact of oxygen is toxic to the organism and this impact progressively increases as the relative humidity decreases below 70% (Benbough 1967). It is worth noting that the relative humidity in the present trials was almost always below 70%. A second death mechanism in air occurs at high relative humidities due to the effect of aerosolisation on *E. coli* RNA synthesis (Benbough 1967). With respect to dilution, the wind speeds during the trials (recorded as 0.6 to 1.3 m s^{-1} on Farm L and 1.1 to 3.2 m s^{-1} on Farm D and S) were generally steady over the cycle period tested. However the occurrence of *E. coli* was very similar across these farms. Thus the die-off or the poor survival of *E. coli* in the air as soon at it reaches the outside aerosol environment seems more important than dispersion or dilution.

Factors other than die-off can contribute to high levels exiting the source but not having the potential to travel to extended distances from the source. For example exhaust air from swine sheds has shown elevated levels of bacteria in these exhausts, with bacterial numbers reaching 190,000 cfu m^{-3} (Heber *et al.* 2001). In the swine operation 75% of the particles were predicted to be greater than 3.3 μm (via modelling) and thus settled early (Heber *et al.* 2001). Large amounts of settled dust were observed in front of the swine exhausts (Heber *et al.* 2001). As large amounts of settled dust was observed in the present study (at distances up to 20 m), this suggests that not all particulate-borne microbes can stay suspended in the aerosol environment for long periods or distances. This may be an additional reason for the low number of *E. coli* detected at distances from the fan. In the current study, the extended distances from 100 to 400 m showed a gradual decrease in the levels of staphylococci in the air.

The use of a suitable indicator organism could play a role in monitoring the impact of both agricultural and waste treatment operations and the surrounding environment.

Clostridium species have been used as an indicator for source tracking biosolids (Dowd *et al.* 1997, Baertsch *et al.* 2007). Thermotolerant clostridia have been used as an airborne indicator of the land application of biosolids (Dowd *et al.* 1997). Thermotolerant coliforms were present in high numbers in biosolids (10^7 g^{-1}) but were undetectable at locations having the greatest potential for aerosolisation (Dowd *et al.* 1997). Thus, the use of suitable and more resilient indicator organisms, depending on the system being studied, can provide valuable information of the dispersion pattern, source related microorganisms and subsequent management and evaluation of risks in the surrounding environment.

In the present trial staphylococci were good indicators of the chicken contributed aerosols due to the close link of this organism to the chicken. Coagulase negative staphylococci are typically isolated from the skin and nares of cattle, pigs, poultry goats and sheep, with these staphylococcal species from farm animals being markedly different from those associated with humans (Devriese *et al.* 1985). The species associated with poultry include *Staph. simulans, Staph. epidermidis, Staph. warnerii, Staph. lentus, Staph. saprophyticus, Staph. hyicus, Staph. xylosus* and *Staph. cohnii* (Devriese *et al.* 1985), all identified with the exception of *Staph. warnerii* from either litter and aerosols in the present study. The latter three species have also been reported by Aarestrup *et al.* (2000) as being common in poultry. Broilers have also been associated with both staphylococci (60%) *Corynebacterium* (18%) and *E. coli* (5%) with the main species identified being *Staph. lentus* (19%), *Staph. simulans* (18%) and *Staph. cohnii* (13%) and *Staph. captis*, *Staph. xylosus* and *Staph. hominis* (around 7%) (Awan and Matsumoto, 1998). The presence of coagulase negative staphylococci was also favoured by high temperatures and low humidity within a sewage treatment environment (De Luca *et al.* 2001).

Clearly the staphylococci isolated from the aerosols in the present study have been sourced from the chicken. In contrast based on the higher percentages of coryneforms isolated from litter in the present study, coryneforms have their origins in litter of wood material, though a smaller percentage can be linked with the chickens (Awan & Matsumoto 1998) as seen on Farm X in the present study. Air tested within saw mills processing both coniferous and deciduous wood resulted in higher levels of organisms being captured in the air associated with mills processing coniferous wood with corynebacteria (and fungi) being isolated (Dutkiewicz *et al.* 2001). The corynebacteria included genera such as *Arthrobacter*, *Corynebacterium,*

Brevibacterium and *Microbacterium* (Dutkiewicz *et al.* 1996). The present study identified two of these genera (*Corynebacterium* and *Microbacterium*) in litter material sourced from pine.

Aerosol studies have also been carried out in poultry processing plants. Defeathering of broiler chicken carcasses resulted in an introduced marker organism being transmitted through feather particulates (Allen *et al.* 2003). The highest concentration of organisms in air occurred in the shackling and defeathering areas (Heber *et al.* 2006) and both *E. coli* and *Enterobacteriaceae* have been isolated in the air in these areas (Whyte *et al.* 2001). Thus, in the present study, the feather material is a likely source of the staphylococci that this study has associated with the chicken.

In a study comparing total airborne bacterial levels on a broiler breeder farm, the levels were higher in the air in the floor-based litter sheds as compared with the sheds in which the breeders were in cages (2,979,252 and 352,629 cfu m^{-3} respectively) (Dreghici *et al.* 2002). Furthermore, staphylococci were isolated at higher levels (2,765,913 cfu m^{-3} - 92.8% of the total bacteria) in the air in the litter sheds than in the air of the cage sheds (19,255 organisms m^{-3} – 5.46% of the total bacteria) (Dreghici *et al.* 2002). Thus, the difference in the production system may be due to a number of reasons, including a greater level of chicken movement in the floor based systems, as well as the obvious difference – the presence of litter material.

In the current study *Staph. aureus* was not a dominant organism in litter or aerosols. A similar absence of *Staph. aureus* was noted by (Awan & Matsumoto 1998) who reported that among 79 staphylococcal isolates from 6 week old broiler chickens only a single isolate represented *Staph. aureus,* the rest being coagulase negative staphylococci. With one exception there are no studies reporting high levels of airborne *Staph. aureus* in poultry environments. The exception is a study by Heber *et al.* (2006) who reported the isolation of "*Staphylococcus aureus* spp." (from Mannitol Salt Agar) in the air within chicken processing plant environments. However they did not confirm their isolates as coagulase positive *Staph. aureus*. It is possible that the organisms reported by Heber *et al.* (2006) as "*Staphylococcus aureus* spp." were in fact the harmless staphylococci reported in the current study as well as the study of Dreghici *et al.* (2002). In the present study *Staph. aureus* was

very rarely observed and was overshadowed by the dominance of other staphylococci. Our results indicate that *Staph. aureus* is not a risk to humans exposed to the aerosols associated with poultry environments.

Thus collectively the staphylococci/coryneform group (as isolated from Baird-Parker Agar) can form the basis of a "marker-organism" capable of indicating the distance travelled by production system sourced aerosols with a contribution from both chicken and litter material from mechanically operated sheds. (Brooks and colleagues (2009) have similarly suggested that elevated staphylococci levels could be a marker of poultry litter contamination as litter levels were around 10^8 g^{-1} of litter.

The levels of bacteria transferred (and surviving) to the outer environment need to be analysed in a comprehensive manner, taking into account a range of interrelated factors (i.e. both biological and physical) linked to the surrounding environment. Overall, the common indicator organism *E. coli* seems to be a poor survivor in the outside environment and did not travel beyond 20 m. Factors such as humidity and temperature play a role in the die-off patterns of this organism. However, staphylococci seem to have a good survival potential. These chicken associated organisms (the staphylococci) when present at elevated levels can act as an indicator of shed generated aerosols, assisting in the understanding and management of such aerosols within and around the chicken production environment.

7.6 Acknowledgements

I acknowledge my co-authors T. Tran, L. Maddock, A. Gale and P. J. Blackall

The financial support of the Rural Industries Research and Development Corporation Chicken Meat Research Program is gratefully acknowledged. The support of the poultry integrator companies and the individual farmers were importants contribution to this work.

Chapter 8

Mechanically ventilated broiler sheds – a source of aerosolized *Salmonella*, *Campylobacter,* and *Escherichia coli*

This Chapter is published in *Applied and Environmental Microbiology*

Citation:

H.N. Chinivasagam, T. Tran, L. Maddock, A. Gale and P.J. Blackall (2009) The aerobiology of the environment around mechanically ventilated broiler sheds, *Appllied and Environmental Microbiology* **75**(23): 7417-7425.

8.1 Summary

This study assessed the levels of two key pathogens, *Salmonella* and *Campylobacter,* along with the indicator organism *E. coli* in aerosols within and outside poultry sheds. The study ranged over a three year period on four poultry farms and consisted of 6 trials across the boiler production cycle of around 55 days. Weekly testing of litter and aerosols was carried out through the cycle. A key point that emerged is that the levels of airborne bacteria are linked to the levels of these bacteria in litter. This hypothesis was demonstrated by *E. coli*. The typical levels of *E. coli* in litter were around 10^8cfu g^{-1} and as a consequence were in the range of 10^2 to 10^4 cfu m^{-3} in aerosols, both inside and outside the shed. The external levels were always lower than the internal. *Salmonella* was only present intermittently in litter and at lower levels (10^3 to 2.3 X 10^5 MPN g^{-1}), and consequently present only intermittently and at low levels in air inside (range of 2.2 to 0.65 MPN m^{-3}) and once outside (2.3 MPN m^{-3}). The *Salmonella* serovars isolated in litter was isolated from aerosols and dust, with *S*. Chester and *S*. Sofia being the dominant serovars across these interfaces. *Campylobacter* was detected late in the production cycle, in litter at levels of around being10^7 MPN g^{-1}. *Campylobacter* was detected only once inside the shed and then at low levels of 2.2 MPN m^{-3}. Thus the public health risk from these organisms in poultry environments via the aerosol pathway is minimal.

8.2 Introduction

Bacterial aerosols can originate from different sources, each representing a unique aerosol environment. The generation of these aerosols can occur during common agricultural practices such as the spray irrigation of wastewater (Donnison *et al.* 2004), and the land application of biosolids (Brooks *et al.* 2004). Biological material in air does not necessarily occur as independent particles (Jones & Harrison 2004) and the survival of particulate matter linked bacteria can vary with particle size and prevailing atmospheric conditions (Lighthart 2000). In addition to the natural variation of bacteria that occur in the general atmosphere (Lighthart 1999), the creation, generation, and disposal of human and animal wastes can increase the potential of microbial pathogens entering the aerosol environment (Pillai 2007). Animal production systems such as broiler farms have been the focus of attention as potential sources of human pathogens entering the general environment and thus eventually the human food chain. Much of this focus has been on the land application of manures (Nicholson *et al.* 2005b) rather than via the aerosol pathway.

The production of aerosols from various sources is generally linked to risks to adjacent communities. In recent times, there has been research into the impacts of bio-aerosols released directly from swine production systems (Thorne *et al.* 2009). Similarly, studies have also been carried out to assess community risk of infection from bioaerosols to residents adjacent to sites associated with the application of biosolids (Brooks *et al.* 2005b).

The poultry production environment is widely accepted as one that is likely to be a source of human pathogens such as *Salmonella* (Gast 2008) and *Campylobacter* (Zhang 2008), with potential for these organisms to enter the aerosol environment during the production cycle. It is also likely that the prevalence of these pathogens within the production environment could vary. Typically broilers demonstrate fecal shedding of *Campylobacter* at around 3 weeks of age and within 2-4 days of shedding, flocks show a 90-100% prevalence rate due to rapid intra-flock transmission rates (Shane & Stern 2003). For *Salmonella*, the estimates of the incidence have been quite variable (Gast 2003). As an example, there was a 42% prevalence for *Salmonella* in 198 US broiler houses (Byrd *et al.* 1999). As a general pattern *Salmonella* can be isolated from a variety of sources (other than the bird) and at various stages of the production cycle (Lahellec *et al.* 1986).

Modern broiler houses reflect considerable progress in design, with majority of poultry houses in countries such as the US and Australia being tunnel-ventilated (Kidd *et al.* 2003). In these systems, large volumes of air are moved through the house, by negative pressure, to provide the optimal temperature for broiler growth (Kidd *et al.* 2003). Clearly, these large volumes of moving air could potentially contain a range of bacteria sourced from the internal environment of the house, including pathogens such as *Salmonella* and *Campylobacter*.

To date, there have been few studies specifically examining the levels of bacteria, including pathogens, in the air either inside or outside tunnel ventilated broiler sheds. In a Bulgarian study of mechanically ventilated sheds, levels of 1.68 X 10^7 bacteria / m^3 of air were found inside the sheds (Baykov & Stoyanov 1999). *Salmonella* has been recovered but not quantified in the air inside a room containing experimentally infected laying hens (Gast *et al.* 2004) and *Campylobacter* has been detected inside and outside broiler houses in UK (Bull *et al.* 2006)9). Other than these few studies, there appear to have been no reports of studies attempting to quantify the levels of key pathogens such as *Campylobacter* and *Salmonella* in the air in and around broiler houses through the production cycle. Such studies would allow an assessment of the quantifiable risks (if any) to public health and the surrounding environments via the aerosol pathway.

The current study, carried out over three years, addresses this issue of aerosolised bacterial pathogens in terms of assessing levels, observing patterns of distribution as well as the possible interrelationships leading to pathogen presence in aerosols. More specifically the study quantified the levels of *Salmonella*, *Campylobacter,* and *E. coli* (the latter as an indicator organism) within the chicken production environment, through whole production cycles, in both internal and external aerosols on four broiler farms.

8.3 Materials and Methods

8.3.1 Basis for selection of farms, shed details and topography

Four farms were randomly selected from two major integrated poultry companies and a total of six trials were carried out over 2005 to 2007 on these farms. The farms were Farm S (November 2005 to January 2006 and April 2006 to June 2006,

with around 32,000 chickens placed in the study shed in each cycle), Farm X (August 2006 to October 2006, with around 33,000 chickens placed), Farm D (March 2007 to May 2007 and May 2007 to July 2007, with around 35,000 chickens placed each cycle), and Farm L (August 2007 to October 2007, with around 32,000 chickens placed). The study shed occurred in a cluster of four to six sheds (depending on the various farms). This shed was randomly selected and was typically approximately 122 to 150 m in length and 14 m in width. Farms D, L, and S had flat land in front of the fans and Farm X had a semi-circular embankment located at around 19 m from the fans.

8.3.2 Overall study design

Each trial started with the commencement of a broiler production cycle, i.e. prior to chicks being placed and continued until final removal of chickens, with a typical cycle lasting around 55 days. Thinning of the flock, i.e. harvesting of some of the chickens, typically occurred around 35 days. Sampling was carried out on a weekly basis during the production cycle, with aerosols (in and out of the shed) and litter being tested on each sampling date. Dust (on Farms D and L) was tested only intermittently. General farm observations and weather data (temperature and relative humidity) were also collected during the sampling of aerosols to aid the interpretation of data.

8.3.3 Aerosol sampling and layout

Outside aerosol samples were collected at 10 m from the fan with the sampler facing the fans. The exception was that on Farm L, the presence of a slight elevation meant that the external samples were collected at a distance of 9 m from the fans. Aerosol samples inside the shed were collected at 10 m from the fan with the samplers facing the non-fan end of the shed in line with the moving air stream (Chinivasagam *et al.* 2010b) This means that the air sampled had moved the length of the shed (from 110 to 140 m). All sampling was done at a height of 1.25 m.

The aerosol sampler used was the Sartorius MD 8 airscan (www.sartorius.com) which holds a gelatine filter with a pore size of 3 µm. The filter has a residual moisture content of 46-49% and a thickness of 250 µm and has almost a 100% capacity to retain both bacteria and phages. The unit has a variable speed ranging from 33.33 to 133.33 l min^{-1} in increments. Following some initial comparisons, the 133.33 l min^{-1} setting was chosen for all samplings.

Aerosol samples were collected in duplicate (one session followed by the other) with two samplers running simultaneously outside the shed. One of the samplers was dedicated to testing *Salmonella* and *E. coli* and the other sampler to *Campylobacter*. Following this sampling run outside the shed, a similar sampling run was carried out in duplicate inside the shed. The mean of the two sampling runs for each organism was used to assess the number of organisms per m^3 of air both outside and inside the shed.

Immediately after sampling (sampling time 35 – 45 min), the filter was aseptically transferred into an appropriate nutrient liquid medium (to minimise any impacts of sampling stress). The filter used for the *E. coli* and *Salmonella* was dissolved into 0.1% peptone water. The filter used for *Campylobacter* was dissolved into nutrient broth. These dissolved filters were transported to the laboratory and analysed immediately.

8.3.4 Sampling of litter and dust inside the shed

Testing of dust was only carried out on Farms D and L while litter was tested on all farms. The litter was collected using a formal random sampling methodology. At each sampling spot, litter to the depth of 40 mm and with a surface area of 400 cm^2 was collected. A total of 20 samples per shed, representing areas under feeder lines and drinker lines, was collected and pooled into a single sample.

In some trials, the levels of settled dust per week (expressed as g/ 50 cm^2) were quantified. In this work, six pre-weighed petri-dishes were placed (open) on the ledge above a min-vent (approx 2.5 m high). One week later, the petri-dishes were collected and sealed. The dishes were then transported to the laboratory and the weight of the collected dust determined. The data for Farm L is presented.

8.3.5 Weather data

A hand held weather station (Kestrel 4000, pocket weather tracker, Nielsen Kellerman, Australia pty. ltd.) was used on site during the microbiological sampling to record and log wind speed, temperature, and relative humidity. Full data logging was used on Farms D and L while individual readings were taken on Farm S.

8.3.6 Preparation of litter and dust samples for microbial analysis

The litter clumps were broken down and careful quartering was performed to achieve uniform samples. Individual samples were transferred aseptically to a sterile bag. Three lots of 25 g were then weighed into separate sterile bags, to which 225 ml of sterile diluent was added. The diluent varied according to the organism tested (see below). The samples were allowed to soak for 15 min, after which they were aseptically blended, using a homogeniser, for 1 min.

The dust was carefully mixed to achieve a uniform and representative sample. An initial dilution in 0.1% peptone water was prepared by placing 1 g of dust in 18 ml of peptone water. This initial dilution was mixed using a magnetic stirrer for 15 min. Tenfold serial dilutions were then prepared in 0.1% peptone water.

8.3.7 Microbiological analysis of aerosols, litter and dust

The dissolved filter (as previously described) was subjected to serial ten fold dilution and appropriate dilutions were tested as described below.

For *E. coli*, dilutions were prepared in 0.1% peptone water and the levels (expressed as cfu g^{-1} of litter or m^{-3} of air) were performed using Chromocult Agar (Merck). For litter and dust samples the minimum detection limit was 20 cfu g^{-1} and for aerosol samples, the minimum detection limit was 5 cfu m^{-3} of air.

A three tube MPN was carried out for *Salmonella.* Appropriate serial dilutions (0.1% buffered peptone) were inoculated into 10 ml buffered peptone water incubated at 37^{o}C overnight. Six aliquots (each of a 30 µl volume) from each incubated broth were inoculated on to a single MSRV (Oxoid) plate and incubated at 42^{o}C overnight. The plates were observed for motile zones from which XLD agar (Oxoid) was inoculated and incubated at 37^{o}C overnight. Positive colonies from XLD were biochemically confirmed using the O.B.I.S. *Salmonella* kit (Oxoid). Confirmed positives were subcultured from the XLD onto Nutrient agar (Oxoid), incubated overnight at 37^{o}C and further confirmed using *Salmonella* O antiserum, Poly A – I and Vi (Difco). Most Probable Numbers (MPN) of *Salmonella* were obtained from MPN tables, with counts being expressed as MPN g^{-1} of litter and dust or m^{-3} of air. The minimum detection limit was 0.3 MPN g^{-1} of litter and dust and 0.22 MPN m^{-3} of air for aerosols. Selected confirmed *Salmonella* isolates were serotyped by the *Salmonella* Reference Laboratory in Adelaide, Australia.

A three tube MPN was carried out for *Campylobacter*. Serial dilutions were prepared in Preston Broth without antibiotics i.e. Nutrient broth No 2 (Oxoid) and 5% lysed horse blood, after which 1 ml of appropriate dilutions were inoculated into 5.5 ml of Preston Broth with antibiotics (Nutrient Broth No 2 (Oxoid), *Campylobacter* growth supplement SR232 (Oxoid), and *Campylobacter* selective supplement SR117 (Oxoid) with 5% horse blood) in triplicate. The broths were incubated under microaerobic conditions using Campygen (Oxoid) gas generating kits for 37^{o}C for 4 h followed by 42^{o}C for 44 h. The broths were then streaked on to CCDA (Oxoid) which were incubated under microaerobic conditions (as above) at 37^{o}C for 48 h. Typical colonies if present were streaked onto Abeyta-Hunt-Bark Agar plates (Food and Drug Administration 2001), and incubated under microaerobic conditions (as above) at 37^{o}C overnight. The isolates were tested for typical motility, cell morphology, oxidase, and catalase reactions. Most Probable Number (MPN) of *Campylobacter* was reported as MPN g^{-1} of litter and dust or m^{-3} of air. The minimum detection limit was 3 MPN g^{-1} of litter and dust and 0.22 MPN m^{-3} of air for aerosols.

8.4 Results

A total of six trials were performed and representative data from four of the six trials are presented for the three different organisms.

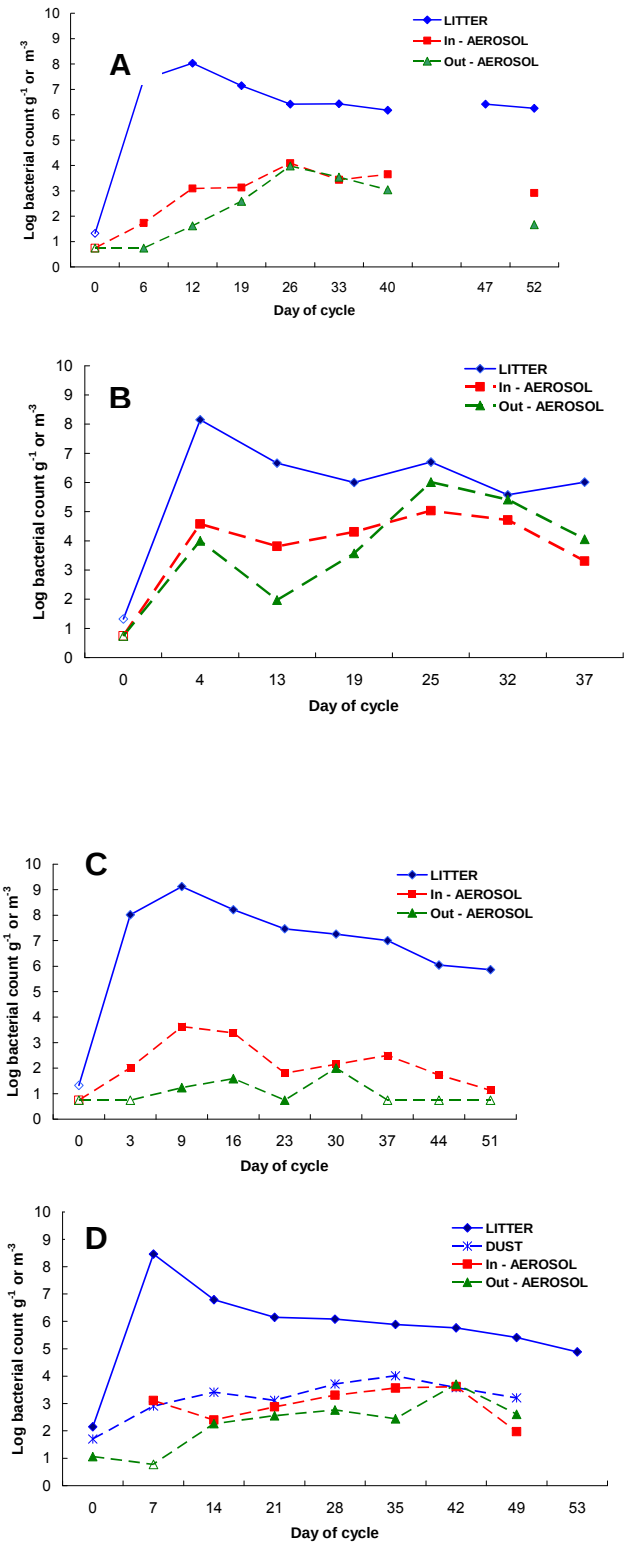
8.4.1 *E. coli*

Fig. 1 illustrates the levels of *E. coli* in litter and aerosols (both inside and outside the shed). The levels of *E. coli* in litter rapidly increased to 10^8 g^{-1} of litter after chicken placement, then remained fairly stable (10^7 g^{-1}) albeit with a slight downwards trend (reaching10^5 to 10^7 g^{-1}), until the end of the cycle (Fig. 1). *E. coli* was not captured in aerosols inside the shed prior to chick placement. However once chickens were placed, typically the levels inside ranged between 10^2 to 10^5 cfu m^{-3}, inside the shed. On all farms, the external air levels at 10 m distance from the fans ranged from not being detected (i.e. below detection limit) to a maximum that varied from 10^2 to 10^4 cfu m^{-3}. The external levels were always below the internal levels with some exceptions. On Farm X, (Fig. 1B) the higher external aerosol levels compared to internal suggested the likelihood that the embankment was causing an accumulation or deflection of airborne microorganisms that was

confirmed by additional testing at day 32 (19, 40 m distance above the embankment) which yielded only very low levels of *E. coli*.

On Farm S two trials were carried out, one over the warmer months and the other spanning across the cooler months. The commonly observed range on Farm S was between 10^2 to 10^4 cfu m^{-3} (warmer months - Fig. 1A) and 10^2 to 10^3 cfu m^{-3} (cooler months Fig. 1C). A notable feature was that in the winter trial, the outside levels were markedly lower (or absent) than the inside levels (Fig. 1C), probably reflecting a seasonality effect.

Figure. 1 Levels of *E. coli* in litter, dust, and air (in and out) during weekly sampling of broiler cycle in Farm S – summer, (Fig. 1 A); Farm X (Fig. 1 B); Farm S - winter (Fig. 1 C); Farm L (Fig. 1 D). Unfilled symbols indicate points where results were negative at a minimum detection limit of 20 cfu g^{-1} (litter); 5 cfu g^{-1} (aerosol).

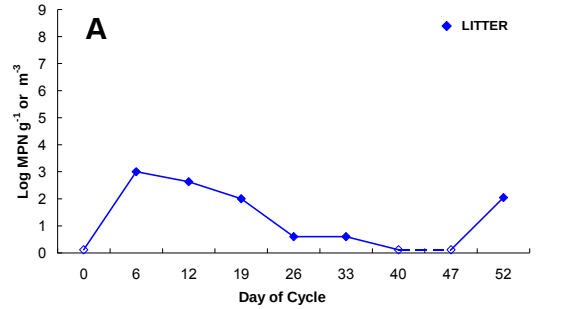


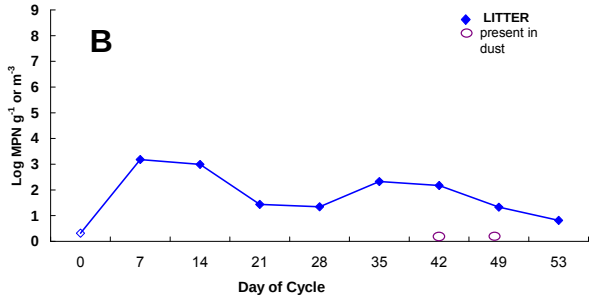
8.4.2 *Salmonella.*

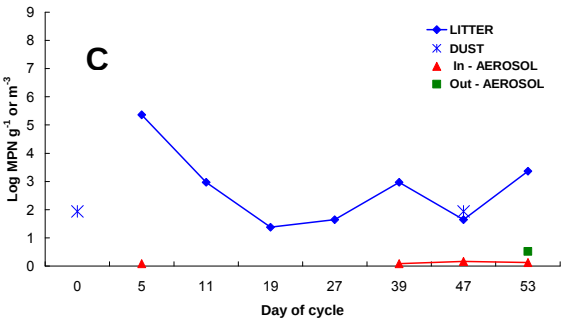
Fig. 2 illustrates the levels of *Salmonella* in litter and aerosols. When present, the *Salmonella* levels in litter ranged from 10^3 to 10^5 MPN g^{-1}, a lower range by around two to three log cycles than that observed for *E. coli*. On Farms S and L where the *Salmonella* levels for litter were sometimes in the range of 10^3 MPN g^{-1}, no *Salmonella* was detected in aerosols inside the shed. In contrast on Farm D where *Salmonella* litter levels were generally higher and ranged above 10^4 to 10^5 MPN g^{-1} of litter (Farm D trials – March to May 2007 and May to July 2007, Fig. 2C and 2D), *Salmonella* was captured in aerosols inside the shed (ranging from 0.22 - 4.4 MPN m^{-3} at various points in the cycle. Farm D was the only farm where *Salmonella* was captured outside the shed, on a single occasion at a low level of 2.3 MPN m^{-3}. Of the six broiler cycles tested in this study, only the two cycles on Farm D yielded *Salmonella* in aerosols, either inside or outside the shed.

On Farm D (Fig. 2C), *Salmonella* was isolated from settled dust within the shed, when no chickens were present presumably as a result of a residual effect of a previous broiler cycle. The levels of *Salmonella* in settled dust reached a maximum of around 10^3 MPN g^{-1} (Farm D). *Salmonella* was also isolated in settled dust on Farm L (days 42 and 49 - data not presented).

Figure. 2 Levels of *Salmonella* in litter, dust, and air (in and out) during weekly sampling of broiler cycle in Farm S (Fig. 2 A); Farm L (Fig. 2 B); Farm D - summer (Fig. 2 C); Farm D - winter (Fig. 2 D). Testing was carried out on all days of the cycle for litter and aerosols. Only positive outcomes have been plotted in the graphs for aerosols, (minimum detection for aerosols 0.22 MPN m^{-3}). Unfilled symbols indicate points where litter was negative (minimum detection limit of 0.3 MPN g^{-1}).







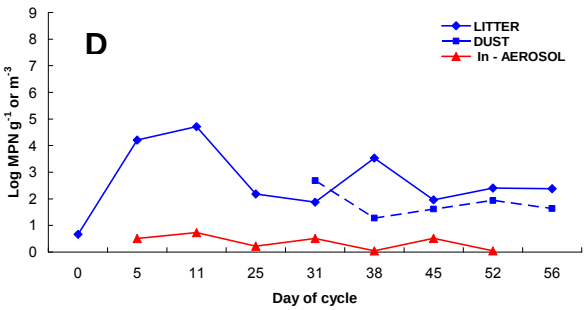


Table 1 presents the dynamics of *Salmonella* serovars present in three phases - litter, dust, and aerosols (both in and out) for cycles 1 and 2 on Farm D. Through these two sequential cycles there is the appearance of five serovars, *S.* Chester, *S.* Senftenberg, *S.* Singapore, *S.* Sofia, and *S.* Virchow. Overall there appears to be a greater dominance of serovars in litter compared to dust. *S.* Sofia (and *S.* Virchow) appear to dominate (in litter, dust and aerosols) during the latter stages of the cycle (days 25 – 27 and onwards) and was not detected prior to day 25 in either trial. In contrast, *S.* Chester, *S.* Singapore, *S.* Senftenberg appear early in the cycle (days 5 – 10) in both cycles and thus were most likely associated with the incoming chicks. *S.* Senftenberg, which was only isolated during days 5 – 10 (cycle 1) was the only serovar never captured in aerosols. *S.* Chester, *S.* Sofia, and *S.* Virchow were the only serovars isolated across both cycles in litter and air inside the shed.

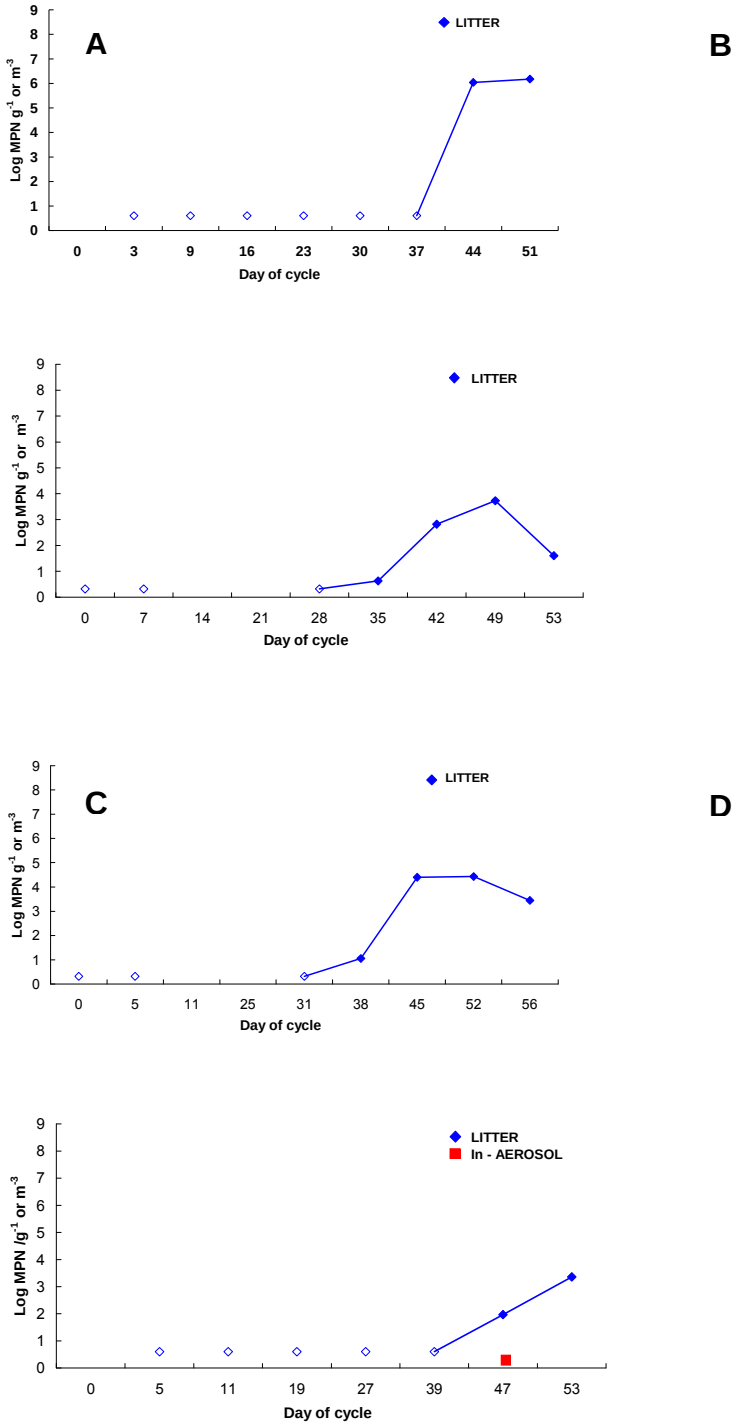
Table 1 Distribution of *Salmonella* serovars in litter, dust and aerosols (in and out of shed) on Farm D during two sequential broiler cycles. Cycle 1 occurred from March to May 2007 and Cycle 2 from May to July 2007.

Day	Cycle	*Salmonella* serovar(s) detected in			
		Litter	Dust	Air In	Air Out
5	1	*S.* Singapore	NP	*S.* Singapore	NP
	2	*S.* Chester *S.* Senftenberg	NP	*S.* Chester	NP
10	2	*S.* Chester *S.* Senftenberg *S.* Singapore	NP	*S.* Chester	NP
11	1	*S.* Singapore	NP	NP	NP
19	1	*S.* Chester	NP	NP	NP
25	2	*S.* Chester *S.* Sofia *S.* Virchow PT 23	*S.* Chester	*S.* Chester *S.* Sofia	NP
27	1	*S.* Sofia	NP	NP	NP
31	2	*S.* Chester *S.* Sofia *S.* Virchow PT 8	*S.* Chester	*S.* Sofia	NP
38	2	*S.* Chester *S.* Sofia	*S.* Chester	*S.* Chester	NP
39	1	*S.* Sofia	NP	*S.* Sofia	NP
45	2	*S.* Chester *S.* Sofia *S.* Virchow PT 8	*S.* Chester *S.* Sofia	*S.* Chester	NP
47	1	*S.* Sofia	*S.* Sofia	*S.* Sofia	NP
52	2	*S.* Chester *S.* Sofia	*S.* Virchow PT 23	*S.* Virchow PT 23	NP
53	1	*S.* Sofia *S.* Virchow PT 23	NP	*S.* Sofia *S.* Virchow PT 8	*S.* Singapore

8.4.3 *Campylobacter*

Campylobacter was detected in litter late in the cycle on all farms (Fig. 3) except Farm X (data not presented) where the organism was absent in the litter until the last sampling date, Day 37 (the latter cycle dates beyond day 37 – 55 were not sampled). The levels of *Campylobacter* in litter reached between 10^3 to 10^7 MPN g^{-1} of litter. *Campylobacter* was only ever detected in the inside air on one occasion (Farm D, Fig. 2 D). The sole detection was at a low level (0.22 MPN m^{-3}) and the levels in litter at that time were also at a low level of 100 MPN g^{-1}.

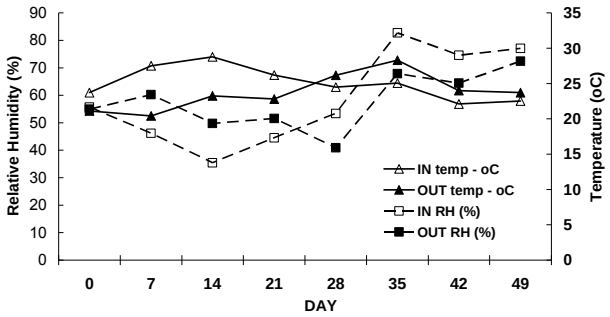
Figure 3 Levels of *Campylobacter jejuni/coli* in litter, dust, and air (in and out) during weekly sampling of broiler cycle in Farm S (Fig. 3A); Farm L (Fig. 3B); Farm D - summer (Fig. 3C); Farm D - winter (Fig. 3D). All farms were tested, aerosols in and outside and points where *Campylobacter* was not detected was not plotted (minimum detection for aerosols 0.22 MPN m^{-3}). Unfilled symbols indicate points where litter was negative (minimum detection limit of 0.3 MPN g^{-1}).



8.4.4 Weather parameters (Farm L)

Fig. 4 illustrates the temperature, humidity and wind speeds for Farm L (inside and outside the shed) and were logged at the point of the sampler. The speeds inside the shed showed a gradual increase over the chicken cycle, reaching a maximum of 1.5 m s^{-1} late in the cycle. The speeds recorded outside at 10 m from the fans also showed a gradual increase over the cycle with the maximum reaching around 4 m s^{-1} (data not presented).

Figure 4 Changes in relative humidity and temperature during a cycle at Farm L.

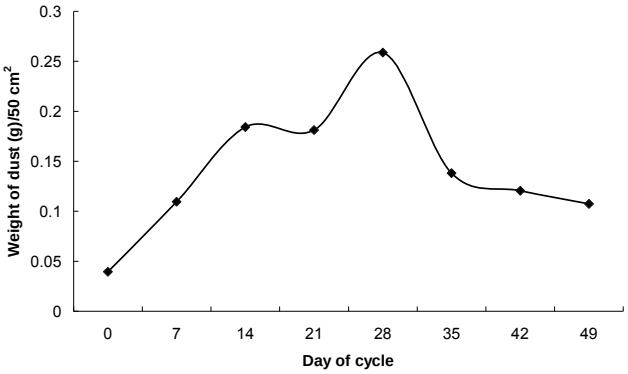


In the early stages of the cycle (until Day 28), the temperature : relative humidity combination ranged from approximately 25 -27^{o}C : 40 -50% inside the shed and 20-23^{o}C : 50-55% outside the shed (Fig. 4). After the 28th day, the internal temperature : relative humidity combination was around 23 – 25 ^{o}C : 75 – 80%. Similarly the external temperature : relative humidity combination was 25 -27^{o}C :65-75% relative humidity. Overall there was an increase in relative humidity with chicken age.

8.4.5 Settled dust levels (Farm L)

Fig. 5 illustrates that there was no sequential increase in settled dust. Increasing weights were observed up to day 28, after which the weights of collected dust on days 42 and 49 returned to the almost the same levels as those recorded on day 7 at the beginning of the cycle.

Figure 5 Change in weight (g) of weekly levels of settled dust during a cycle per 50 cm^2 at Farm L.



8.4.6 Farm related factors

General farm and shed related factors were also recorded (data not presented) and the number of fans in operation across the sheds on the different days did not seen to have a major impact on the *E. coli* litter – aerosol relationship observed in the various trials or the patterns observed with *Salmonella* or *Campylobacter* detection across the trials. The overall comment on these farm /shed related factors (such as number of fans in operation) is that these subtle variations seem to have a minimal impact on the general trends of the distribution patterns of *E. coli*, *Salmonella,* and *Campylobacter* in litter, dust, and aerosols. Despite variations in farm management practices, the varying pathogen status of the flock (presence or absence of *Salmonella* and *Campylobacter)*, seasonality and topography, we could detect general patterns of survival of the pathogens across the different trials carried out on the study farms.

8.5 Discussion

The sources of *Campylobacter*, *Salmonella,* and *E. coli* in air are the chicken, either directly or indirectly from the feces, and dust originating from the production system. In automated chicken egg layer management systems, the main sources of aerosols were the live birds (Venter *et al.* 2004) with both the feces and the birds linked to the contribution of both *Salmonella* and *E. coli* to aerosols (Venter *et al.* 2004). Air circulation within the poultry housing environments provides opportunities for the transfer of these pathogens to the surrounding air environment. This transfer, the aerosolisation process, is a traumatic process for most microorganisms and survival can be dependent on the mechanisms of aerosolisation and the climate into which

these organisms are launched (Wathes *et al.* 1986). In the present study, this transfer process was seen to occur via the litter-dust-air interface within the shed environment. The present study has demonstrated a more or less common pattern across all the farms studied in terms of the levels of these organisms captured both inside and outside the shed. Among the three organisms tested, *E. coli* was the dominant organism in aerosols both inside and outside the shed. The pattern of distribution of this organism clearly demonstrated a litter – aerosol relationship across trials which spanned a period of 3 years. Consistently, levels of *E. coli* were high in litter and, thus in the aerosol, which was not the case for either *Salmonella* or *Campylobacter*.

Studies relevant to the presence and levels of air-borne *E. coli*, *Salmonella* and *Campylobacter* inside poultry shed environments are very limited. However analogies can be drawn from the survival patterns of these organisms in other aerosols environments such as processing plants or hatcheries. *E. coli* K12 was able to survive in aerosols or large droplet particulates during de-feathering of poultry (Allen *et al.* 2003). The levels of *E. coli* in processing plant air have been reported to range from $\log_{10}$ 1.18 to 1.67 cfu/15 cubic feet (de-feathering areas) (Whyte *et al.* 2001). In another study, the levels were in the range of 100 to 624 cfu m^{-3} with the highest levels in picking and shackling areas (Heber *et al.* 2006). The present study has demonstrated higher levels of *E. coli* in air than these processing plant studies, with levels typically ranging from 10^2 to 10^4 cfu m^{-3} (maximum 10^5 cfu m^{-3}) across various trials. Lower levels of *E. coli* have been captured in aerosols in pig sheds (23 cfu m^{-3}) (Chinivasagam & Blackall 2005). These higher levels of *E. coli* in aerosols found in broiler sheds in comparison to other studies (processing plants and pig sheds) probably reflect the differences in the very nature of the aerosol environments and the waste generated by high numbers of live birds present within a ventilated enclosed shed environment compared to these other environments.

Temperature (and relative humidity) can have an impact on survival of *E. coli* in the aerosol environment. This link has been demonstrated in a study associated with infection in pigs raised under climate controlled conditions where the highest number of infections occurred at 15 ^oC and no infections occurred at 30 ^oC (Wathes *et al.* 1986). Laboratory studies to address this issue found a rapid die-off of this organism during aerosolisation at 45-65% relative humidity and a temperature of 30^oC, with both factors having a marked impact on survival (Wathes *et al.* 1986).

Death rates of the organism at 30°C were four times faster than at 15°C. These studies (Wathes *et al.* 1986) showed that a warm dry atmosphere with a temperature around 30°C and a <50% relative humidity will favour the rapid death of *E. coli* in air. The above results supported the relative importance of ventilation rates in terms of microbial death as a means of improving air quality within a piggery (Wathes *et al.* 1986). It is possible that the ventilation rates occurring in the chicken sheds could also be contributing to significant *E. coli* die-off. In the present study internal temperatures were around 30 °C and a relative humidity of around 50% during the initial stages of the cycle, similar to conditions in study of Wathes *et al*. (1986) and 25 °C and 70% during the latter stages of the cycle. These differences in both temperature and relative humidity could impact on the levels of *E. coli* that survived in air at various times. Overall, *E. coli* has shown the potential to be distributed in the aerosol environment, the levels of which seem to be dictated by the very nature of those environments. This information forms the basis for creating an understanding for the aerosol survival of both *Salmonella* and *Campylobacter*.

In the present study *Salmonella* was isolated at lower levels than the *E. coli* in litter and as a consequence at lower levels in aerosols inside and outside the shed. Importantly, the detection of *Salmonella* in aerosols only occurred at intermittent occasions of a cycle. The key point is that the levels in litter were simply not high enough (as with *E. coli*) to cause a dominance of this organism in aerosols inside the shed. Moreover, though present in litter on some farms, *Salmonella* was never captured in aerosols on these farms.

There are few studies on aerosolised *Salmonella* in poultry environments. Air-borne movement of dust and fluff have been implicated in the transfer of this organism in layer houses (Davies & Wray 1994). *Salmonella* was isolated (63 of the 206 samples) in aerosols in processing plant environments (picking areas) at levels ranging from 2 to 598 cfu m^{-3} (Heber *et al.* 2006). *Salmonella* has shown to be viable in laboratory generated aerosols for more than 2 h (McDermid & Lever 1996). As well, it was shown that the death rate of *Salmonella* was influenced by the protective nature of the media during aerosolisation along with overall prevailing relative humidity and temperature of the air (Stersky *et al.* 1972). The D values for *S.* Newbrunswick aerosolized in skim milk at 10°C ranged from 245 min to 404 min at 90 and 30% relative humidity and at 21°C from 164 to 470 min (Stersky *et al.* 1972). This work, though carried out under laboratory conditions, does emphasise the link between environmental parameters (whether it be the internal poultry shed

or the external atmosphere) and the impact on survival of air-borne organisms and thus contributing the low levels observed within the shed.

Campylobacter was only ever isolated once (from air) inside the shed and never outside, despite the numerous samplings performed in this study. It appears that *Campylobacter* does not survive well in the aerosol environment, despite the high levels isolated from litter. *Campylobacter* is very sensitive to drying (Murphy *et al.* 2006) and thus would be a poor survivor in the air environment. *Campylobacter* has been detected in 46.7 to 70% (defeathering) and 6.7 to 70% (evisceration) of the aerosols in plant environments at levels from 0 to 60 cfu/ 15 cubic feet of air (Whyte *et al.* 2001). Similarly, *Campylobacter* was prevalent in the aerosols, droplets and particulates, mainly in the evisceration areas and the relevant carcases, at the time, had high counts (log_{10} 5 to 7.8) (Allen *et al.* 2007). In the present study though *Campylobacter* levels were high (10^5 to 10^7 organisms g^{-1} in litter), the organism was not a common inhabitant of either the internal and external aerosols. Since *Campylobacter* is sensitive to dry conditions, high relative humidity and/or precipitation contribute to survival in the environment (Patrick *et al.* 2004). Though a high relative humidity (75 to 80% at 23 to 25 ^oC) did occur at the latter stages of the broiler cycle it would appear that other factors resulted in the low levels of *Campylobacter* seen in the current study. For example, key regulators of the stress defence systems found in *Salmonella* spp. and *E. coli* are not present in *C. jejuni* (Murphy *et al.* 2006). Hence in the current study, even though *Campylobacter* was present in litter at high levels it is possible the organism was already stressed when present in the litter phase and simply could not survive further aerosolization stress. In contrast, a processing plant may have different internal environmental conditions than in a shed (more humid and moister) due to the very nature of the activities undertaken. It appears that *Campylobacter* can possibly survive better in processing plant environments than those in poultry sheds.

Extensive testing in the current study across several farms resulted in *Campylobacter* not being isolated in the outer environment, even with a low detection limit of 0.2 MPN m^{-3}. Bull *et al.* (2006), in the context of studies on the possibility of air transmission routes on-farm being a source for flock infection, detected *Campylobacter* inside the shed in 6% (from a total of 248 samplings) of the air samples (originating from five flocks) and on four occasions from 18 samplings (originating from two of the five flocks), up to 30 m downwind of the broiler house. As the internal sampling included settle plates this means that some of the positive

results could have been associated with large particulate matter, not typically regarded as an aerosol component. Given the low isolation percentage of *Campylobacter* reported (from air) inside the house by Bull *et al.* (2006), and our study which isolated (from air) the organism only once inside the house, there is a remote likelihood of this organism being present in significant numbers in the air outside the shed.

The present study saw a decrease in survival of *E. coli* in external aerosols linked to the colder months, suggestive of seasonality impacts such as humidity. Reduced outdoor survival of *E. coli* has been linked with increasing temperature and direct sunlight but not wind direction or air quality (Handley & Webster 1995). Single bacteria are not common in the atmosphere, but are more commonly “clumped or `rafted' on pieces of plant or soil debris in the atmosphere (Tong & Lighthart 1998). Greater bacterial survival following solar radiation of larger particles (compared to small) supported the hypothesis of a dominance of “larger-sized viable bacteria-associated particles” in the atmosphere during clear sunny days (Tong & Lighthart 1998). *Campylobacter* has also been suggested to be sensitive to UV radiation in the form of sunlight (Murphy *et al.* 2006) and thus would have problems surviving in the open atmosphere while being transported as aerosols. A Swedish study assessing the correlations between *Campylobacter* spp. prevalence in the external environment and broiler flocks found this organism to be more frequently present in the surroundings on rainy days than when compared with sunny days (Hansson *et al.* 2007). This suggests that atmospheric conditions can have an impact on supporting the organisms’ presence either in the environment or air. In the present study the only organism of significance found in the external environment (at 10 m) was *E. coli*, which could be a result of the better survival potential, together with the high numbers being expelled and perhaps travelling in larger particles affording some protection.

Studies (Kwon *et al.* 2000) on the recovery of *S.* Typhimurium from infected chickens demonstrated that sampling aerosols was a representative way of understanding overall flock contamination, alleviating the need to examine large numbers of litter samples. This earlier study (Kwon *et al.* 2000) clearly demonstrated a litter – aerosol link, a link also observed in the present study. In a cage production situation, excreted *Salmonella*s have been suggested to show increased survival in nest boxes due to body heat of the birds and protection via the organic matter in feed or dust (Davies & Wray 1994), a possible reason for the good

survival of *E. coli* in litter in the present study. Similarly, the temperatures within litter may have a role in supporting the continued survival of the organism.

Settled dust can be a source of pathogens that enter the aerosol environment. *E. coli* has been isolated from settled dust collected in residential environments (Rosas *et al.* 1997). Litter has ultimately a role in the generation of dust that can accumulate on walls, ceiling and other equipment, with micro-organism adhering to this dust (Richardson *et al.* 2003). Studies involving laying hens raised in houses with litter and in cages have shown that there was 1.6 times more dust and 2.4 times more bacteria in the air of litter based poultry houses than the cage based technologies (Vaicionis *et al.* 2006). In the present study *Salmonella* was isolated from settled dust. The levels of dust peak during the middle of the cycle, thus showing a link to chicken activity.

Various factors can contribute to the continued survival of *Salmonella* in source material such as dust or litter. It is possible that some serovars can be more resilient that others in a poultry environment. A Danish study found a *S*. Senftenberg clone persisted for more than 2 years, despite cleaning, disinfection, desiccation and depopulation, and was subsequently able to infect newly placed *Salmonella*-free layers (Broennum Pedersen *et al.* 2008). In the present study, the fact that all serovars (except *S.* Senftenberg) were detected in litter and aerosols, suggest that those serovars detected in the air were resilient enough to be captured in the aerosol environment as well as litter. Overall *S.* Chester was by far the dominant serovar captured in all three intefaces through the two consecutive cycles and thus, in the present study was by far the most resilient serovar in terms of entering and surviving in the aerosol environment.

Ultimately the direct risk of acquiring infections from aerosols containing these pathogens is what matters. Air-borne particles of >7 μm are trapped in the upper respiratory tract regions, nose and throat (and can thus gain access to the gastrointestinal tract), while smaller particles (< 1 μm) can pass down to the alveoli of the lungs (Hatch 1959). Hence, in terms of the risk of direct illness, it is only that fraction of the air-borne pathogens that are capable of being swallowed that pose an infection risk. It is generally accepted that for the para-typhoid *Salmonella* (i.e. the types of *Salmonella* present in chickens) the infectious dose is around 10^5 to 10^6 organisms (Shuval & Yekutiel 1986). In contrast, the infectious dose for *Campylobacter* has been reported to be around 500 organisms (Robinson 1981).

Overall, when considering the relevance of the levels, if any, of *Salmonella* and *Campylobacter* in air, it is important to include recognition of the difference of the infectivity of these two species.

This study has identified the dynamics of pathogen transfer within Australian mechanically ventilated production systems (Australian Chicken Meat Federation 2007) which do not vary markedly to the US production systems (University of Georgia 2007). Given the overall similarities of the production systems, there is likely to be little difference in the way both *Salmonella* and *Campylobacter* behave within the relevant production environments (including the aerosol environment), which is ultimately based on their presence and levels.

In conclusion, this study has shown that, as a direct consequence of the association of both *Salmonella* and *Campylobacter* with chickens, these organisms can be inhabitants of the immediate poultry environment. However, the levels of these organisms transferring into both the internal and external environment as aerosols are of little significance in terms of human infections. A combination of factors dictates the survival potential of these organisms in the environments studied. The very low levels of capture as aerosols both inside the shed and at close distances from the fan outside the shed across a three year period studying varying farms means there is minimal risk of encountering high levels of these organisms under normal prevailing operating conditions on poultry farms.

8.6 Acknowledgements

I acknowledge my co-authors T. Tran, L. Maddock, A. Gale, and P. J. Blackall

The financial support of the Rural Industries Research and Development Corporation Chicken Meat Research Program is gratefully acknowledged. The support of the poultry integrator companies and the individual farmers was an important contribution to this work.

Chapter 9

Conclusions and implications

9.1 Introduction

9.1.1 Waste, food and water-borne outbreaks

The challenges linked to livestock waste in the environment have always been of concern due to the potential environmental reservoir of zoonotic pathogens originating from intensive animal farming. In the current climate most animal waste has a role in agriculture, specifically farming food crops. Therefore, there is need for agriculture to be sustainable without concerns of environmental pollution with zoonotic pathogens (Donham 2000, Gerba & Smith 2005). Studies in the United States have been carried out on both the direct and indirect impacts of zoonotic pathogens on human health linked to concentrated animal feeding operations (CAFO) (Hill 2003, Carlson *et al.,* 2011).

The growing number of food and water-borne outbreaks has triggered concerns about the impacts of animal waste on human health. For example a major water-borne outbreak of *E. coli* 0157 and *Campylobacter* occurred in 2000 in Walkerton in Canada, with 2300 persons being taken ill and 7 deaths (Hrudey *et al.* 2003). The organisms implicated originated at an adjacent cattle farm (Clark *et al.* 2005) contaminated the city water supply. In 2007, contaminated spinach resulted in a food-borne outbreak across 26 states and the Centre for Disease Control (USA) estimated the possibility of 4000 cases based on those that reported the illness. The implicated spinach was grown on a 2000 head cattle ranch where *E. coli*, which is thought to have originated from cattle faeces (Anon 2007), contaminated the irrigation water.

It is also possible that a source of contamination can be ongoing and originate from multiple sources. During 1995 – 2006, the Salinas watershed in California was implicated as a source for *E. coli* 0157:H7 (from animal faeces) being responsible for 22 produce outbreaks involving mainly spinach and lettuce. These outbreaks were linked to various point source contaminations along the watershed following heavy rains (Cooley *et al.* 2007). *E. coli* 0157:H7 is primarily associated with cattle. The main reservoir for *Campylobacter* is warm-blooded animals such as poultry though the organism has been isolated from surface waters (Koenraad *et al.* 1997) possibly because of run-off illustrating the role of the environment.

Salmonella is a zoonotic pathogen which has also been responsible for large outbreaks involving food crops. In 2008, jalapeno peppers were the vehicle for an outbreak across 43 states in the USA and 1442 people were ill. The cause was *Salmonella enterica* subsp*. enterica* serovar Saintpaul although the source of this infection remains uncertain, (Anon 2008). There is thus need for work targeting the environmental transmission of these key pathogens during the use of waste (such as effluent, litter and manures) as a consequence of intensive pig and poultry farming.

9.1.2 Waste, aerosols and zoonotic pathogens

Arising from solid and liquid wastes, aerosols are another source of pathogen transfer due to either the nature of the farming practice (e.g. tunnel ventilation) or the re-use of waste (e.g. irrigation). Such aerosols from intensive animal operations may be generated by liquid droplets or dry material and transfer in air either individually, as clusters, or on organic particulate matter (Millner 2009). Studies undertaken in New Zealand tested the ability of microorganisms to survive in aerosols originating from treated human effluent and demonstrated the detection of *Serratia entomophila* used as a tracer at 100m and 200m during low and high-pressure spraying respectively (Donnison *et al.* 2004). Thus piggery effluent could be a source of pathogen transfer when used as a source of irrigation for spraying agricultural crop production that can occur adjacent to pig farms in Australia.

Similarly, pathogens can also move, via aerosols, from a concentrated source e.g. composts to the environment and cause illness in humans via direct ingestion (Déportes *et al.* 1995). Studies on both pig and broiler sheds in Australia (Banhazi *et al.* 2008b, Banhazi, 2008 *et al.* 2008c) assessed the airborne concentration (both viable (bacterial) and non-viable particulates) from the internal shed environment. Most pig and poultry sheds in Australia house large number of animals and are mechanically ventilated with the movement of large volumes of air to the exterior environment. Banhazi *et al.* (2008b) measured the airspace surrounding 17 Australian poultry buildings (2000 – 2001) and noted that the older buildings appeared to have lower airborne particle concentrations than the modern mechanically ventilated buildings.

The above studies found that the maximum levels of total bacteria in air in poultry and pig sheds were 5.27×10^5 and 1.17×10^5 cfu /m^3 respectively. These levels are high however the number of the key pathogens *Salmonella* and *Campylobacter,*

within the reported total count by Banhazi *et al.* (2008b) is not known. Studies in the USA have detected both coliforms and *E. coli* in aerosols (2 log10 to 10^2 MPN m^3) at distances of a biosolid loading operation (Brooks *et al.* 2005b) illustrating the potential for such organisms to travel distances and thus a risk.

As with animal waste, wastewater from sewage treatment contains human and animal pathogens that can cause viral, bacterial, or parasitic infections by direct contact or contamination of food crops (Godfree & Farrell 2005). The re-use of human effluent is a widely researched area both internationally and in Australia (Rudolfs *et. al.* 1950, Gardner, *et al.* 2001, Chinivasagam *et al.* 2008, Ross *et al.* 1992, Adams & Spendlove 1970, Benarde 1973). There was thus the need in Australia to undertake similar studies with piggery effluent and poultry litter.

9.2 Objectives of research

Intensive animal operations in Australia are large consisting of 22,522 million pullets and layers in 2011 (Anon 2012), 550 million in 2009 – 2010 (Australian Chicken Meat Federation 2012) and 5.4 million pigs in 2007 – 2008 (Anon 2009a). These are predicted to grow in the future. However, there are increasing demands for ethically produced safe food with minimal interventions. All these factors have contributed to on-going environmental concerns linked to large-scale intensive animal farming both in Australia and overseas. Such concerns also surround the practices of re-use of animal waste for various purposes including food agriculture. This study has provided data previously unavailable in Australia, on pig and poultry farming and opened avenues for further research in this area.

9.2.1 What are the types and levels of zoonotic pathogens present in Australian pig and poultry waste?

The initial objective of this study was to investigate the extent of the significant pathogens in farm wastes i.e. piggery effluent and chicken litter. Re-use of piggery effluent and chicken litter from both pig and poultry farming is an ongoing and growing Australian practice, especially in Queensland. The first and second studies evaluated the extent of zoonotic pathogens in both piggery effluent and chicken litter respectively. Among the zoonotic pathogens studied were the key food-safety pathogens *Salmonella* and *Campylobacter* which were present in both pig effluent and chicken litter and thus have the potential to transfer via various environmental pathways.

More specifically, the first study via a survey of piggery effluent across 13 widely distributed pig farms in S. E. Queensland, determined the extent of zoonotic pathogens, of pig origin associated with ponded pig effluent. The results showed that *Erysipelothrix rhusiopathiae* and rotaviruses were not present in piggery effluent. The study also provided baseline levels of two key pathogens, *Salmonella*, *Campylobacter* and *E. coli,* the latter an indicator, to enable the management of risks associated with the re-use of ponded effluent. *Campylobacter* was more of concern than *Salmonella* due to its higher levels in effluents studied.

The second study surveyed broiler litter across 28 farms in three Eastern states; Queensland, NSW and Victoria to understand the extent - levels of the main zoonotic pathogens. The levels of pathogens in both multiple-use and single-use bedding were compared across the farms surveyed. The survey demonstrated the absence of *Listeria monocytogenes*, (a major zoonotic pathogen that can survive in soil environments) in poultry litter. However, the key pathogens *Salmonella* and *Campylobacter* were present at appreciable levels across the litter sourced from various states, irrespective of it being single or multiple-use. *Salmonella* was present in the majority of litter samples (20 of 28) but only at low levels across both single and re-use litter farms. However, the majority, 70% of the *Salmonella* isolated across all the farms were S. Sofia, which is apparently of low pathogenecity to humans. Serovars of concern to humans represented the rest. These outcomes can have a lower impact on the presence of *Salmonella* from food-safety perspective to the broiler industry.

9.2.1 What is the potential for movement of key food-safety pathogens *within* the farming systems?

Prior to re-use within the farming system both litter and piggery effluent undergo a simple and cost effective treatment process targeted at pathogen reduction. Used litter destined for the next farming cycle is piled-up in-shed to aid in shed clean-out prior to placement of the next cycle. This simple process is in effect, composting and it generates heat that contributes to both *Salmonella* and *Campylobacter* die-off (not reported in this thesis). Similarly piggery effluent is stored in anaerobic ponds for a period where natural die-off results in pathogen reduction.

The third study examined the changes in *Salmonella* serovars during litter re-use across a farming cycle based on a current Australian practice. During this practice,

new bedding is placed at the front end of the shed and re-used bedding at the back of the shed. The study demonstrated that was a trend for lower levels of *Salmonella* (and a lower *Salmonella* serovar diversity) in re-used litter environment as compared with the new litter environment. There is therefore a possibility that re-used litter may inhibit *Salmonella*. The re-used litter thus did not contribute to the build up of *Salmonella* and thus be a source entry to "*Salmonella* free" new flock with concerns to food-safety.

The fourth study addressed the direct risks of pathogens to humans (inhalation by farm workers) due to the re-use of piggery effluent within the pig shed for flushing and cleaning. Aerosol droplets generated during this process can be a source of pathogens (in a similar manner to spray irrigation of a crop). The study addressed the risks caused to farm workers exposed to such aerosol droplets. By studying *E. coli,* an indicator organism in piggery effluent, this study demonstrated that based on *E. coli* levels both prior to and after shed flushing; there was no apparent risk to humans.

9.2.3 What is the potential for movement pathogens, *external* to shed environments, such as soil?

Piggery effluent is re-used on-farm for the irrigation of crops food and pasture. There is thus the the potential for pathogen build-up in soils and the contamination of crops, or transfer via soil to waterways during heavy rain. *Arcobacter butzleri*, is an emerging food-borne pathogen also known to be present in pigs, though an area with limited research. This study demonstrated that *Arcobacter* spp. levels in the effluent ponds were high (even when compared with both *Salmonella* and *Campylobacter*) and varied from 6.5 X 10^5 to 1.1 X 10^8 MPN 100 $ml^{-1.}$ The *Arcobacter* levels in freshly irrigated soils ranged from 9.5 X 10^2 to 2.8 X 10^4 MPN g^{-1} in all waste disposal sites tested. *A. butzleri, A. cryaerophilus* and *A. cibarius* were isolated from both piggery effluent and effluent-irrigated soil, at levels suggestive of good survival in the effluent pond. This is the first international study to provide quantitative information on *Arcobacter* spp. levels in piggery effluent and also associates a newly described species, *A. cibarius* (Houf *et al.* 2005) with pigs and piggery effluent environments There could be potential risks from this pathogen of which the pathogenic potential to humans is unknown.

9.2.4 What is the potential for movement of key food-safety pathogens via aerosols?

Modern poultry operations are a source of large volumes of air and dust (~50,000 chickens/shed, several sheds/farm) expelled due to the mechanical ventilation of sheds. The sixth and seventh studies addressed this aspect using two approaches; the first by identifying an indicator organism to assess the distance expelled broiler shed air travelled and the second by determining specific zoonotic pathogens in aerosols. Staphylococci, (commonly associated with chickens were present in the air around mechanically ventilated broiler sheds. This study thus used staphylococci as an airborne 'marker organism' to detect poultry derived aerosols in the surrounding environment of broiler sheds. It was demonstrated that staphylococi levels reached background levels at 400m from the fans, possibly due to dilution and dispersion of aerosols over distance. Thus, aerosols emitted from broiler sheds could be monitored, and managed to address neighbour complaints by examining the levels of staphylococci at various distances from the sheds.

The final study in this series continued to determine the risks that could be attributed to aerosols from large mechanically ventilated sheds by assessing the extent that key pathogens can be present in aerosols both within the shed and consequently emitted from the shed via ventilation fans. The study was undertaken over a 3-year period on four poultry farms. The levels of two key pathogens, *Salmonella* and *Campylobacter*, were assessed along with the indicator organism *E. coli,* in aerosols within and outside poultry sheds. *Salmonella* was detected (at low levels) both inside and outside the shed (at 10 m). The *Salmonella* serovars isolated in litter were generally also isolated from aerosols and dust within the shed. *Campylobacter* was detected only once inside the shed during the 3-year period at low levels. A key point that emerged is that the levels of airborne bacteria appear to be linked to the levels of these bacteria in the litter and that managing the levels of these organisms in litter would manage the levels in aerosols. The study showed that based on the outcomes of the study there was minimal risk to humans.

9.3 Contributions to research

These series of studies have contributed knowledge by a scientific and an industry / environmental perspective providing seven peer reviewed international scientific publications (Appendix 1) on several aspects of food-safety pathogens and intensively farmed pigs and poultry. There was a lack of data on the extent of the key pathogens in both piggery effluent and chicken litter, with that lack of data reducing confidence in the capacity of Australian agriculture to re-use these by-products. There were also safety concerns with regards to pathogen transfer from pathways other than re-use such as the the air environment via aerosols from intensive farming practices.

Re-use of poultry litter is not a common practice internationally and some of the outcomes of our trials provide a baseline that can support further research on litter re-use in a manner adopted in Australia. The significance of the outcomes of the work on litter has been recognised by the Australian chicken meat industry, which has funded two subsequent studies on litter re-use (one now completed and the other in progress). These subsequent studies will provide further data on the impact of litter re-use associated with the two key food-safety pathogens - *Salmonella* and *Campylobacter*.

This thesis has contributed to a greater knowledge of pathogens associated with piggery effluent. In particular, *Arcobacter butzleri* is considered an emerging food-borne pathogen. This is the first report measuring *Arcobacter* levels in piggery effluent. In addition, this was the first international study recognising that the new species *A. cibarius* can be present in piggery effluent and in soils irrigated with piggery effluent. Further work investigating the potential impact of both *Arcobacter butzleri* and *Arcobacter cibarius* is now warranted.

These studies have also contributed to research on aerosols which is, especially relevant to intensive poultry operations. Prior to the work described in this thesis there was limited knowledge on the extent and dispersion of pathogens via poultry derived aerosols in the surrounding environment. This research introduced, for the first time, the possibility of using a “marker organism” i.e. staphylococci to “track” that aerosols from poultry sheds do travel. Moreover, the presence and levels of the key organisms, *Salmonella* and *Campylobacter,* in aerosols were determined and the potential risk to humans assessed.

A major limitation to litter re-use has been concerns that the level of food-borne pathogens in both chickens raised on the re-used litter and in the shed environemnt in general) will rise with re-use. The present study showed that detailed information on food-borne pathogens aspects when litter is either re-used within the shed or externally in the environment. The outcomes of this study will allow the industry to make relevant, sustainable decisions on litter management. This study has provided baseline levels of pathogens in litter and assessed litter re-use through the chicken farming cycle. Such outcomes support re-use thus ensuring that the poultry industry has a range of clear scientific data to support management options that benefit the industry. Research from the current study and a subsequent follow-on study funded by industry are being incorporated into new draft industry guidelines on litter re-use.

Similarly, the research on piggery effluent assisted in the development of guidelines to ensure the safe and sustainable re-use of piggery effluent in and around piggery farms. This study has provided baseline information on the extent (or levels) of pathogens in piggery effluent and aerosols. Thus during re-use appropriate risk assessments or decision trees can be formulated depending on end use (e.g. raw food crop or pasture). In particular, the Australian pig industry now has guidelines (Australian Pork Limited 2010) on effluent re-use based the results on the presence of pathogens generated in the current study.

The knowledge provided by the study that piggery effluent can be re-used for washing of sheds is a key contribution. Such re-use back into the shed (shown in this study to not cause any risks to piggery staff) can result in savings of large volumes of fresh water that would otherwise be required. Such savings on water consumption also apply to the use of piggery effluent as an irrigation fluid – with the data from this study providing specific information on soil survival of food-borne pathogens. Quantitative Microbial Risk Assessment using the airborne levels of *E. coli* inside pig sheds during flushing/washing showed that for every 10,000 workers who spend 3.8 years of continuous work in a grower shed, one would become infected due to the exposure to *E. coli* in the aerosols. (Blackall 2001).

The research from this thesis is also of relevance to the broader community. The study provides detailed data that will ensure that litter management will be undertaken with a full knowledge of the impact of these practices on food-safety

pathogens as determined in this thesis in the chicken and in the environment. Overall, the community benefits from a safer product and a heathier environment.

The study has had a positive impact on ecological sustainability and biodiversity. Using the chicken meat industry as an example, the study outcomes (scientific data on the impact of litter management on food-borne pathogens levels in litter) will allow the industry to implement litter management practices that reduce the load of food-borne pathogens entering the environment, the load of the same pathogens entering the human food chain and at the same time reducing the demand for fresh bedding. Hence, with less demand for fresh bedding and with a lowered level of food-borne pathogens in the spent litter entering the general environemnt, the overall ecological sustainability of the chicken meat industry will be considerably improved.

Overall this study has had significant scientific outcomes, resulted in industry changes to ensure better and safer food production systems and has helped improve the ecological sustainability of key food production industries.

9.4 The future

The sequence of outcomes from this thesis and the associated publications has demonstrated that there will be on-going transfer of zoonotic pathogens from farming to the surrounding environment. These environments are soil, crop and water, which have a subsequent link to the food chain. In a recent review by Jacobsen *et al.* (2012) it was suggested that though areas of animal production are recognised as high risk, the specific pathways remain unconfirmed. In a recent review entitled “food-borne diseases — the challenges of 20 years ago still persist while new ones continue to emerge," Newell *et al.* (2010), emphasised that organisms such as *Salmonella* spp. and *E. coli* seem to evolve to exploit novel opportunities within environments such as fresh produce and thus generate new public health challenges.

It is thus possible that pathogens such as *Salmonella* will exploit and evolve to survive in various micro-environments or “niches” within soil and crop including water. An understanding of the survival of *Salmonella* in soil, water and fresh produce is required to control this organism (Jacobsen & Bech 2012). Jacobsen *et al.* (2012) in a recent review suggest that once introduced to soil, *Salmonella* spp. survival is influenced by the method of introduction, temperature and external

factors such as the predation by soil protozoa. Thus, such complex microbial interactions within various soil ecosystems need to be evaluated to minimise *Salmonella* transfer from the environment to the food chain.

Salmonella has the potential to attach or internalize into vegetables and fruits (Hanning *et al.* 2009). Experimental studies under tropical field conditions have demonstrated that the cabbage rhizosphere can enhance the persistence of *S*. Typhimurium and *E. coli* O157:H7 in manure-amended soil, which influences the long-term contamination of the leaves (Ongeng *et al.* 2011). Experimental studies have also shown that irrigation of tomato plants with contaminated water plus inoculated seed both resulted in detectable *Salmonella* populations in the phyllosphere (Barak *et al.* 2011). Furthermore, this study showed that the *Salmonella* population levels on tomato leaves were “cultivar dependent” (Barak *et al.* 2011).

It is yet not conclusive as to how and to what extent non-typhoidal *Salmonella* and enterovirulent *E. coli* have evolved to use plants as alternate hosts and if plants benefit from such associations or the limitations plants may impose on such colonizations (Teplitski, *et al*. 2012). This means that the identification of such factors supporting these interactions between human enteric pathogens and plants will have major implications in the approaches to produce safety (Teplitski, *et al*. 2012). Thus more work is required under both laboratory and commercial agriculture cropping conditions when re-using water, or animal waste, for the purpose of irrigation or soil conditioning, due to their link with these key zoonotic pathogens.

Future work should also examine how *Salmonella* populations naturally adapt and thrive in the soil and water environments, contamination and internalisation within food crop especially those eaten raw due to potential food safety risks. More so, the virulence of such environmentally adapted populations in comparison to those that have been derived from livestock or poultry needs to be elucidated. Would environmentally adapted populations be of significance from a disease causing perspective? The potential to use vegetable cultivars that are resilient or resistant to *Salmonella* spp. is an area of interest. Especially when cultivated adjacent to animal farms, that either re-use waste or when waste is used a fertiliser with minimal treatment. If future research suggests that organisms such as *Salmonella* have evolved to utilize plants as transient hosts, then cultivar selection, modification of production practices and manipulation of the crop-associated micro-biota will need

to be incorporated into farm-to-fork safety programs (Teplitski, *et al*. 2012). Such measures can assist in the reduction of the environmental footprint of intensive animal farming operations as a source of transfer of these organisms to the food chain via the environment.

Current advances in genomics can provide a better understanding on *Salmonella* or *Campylobacter* populations that may be adapted to the environment. Genomics driven studies can provide information on the ability of such pathogens to adapt and survive in various environments by the identification of "functional genes" linked to niche diversity and a molecular basis for bacterial diversity (Begley & Hill 2010). Such future studies may enable the evaluation of any potential risks in the food chain associated with potential build up of environmental populations versus the host-adapted populations transferring to the environment.

One of the major challenges that can influence the survival and subsequent movement of pathogens in current times is climate change. The consequences to soil environments due to climate related factors can affect pathogen survival and thus food-safety through multiple pathways. These include changes in temperature, precipitation patterns, increased frequency / intensity of extreme weather events (e.g. floods) (Tirado *et al.* 2010). *Campylobacter,* though a poor survivor in the environment is isolated from waters (Jones 2001) perhaps through run-off as a consequence of weather events such as flooding where it persists in cool water.

The consequences of climate change on the ecology of pathogens are difficult to forecast and changes affecting microorganisms are likely to involve already known evolution or adaptation mechanisms of the various organisms of concern (Carlin *et al.* 2010). These changing environmental factors and the adaptation of pathogens to these changes will make the prediction of risks to food-safety a challenging issue for the future. As previously mentioned, future work should explore potential environmental niches of both *Campylobacter* and *Salmonella*, which originate from both pig and poultry farming.

Overall, there is a need to understand pathogen survival (and hence numbers) during farming to be able to address other issues of key concern such as antibiotic resistance. Microbial contamination of poultry litter should be reduced, or eliminated prior to re-use to minimize environmental health risks to humans from antibiotic-resistant bacteria (Kelley *et al.* 1998). Intensive swine feeding operations can be a

pathway for airborne transmission of multidrug resistant bacteria from swines to humans (Chapin *et al.* 2005). An increase in multiple resistance of *Campylobacter* from food production environments has been reported in some EU-member states, (Mozina *et al.*). Antibiotic resistant enterococci have been isolated from the air within poultry sheds in the USA (Brooks *et al.* 2010). International initiatives such as the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) monitor resistance from animals to humans (Bager 2000) and such initiatives require pathogen data from both farming and environmental sources. Australian work is required in this area, to be able to address any future concerns linked to the environment and antibiotic resistance.

Campylobacter is responsible for a large number of gastrointestinal outbreaks in Australia and is closely associated with poultry, as with *Salmonella*. There is a need to minimise the movement of these organism to the environment (and carcass) by reducing levels of organisms at the source or the farming system. Several options for the purpose such as vaccination, probiotics, passive immunisation and antibiotic dissection are available (Hermans, *et al.* 2011). All such treatments however, have the potential to have implications of residues both on-farm (chicken / litter) and off-farm (waste re-use environments). There is a need to develop more environmentally friendly measures within farming practices.

Bacterial viruses that target *Campylobacter* (bacteriophages) could be considered as an environmentally friendly control option provided host range trials demonstrated specificity. Future work should thus focus on the natural survival patterns of these two key pathogens within the farming system. Bacteriophages against *Salmonella* (Callaway *et al.*) have been isolated from pig farms and those against *Campylobacter* from poultry farms (Connerton *et al.* 2004). The understanding of such “on-farm bacteriophage distribution patterns” would enable the use or manipulation of such natural on-farm systems to minimise targeted pathogens such as *Salmonella* and *Campylobacter* within the farm environment.

Overall, this study has provided a basis for understanding and managing zoonotic pathogens in the environment. However, there is a need for further research to understand zoonotic pathogens due their continuous transfer, potential to adapt, survive and eventually multiply in environmental sources such as water and soil and the phylosphere. Such studies will help to manage and minimise risks of these

pathogens that originate from intensively farmed operations and circulate in the environment with entry to the human food chain.

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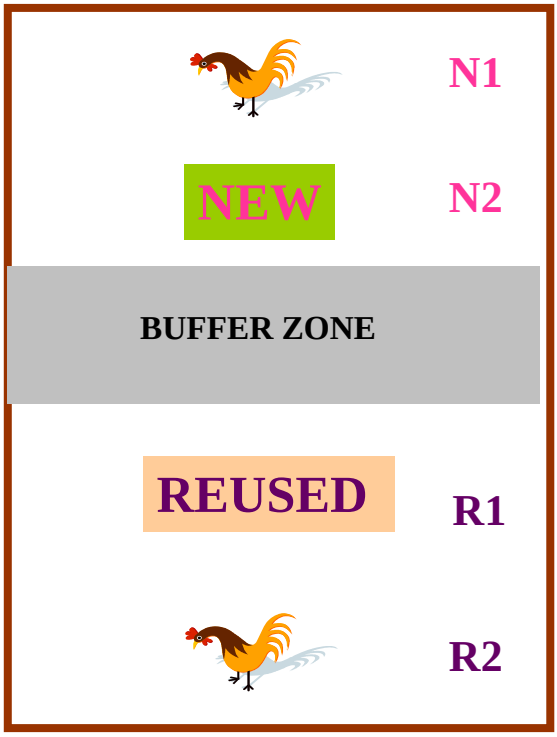
Appendix 1
4.2.2 Litter and surface dust sampling

Sampling plan of shed



4.2.2 Litter and surface dust sampling

Litter sampling tool

The litter sampler had a set surface area and was in two parts.

- The outer housing (with litter depth marked)
- A plate that was sharp enough to cut into the shed litter surface

It collected litter at a 40 cm depth over 400 cm^2

The sampler was place on the liter surface at a uniform depth (the housing had a sharp edge)

The inside unit was pushed to the end of the housing that was within the litter. The plate was then removed with the litter (see photo)

This ensured that litter was always collected from a uniform surface area and depth to enable the comparison of bacterial levels across litter