POULTRY CRC LTD

FINAL REPORT

Sub-Project No: 2.2.3

PROJECT LEADER: Professor Stephen Walkden-Brown

Sub-Project Title: Methods to quantify and inactivate viruses in poultry litter

DATE OF COMPLETION: December 2014

© 2014 Poultry CRC Ltd
All rights reserved.

ISBN 1 921010 82 7

Final Report. Poultry CRC Project 2.2.3 "Methods to quantify and inactivate viruses in poultry litter"
Sub-Project No.2.2.3

The information contained in this publication is intended for general use to assist public knowledge and discussion and to help improve the development of sustainable industries. The information should not be relied upon for the purpose of a particular matter. Specialist and/or appropriate legal advice should be obtained before any action or decision is taken on the basis of any material in this document. The Poultry CRC, the authors or contributors do not assume liability of any kind whatsoever resulting from any person's use or reliance upon the content of this document.

This publication is copyright. However, Poultry CRC encourages wide dissemination of its research, providing the Centre is clearly acknowledged. For any other enquiries concerning reproduction, contact the Communications Officer on phone 02 6773 3767.

Researcher Contact Details

Prof Stephen W Walkden-Brown
Animal Science W49
University of New England
Armidale
NSW 2351

Phone: 02 6773 5152, 0413 107 973
Fax: 02 6773 3922
Email: swalkden@une.edu.au

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in May 2015

Table of Contents

Executive Summary

This project builds on earlier CRC work to better manage the disease transmission risks associated with reusing litter for multiple batches of chickens. The broad aims were to:

- Develop methods for quantification of viral genome copy number in litter (Strand 1);
- Investigate factors affecting the temperatures achieved in heaped litter and develop standard recommendations on in-shed pasteurisation of litter that will provide reliable and predictable pasteurising temperatures (Strand 2);
- Define the association between viral genome copy number and virus infectivity determined by a chick bioassay under a wide range of temperature conditions to determine if the former can replace the latter (Strand 3); and
- Use the information obtained in Strands 1-3 above to produce practical outcomes for industry including a decision support tool for litter pasteurisation.

Taqman® real-time quantitative PCR tests and viral nucleic acid extraction methods for the successful quantification of viral genome copy number of Fowl adenovirus (FAdV), Infectious Bursal Disease virus (IBDV), Infectious laryngotracheitis virus (ILTV), chicken infectious anaemia virus (CAV) and Marek's disease virus (MDV) in poultry litter and faeces were successfully developed and reported in Section 4 of this report. Hardwood shavings were shown to contain an inhibitor of the PCR reaction that was not present in softwood or rice hull litters. The inhibition could be readily overcome by and additional step in the process.

Three major on-farm studies investigated the effects of covering of heaped litter, addition of moisture, turning and heap size and shape on the temperature kinetics of heaped broiler litter (Sections 5, 6 and 7). Taken together these finding indicated that the use of covers and/or additional moisture are unlikely to produce benefits except under some very specific conditions of very dry litter in small heaps under very cold conditions. The work has shown that high temperatures can be achieved as close as 5cm from the surface of the heaps and that for short pasteurisation periods of a week or so, turning of the litter is unlikely to have major benefits due to the significant temperature decline in the day following turning. It also showed that smaller heaps heat up and cool down more quickly, and should be used when the time available for pasteurisation is limited. These findings, and others from the literature were used to produce a set of guidelines and Standard Operating Procedure for litter pasteurisation between batches (Section 8). A small experiment reported in Section 6 also investigated the effects of litter chemical amendments and moisture content on temperatures achieved and ammonia production during litter partial composting.

Another series of experiments investigated the shedding pattern of the FAdV, IBDV, ILTV and CAV in faeces and subjected virus-contaminated litter from these experiments to a range of temperatures for different periods before testing the litter for virus infectivity in a chick bioassay and for viral genome copy number by qPCR. Major differences in the timing, amount and duration of shedding of the different viruses in faeces were observed in SPF chickens and broiler chickens infected at two different ages (Section 9). The differences are mostly attributable to the presence of maternal antibody in the broiler chickens. High levels of ILTV, a respiratory virus, were found in faeces, a novel finding with potentially significant implications on our understanding of the epidemiology of this virus.

Virus-contaminated litter from one of the virus shedding experiments was subjected to temperatures of 25, 35, 45, 55 and/or 65 ˚C for 5, 10 or 20 days before being placed in isolators with uninfected non-SPF or SPF chickens to test for infectivity for each of the 5 viruses under test in this project (Section 10). The treated litters were also subjected to qPCR tests to enumerate viral genome copies in them. A failure of a SPF chick hatch had adverse effects on this experiment, necessitating the freezing of the virus-contaminated litter and conducting two rather than one bioassay experiments, in non-SPF and SPF chicks respectively. Nevertheless some useful temperature–time relationships for inactivation of virus in litter were determined. CAV alone appeared unaffected by the litter treatments and there were apparent adverse effects of prolonged freezing on the double stranded DNA viruses FAdV, ILTV and MDV. Viral genome copy number in litter was generally a poor indicator of viral infectivity, because viral nucleic acids were frequently detected in treatments in which no infectivity was demonstrated. Nevertheless, in general, detection of viral nucleic acids decreased with increasing temperature and time.

Two further on-farm studies aimed to determine the rate of viral nucleic acid decay in normal heaps of broiler litter but unfortunately none of the viruses of interest were present or detectable in the litter (Section 11). These studies provided further useful information on the temperature kinetics of heaped litter, particularly close to the surface.

Data from 8 detailed on-farm studies investigating temperature kinetics in heaped litter and factors influencing them, including those from this project, were compiled into a single data set from which a detailed mathematical model is in the late stages of development and validation (Section 11). The model will allow users to input various conditions about their litter, the environment and any additional litter pasteurisation management factors to receive detailed information on the temperature conditions in the whole heap or any part of it at any time or over a given time period. The decision support tool is being created in an excel spreadsheet and will be made available to the CRC and users to improve decision making on litter pasteurisation together with the SOP and litter pasteurisation guide.

1 Introduction

This project was a development from the findings of Poultry CRC project 06-15 “Optimising methods for multiple batch litter use by broilers” which was conceived during 2005 and funded for 2.5 years between 2007-2009 (Walkden-Brown *et al.* 2010a). That project and a small CRC project 09-34 “Temperature inactivation of viral pathogens in litter” Walkden-Brown and Islam (2010) had demonstrated:

a) Marked spatial and temporal variation in the temperatures achieved following heaping of litter in the shed between broiler batches;

b) Such heaping induced time-dependant reductions in infective viral load of several important poultry viruses; and

c) There was variation between viruses in the ability to withstand the effects of such heaping; and

d) Detection and quantification of viral DNA from contaminated litter was possible

The Australian broiler industry has a well-recognised problem with both the supply of bedding material in some regions, and with the disposal of spent litter. Runge *et al.* (2007) estimated that the annual requirement for bedding material was 0.957 million m^3 costing $10.78 million with the industry producing 1.66 million tons of spent litter. As the volume of spent litter has increased it has become more difficult to dispose of, primarily due to food safety concerns with direct application in the horticulture industry. The industry is quite rightly investigating alternative sources of bedding material and improved treatments and value adding of spent litter. This project however was aimed at providing tools for increasing litter re-use for chicken production. The potential consequences of litter re-use for chickens are large, with each re-use almost halving the requirement for fresh litter materials and the amount of spent litter to be disposed of (Coufal *et al.* 2006). Partial composting of litter between batches of chickens also significantly reduces litter moisture content, improves friability and reduces the availability of phosphorous and other nutrients because as the litter ages, more nutrients become complex in degraded organic matter, decreasing the solubility of the nutrients and reducing the environmental risk of water contamination (Lavergne 2008).

The broiler industry produces 1.2 to 1.5 tons of waste products in the form of poultry litter per 1000 broiler chickens when reared on a single batch of litter (Coufal *et al.* 2006). In Australia 85% of chickens are reared on fresh litter materials following complete shed cleanout between batches (East 2007). The remainder are reared on previously used litter using a variety of systems.

The main reasons for the low rate of litter re-use in Australia are concerns about chicken health, biosecurity and performance. The principal concerns are with carryover of poultry pathogens in the litter, particularly viruses (Groves 2002), and increased ammonia concentrations during growout particularly in winter when heating is being used and ventilation rates are lower. There are also concerns about the carryover or accumulation of zoonotic bacteria but this has been dealt with in separate projects. This project addressed the risk of poultry viral pathogen carryover between batches with the aim of minimizing this risk.

Under Australian conditions the most practical means of reducing viral pathogen load in litter between batches is through partial composting of litter in static heaps or windrows. As this process is known to reduce bacterial and viral load in litter it will be referred to as "litter pasteurisation" in this report. The general principles of composting are well understood and it has always had an important role in the inactivation of human, animal and plant pathogens and weed seeds. It is clear that the inactivation is due primarily to the effects of temperature during the thermophilic stage of composting (Haug 1993a; Bohm 2007) although ammonia generated during litter composting could also contribute to inactivation of some viruses (Burge *et al.* 1983; Cramer *et al.* 1983). Temperature effects are moisture and time dependant, and for many of the most important bacteria, the temperature-time relationships for inactivation are well understood. Some regulatory processes specify time temperature relationships. For example for sludge-based composted in the USA the EPA Class A requirements allowing marketing to the public can be by specific enumeration of key bacteria, or by demonstrating conformity with time temperature equations (Haug 1993a). These, for example correspond to 53˚C for 5 days, 55˚C for 2.6 days or 70˚C for 30 minutes. The EPA also recognizes composting as a composting as a "process to further reduce pathogens" (PFRP) in pre-treated biosolids (Class B) under the 40CFR.503 regulations (503 or Biosolids Rule) (Lavergne 2008). The PFRP relies on a time/temperature relationship under which composting materials are required to exist above 55˚C for various periods of time; 72 hours in static piles and in vessel composting technologies or 15 days (with five turnings) for turned windrow composting.

For viruses temperature-time data are less complete and are more variable as viruses are non-cellular and in the environmental phase are relatively inert combinations of nucleic acids and proteins without the soluble, generally thermolabile enzyme systems required to maintain survival in vegetative bacteria. Detailed temperature-time information for inactivation is required for accurate prediction of inactivation of any pathogen using variable heat processes including inactivation of poultry viruses during partial composting. It is clear from the review of Islam and Walkden-Brown (2010) that there is not a universal set of time-

temperature relationships for the different poultry viral pathogens, which have widely divergent thermolability.

In recent years some progress has been made in defining temperature-time inactivation of poultry viruses during composting. Guan *et al.* (2009) described the change in nucleic acid detection and loss of infectivity over time for Newcastle disease virus (NDV) and Avian influenza virus (AIV) in litter at ambient temperatures and during composting. Giambrone *et al.* (2008) reported on similar work with ILTV. Under Poultry CRC Project 06-15 "Optimising methods for multiple batch litter use by broilers" a chick bioassay for detecting virus infection in litter was developed (Islam *et al.* 2013a) and used to measure the efficacy of different types of between-batch litter composting on inactivation of Marek's disease virus (MDV), Chicken anaemia virus (CAV), Fowl Adenovirus (FAdv), Infectious Bronchitis virus (IBV) and Infectious Bursal Disease virus (IBDV) at different stages of the composting process (Islam *et al.* 2010; Walkden-Brown *et al.* 2010b). However these studies failed to produce a comprehensive set of temperature x time interactions for inactivation for a range of reasons, mostly associated with measuring infectivity of pooled material subject to widely varying temperatures due to location within the compost heap, and in the latter case, because the cost of measuring infectivity precluded testing a very wide range of samples. This project aims to overcome the first limitation by applying a range of temperatures to infective litter under controlled laboratory conditions. It aims to overcome the second limitation by developing and validating cheaper and more rapid PCR based measures of infectivity to replace the use of the chick bioassay.

Once temperature time relationships are defined for the key poultry viruses, it is important that industry has composting methods that reliably produce temperatures capable of reducing viral infectivity to acceptable levels. Our understanding of how factors such as heap size, shape, moisture content, covering, turning and external temperatures influence the temperature profiles within heaps is incomplete despite a number of Australian studies in which temperatures within heaped or windrowed broiler litter have been measured (Wilkinson *et al.* 2003; Chinivasagam 2009; Walkden-Brown *et al.* 2010b). There are also a number of reports from the USA (Kwak *et al.* 2005; Lavergne *et al.* 2006; Macklin *et al.* 2006; Macklin *et al.* 2008). In order to better predict the temperatures achieved in heaped litter windrow, and to model the influence of factors such as litter material, windrow size, moisture level, and turning frequency the project undertook on farm experimentation aimed at developing standard procedures for between batch litter composting that will reliably produce the required temperatures. It is also proposed that the results of such experimentation be used to develop a predictive model of heaped litter temperatures.

2 Objectives

2.1 Broad objectives

The broad rationale for the project is outlined in the Introduction. More narrowly, the specific objectives of the project were to:

- Optimise the methods for recovery of nucleic acids of 5 viruses (Marek's disease virus (MDV), infectious laryngotracheitis virus (ILTV), Fowl adenovirus (FadV), chicken anaemia virus (CAV) and infectious bursal disease virus (IBDV) from chicken litter to ensure high levels of accuracy, reproducibility and sensitivity in PCR assays;
- Develop standard recommendations on in-shed partial composting of litter that will provide reliable and predictable pasteurising temperatures;
- Define the association between decline in qPCR quantification of viral nucleic acid and decline in infectivity as determined by chick bioassay of litter contaminated with the 5 viruses listed above under a wide range of temperature conditions; and
- Use the information obtained at 1-3 above to produce practical outcomes for industry including:
    a. Clear guidelines for litter in-shed composting that produce reliable temperature profiles and adequate levels of pathogen inactivation;
    b. Clear information on the factors that will influence temperature profiles in litter windrows and methods for estimating the magnitude and consequences of such effects;
    c. Clear information on the temperature-time relationships for inactivation of 5 high risk viruses for litter re-use; and
    d. New practical methods to accurately and sensitively quantify viral pathogen nucleic acids in poultry litter. The most likely applications of these methods are:
        i. To determine the efficacy of litter partial composting treatments on viral pathogen inactivation; and
        ii. To routinely monitor end of batch litter for viral pathogen load as part of decision making relating to litter reuse.

2.2 Research strands and hypotheses

To meet these aims, the project grouped the research under 4 strands, each addressing one of the objectives. These strands, and the general hypothesis under test in each of them were:

Strand 1. Optimisation of detection and quantification of viral nucleic acids from litter chicken litter of different types

Hypothesis: Based on the published development of reproducible qPCR assays for pathogen DNA and RNA in soil, faeces, poultry dust and poultry litter, we will be able to develop sensitive, accurate and reproducible assays for viral DNA and RNA.

Strand 2. Optimisation of on-farm in-shed partial composting of litter to provide predictable and effective litter pasteurisation

Hypothesis: Understanding of factors affecting thermophilic composting will enable development of standardized protocols for litter in-shed treatments that produce predictable and effective thermal outcomes.

Strand 3. Correlation between qPCR-based detection of viral nucleic acids and decay in infectivity

Hypothesis: Under warm moist litter conditions such as those seen during partial composting of litter, decay of detected nucleic acids and infectivity will be closely correlated. Under drier, cooler conditions the correlation will be weaker.

Strand 4. Practical application of laboratory findings

Hypotheses: a) Decay in detectable viral nucleic acids will be a useful tool for assessing decay in litter infectivity under litter composting conditions in the field

b) The temperature conditions within windrowed litter can be modelled with comparatively few inputs

c) Routine PCR monitoring of viral pathogen load in normal litter will also prove to be a useful disease-monitoring and management tool in broiler flocks even if it is not directly associated with current infective risk.

3 Methodology

3.1 Institutions and personnel

Project activities were organised into 4 research strands as identified in the section above. The experimental work is shared between the collaborators at UNE, CSIRO and DAF (Qld). These were:

UNE Prof Steve Walkden-Brown, Dr Fakhrul Islam, Dr Katrin Renz, Dr Yan Laurenson, Prof Parimal Roy (visiting scientist) Ms Sue Burgess (Technician), Mr Robin Achari (PhD student), Ms Kanchana Jayasundara (PhD student), Mr Mamdouh Alsharari (PhD student), Mr Hai Tran Minh (Masters Student)

CSIRO Dr Peter Hunt and Ms Jody McNally (Research Assistant).

DAFF (Qld) Mr Mark Dunlop

The project was overseen by an **Industry Steering Committee** comprising Rod Jenner (Golden Cockerel), Gary Sansom (Australian Chicken Growers' Council Limited), Margaret McKenzie (Inghams Enterprises P/L), Jorge Ruiz (Baiada Poultry), Pat Blackall (Poultry CRC), Tim Walker (Poultry CRC).

3.2 Viruses selected for investigation

Details of the viral pathogens suggested for evaluation in the project are shown in Table 3-1. The final viruses to work on were selected by the Industry Steering Committee.

Table 3-1. Details of the viral pathogens included in this project.

Disease	Causative virus and classification	Virus abbr.	Nucleic acid type and no of strands*	Route of virus shedding	Risk of litter transmission
Marek's disease	Herpesviridae *Gallid herpesvirus 2*	MDV	DNA ds	Feather dander	High
Infectious laryngotracheitis	Herpesviridae *Gallid herpesvirus 1*	ILTV	DNA ds	Respiratory tract, faeces?	Moderate
Inclusion Body Hepatitis and Hydropericardium Syndrome (IBH/HS)	Adenoviridae *Aviadenovirus*	FAdV	DNA ds	Faeces also respiratory and oral secretions	High
Chicken infectious anaemia	Circoviridae *Gyrovirus*	CAV	DNA ss circular	Faeces	High
Infectious bursal disease	Birnaviridae *Avibirnavirus*	IBDV	RNA ds	Faeces	High

* ds double stranded, ss single stranded

3.3 Organisation of research strands and methods used

Details of the 4 research strands and an outline of the general approach and methods used in each is provided below. Specific details of some methods are provided in context in the experimental chapters. Much of the project was about method development and application so the methods are an output of the project. An overview of each of the experiments carried out on the project is provided in Table 3-2.

3.3.1 Strand 1. Optimisation of detection and quantification of viral nucleic acids from chicken litter

The first task was to develop fully quantitative Taqman® real-time qPCR assays for each virus. At the commencement of the project we only had such an assay for MDV. Initial assay development occurred at CSIRO and involved adapting published PCR assays for FAdV, ILTV and CAV to the Taqman platform, and developing a completely new assay for IBDV. Then plasmid produced target sequence was used to develop and validate standards allowing absolute quantification of viral genome copy number.

The next task which was carried out at both CSIRO and UNE to develop effective, sensitive methods for the extraction of viral nucleic acids from litter materials. For this we drew on previous work on extracting viral DNA from litter or poultry compost (Lu *et al.* 2003b; Guan *et al.* 2008; Walkden-Brown and Islam 2010) and isolation of microbial DNA and RNA from other environmental samples (Schwab *et al.* 1995; Kuske *et al.* 1998; Griffiths *et al.* 2000; Hurt *et al.* 2001; Ophel-Keller *et al.* 2008).

Under this task there were two key activities

Activity 1.1. Optimisation of qPCR detection of known amounts of MDV (DNA) and IBDV (RNA) virus in wood shavings litter (optimising assay sensitivity). For this work a range of sample preparation and extraction methods were compared using virus free litter, litter naturally contaminated with virus, and litter contaminated with known amounts of virus with the latter enabling calculation of recovery/detection rate of added virus by each method.

Activity 1.2. Test for inhibitors of PCR in litter samples. Extracted material from different litter types (softwood shavings, hardwood shavings and rice hulls) was spiked at different concentrations with known amounts of virus, then extracted and recovery of spiked virus compared to water controls. Inhibition is demonstrated when recovery/detection fails, usually at high concentrations of extracted material.

Activity 1.3. Extension of Activity 1 to other viruses and litter types. The optimised methods developed under activity 1.1 were then evaluated for the other viruses.

3.3.2 Strand 2. Optimisation of on-farm in-shed partial composting of litter to provide predictable and effective litter pasteurisation

In this strand we worked with industry partners to develop and test standard operating procedures (SOPs) for litter in-shed treatments and investigate the effects of systematic variations to the SOPs such as size of windrow or heap, moisture content, frequency of turning and use of covers as an alternative to turning. The only measured variable was temperature distribution within the heaped litter over time. In earlier work the shallowest depth below the surface at which temperature was recorded was 20-25 cm. In the present experiments we extended our understanding of temperature kinetics by examining this region of the heap in more detail, with temperatures at 5 and 10 cm depths also measured.

We conducted 3 on-farm studies (Experiments 2.1-2.4) in this strand, one each at UNE, Sydney and Tamworth in which various aspects of the litter pasteurisation process were varied and their effects on temperature profiles at different depths within the heap measured. These are reported individually in the experimental chapters and suggested guidelines and an SOP are presented in Section 8 of the report.

3.3.3 Strand 3. Correlation between qPCR-based detection of viral nucleic acids and decay in infectivity

This was the most difficult and challenging work in the project. We had to generate litter known to contain the 5 viruses of interest, shed naturally into the litter by infected birds, and then measure both litter infectivity (using the chick bioassay) and qPCR quantification of the 5 viruses following a range of heat and moisture treatments. Four major experiments were carried out in this strand, all at UNE. Each is described in detail in the experimental chapters.

Expts 3.1 and 3.2. Refinement of model for producing infective litter. In past experiments we had produced infective litter by infecting broiler chickens with low virulence or vaccinal strains of the organisms of interest that are known to transmit. Evidence of viral shedding was only determined indirectly by seroconversion of the shedder chickens and/or recipient chickens in the chick bioassay. Results could be influenced markedly by the presence of maternal antibody directed against the challenge virus. In these two experiments we set out to define the precise viral shedding patterns of the viruses of interest following challenge in both SPF

birds free of maternal antibody (Expt 3.1), and commercial broiler chicks (containing maternal antibody against most of the challenge viruses), at two ages (Expt 3.2).

Expts 3.3 and 3.4. Effects of temperature on loss of infectivity and qPCR detection of MDV, FAdV, CAV, IBDV, and ILTV over time and correlation with loss of detection of virus by qPCR. Two experiments were carried out to assess this on infective litter produced in Expt 3.2, the first in non-SPF layer cockerels and the second in SPF chickens. Chicks were exposed to infective litters collected from Expt 3.2 treated by exposing them to a range of different temperatures between 24˚C and 70˚C for different periods of time (0, 5, 10 and 20 days). At day 35 post litter exposure, birds were bled, sera retained and assayed for seroconversion to the virus of interest using ELISA. Litter samples before and after heat treatment were subsampled and viral load in the litters determined by qPCR.

3.3.4 Strand 4. Practical application of laboratory findings

This strand involved on-farm validation of the experimental findings under the following activities.

Activity 4.1 On farm validation of experimental findings. Two experiments were conducted on commercial broiler farms in Sydney (Expts 4.1 and 4.2) to assess temperature profiles and viral decay as determined by qPCR and see if the results were consistent with those produced experimentally (litter heated in ovens). As the experiments were conducted on commercial farms, the presence of the viruses of interest could not be guaranteed.

Activity 4.2 Development of decision support tool for in-shed litter composting. Litter temperature profiles from Strand 2 research, previous Poultry CRC project 06-15 (Walkden-Brown et al., 2010) and the PhD work of Poultry CRC supported scholar Michael Cressman (Cressman, 2014) were used by Yan Laurenson to develop a predictive model of temperature distribution and kinetics in litter heaps in consultation with Mark Dunlop at DAFF (Qld) and his fellow collaborators at UNE.

Activity 4.3. The value of routine monitoring of viral nucleic acids in litter as a disease/biosecurity management tool was also to be assessed and this is done in Section 12 of the report.

Table 3-2. Summary of the major experiments carried out on the project by research strand.

Expt No*	Location	Type
2.1	Sydney (Refalo)	Litter heaping treatments
2.2	UNE (Kirby)	Litter heaping treatments (3 experiments)
2.3	Tamworth (Hebblewhite)	Litter heaping treatments
3.1 (9.1)	UNE	Effect of bag type and temperature on moisture loss from litter during storage and detection of viral nucleic acids
3.1 (9.1)	UNE Isolators	Challenge and shedding profile of ILTV, FAdV-8, CAV and IBDV in SPF chickens
3.2 (9.2)	UNE Isolation pens	Challenge and shedding profile of LTV, FAdV-8, CAV and IBDV in broiler chickens and generation of infective litters
3.3 (10.1)	UNE isolators	Chick bioassay 1 – non SPF. Effect of heat x time treatments on litter infectivity for 5 viruses
3.4 (10.2)	UNE isolators	Chick bioassay 2 –SPF. Effect of heat x time treatments on litter infectivity for 5 viruses
4.1 (11.1)	Sydney (Cauchi)	Field validation expt 1. Inactivation of viruses in heaped litter.
4.1 (11.1)	Sydney (Sultana)	Field validation expt 2. Inactivation of viruses in heaped litter.

* For convenience later experiments are numbered for the section of the report they are in, rather than the strand of the project.

3.4 General research facilities and methods

3.4.1 Measurement of litter temperatures

Temperature in litter heaps was measured at hourly intervals in heaped litter using iButton dataloggers iButton® DS1921 data loggers (Evolution Education Ltd, Bath, UK) inserted into litter heaps at various depths relative to the surface of the heap . For field experiments 80 – 150 iButton data loggers were deployed across the various treatments and depths to provide accurate estimates of mean temperature. In most experiments replication was achieved at both the level of heap treatment and iButton within heaps. Details are provided in each experimental chapter.

Figure 3.1. Litter heap showing tethered iButton dataloggers inserted at different depths.

3.4.2 Measurement of litter dry matter

Litter samples (200-300 g) were placed in aluminium foil trays and weighed. The weight of each sample was recorded. They were then placed in a drying oven at 100 °C, for 24 hours. The samples were then taken out of the oven, left to cool then weighed again. From the difference between the original weight and the dried weight, the moisture content (MC) was calculated and expressed as a percentage.

3.4.3 Animal Facilities at UNE

3.4.3.1 UNE isolator facility

The SPF challenge experiment and two chick bioassay experiments were conducted in the main 24-isolator facility at UNE. The isolators are housed in a biological PC2 laboratory under constant negative pressure and with all outgoing air HEPA filtered. Each isolator has a length of 2.05 m, width of 0.67 m and height of 0.86 m with a stainless steel frame. Two types of flooring were deployed. For experiment 3.4 the floor was 2.5 mm stainless steel (304 2b) with 12.7 mm holes punched out with centres 17.45 mm apart staggered providing 49% open area. For experiments 3.1 and 3.3 galvanised metal trays were placed on the punched floor and filled with litter, so the birds were effectively raised on litter. The Isolators are positive-pressure soft-bodied with disposable plastic linings, gauntlets and gloves, disposed of after every experiment. Isolators are provided with temperature-controlled HEPA-filtered air via a central air supply system and air is scavenged from each isolator via a series of scavenger ducts and HEPA filtered on exit. Both inlet and outlet air supplies are under manual control via a variable speed controller, giving complete control over air-flow and isolator pressures. Isolators are individually fitted with heat lamps under separate thermostatic control, automatic waterers and feeders. The entire feed supply for each experiment is loaded into a large feed hopper for each isolator and sealed for the duration of the experiment. Temperature in each isolator is monitored constantly via a data logger and displayed on a computer screen in the facility. The entire facility has automated power backup via a 13 KVA generator. At the time of writing, nine major experiments have taken place in the facility without breakdown of biosecurity or other major problems. Photographs of the facility are included in Figure 3.2 and Figure 3.3.

3.4.3.2 UNE isolation pens

For generation of infective litter broiler chicks infected with the different viruses were housed in isolation pens on a hill on the windward side of the UNE campus. The pens are spaced 20 – 30 m apart and have internal dimensions of 1.5m x 2.5m with raised solid wooden floors on

which bedding is placed. Watering is by a nipple system and feeding by suspended feed bins. Photographs of these are provided in Figure 3.4 and Figure 3.5.

Figure 3.2. Interior of isolator facility at UNE showing 24 isolators and main air inlet duct. This carries HEPA filtered, heated air to each isolator. Note the green feed hopper above each isolator.

Figure 3.3. Exterior of the isolator facility at UNE showing the plant room on the right and the main isolator facility in the middle with the air extraction and filtration system next to the people.

Figure 3.4: Two isolation sheds.

Figure 3.5: Chickens within an isolation shed.

3.4.4 Chick bioassay to test litter infectivity (UNE)

The development and validation of this test in SPF chicks is fully described by (Islam *et al.* 2013a). Briefly, groups of 10–12 SPF chickens (SPAFAS, Australia) were placed in positive pressure isolators and exposed to approximately 9 litres of chicken litter in two plastic “kitty litter” trays. Chickens tended to actively explore in the litter as soon as it was introduced and

many would sleep on it. Litter remained in the trays for approximately 3 weeks in decreasing amounts. At day 35 post exposure to litter, chickens were bled and sera examined for antibodies indicating exposure to infective virus. Other measurements were sometimes made at this point or at a common age near this point as well.

Figure 3.6. Chick bioassay to test litter infectivity in positive pressure isolators. SPF chicks soon after exposure to litter (*left*) and close to day 35 post exposure to litter (*right*).

3.4.5 Challenge viruses used

A summary of the challenge viruses used in Strand 3 of the project is provided in Table 3-3

Table 3-3 Summary of virus isolates used in Strand 3 experiments

Virus	Isolate	Type/Origin	Expts used in
MDV	Various	Wild type	3.2
ILTV	A20	Vaccine CEO/TCO	3.1
	SA2	Vaccine CEO	3.1 and 3.2
FAdV-8	EMAI Strain	EMAI	
CAV	269/7 P4	Wild type. CSIRO	3.1
	Steggles 3311	Vaccine	3.1
IBDV	06/95	Wild type - classical Australian. CSIRO	3.1
	02/95	Wild type - variant Australian. CSIRO	3.1 and 3.2

3.4.6 Molecular analysis of samples

3.4.6.1 Viral nucleic extraction

Following sample preparation by a variety of methods as described in the experimental chapters, DNA/RNA was extracted from the samples using a range of commercial kits depending on sample type and nucleic acid involved. These included:

- Magnetic bead method (MagMaxTM Total RNA isolation kit Ambion). DNA and RNA
- Automated Kingfisher Flex 96 (Thermo Fischer Scientific, USA). DNA

- Automated X- tractor Gene (Corbett Robotics, Australia). DNA
- Qiagen QIAmp DNA stool extraction kit (Qiagen, Valencia, CA, USA)
- BIOLINE ISOLATE II RNA Mini kit (Bioline Aust) Pty Ltd, Alexandria Aust.) (RNA)
- BIOLINE ISOLATE genomic DNA mini kit (Bioline, Australia)
- GeneJET viral DNA and RNA purification kit (Thermo Fisher Scientific Inc, USA (DNA and RNA)

3.4.6.2 Taqman qPCR assays

Fully quantitative Taqman® qPCR assays with plasmid-based standard curves were used to quantify the 5 viruses. The origins of each assay are provided below.

- Marek's disease virus (MDV). As described by (Islam *et al.* 2004; Islam *et al.* 2006). Specific for serotype 1 MDV.
- Infectious laryngotracheitis virus (ILTV) As described by (Callison *et al.* 2007). Generic for ILTV (single serotype only).
- Chicken anaemia virus (CAV). Adapted from (Zhang 2009). Generic for CAV.
- Infectious bursal disease (IBDV). Developed from scratch based on the VP2 sequence data (Ignjatovic and Sapats 2002). Generic for IBDV.
- Fowl adenovirus (FAdV). Initial assay based on modified HEX-S target (Steer *et al.* 2009) but subsequently replaced by an SYBR Green real-time PCR of (Günes *et al.* 2012). Generic for fowl adenovirus of all species. A serotype-8 specific assay was also designed by the project targeting a unique region of the ORF33a gene (Grgić *et al.* 2011).

3.4.6.3 Cloning of target sequence to produce plasmid standards and enable absolute quantification

Cloning of target sequence to produce plasmid standards for each target sequence was by the pGEM T-easy vector system (Promega, Madison, WI, USA) following the manufacturer's instructions. Plasmid quantity was assessed spectrophotometrically using the NanoDrop® ND-1000 UV–Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA) after verification on gels. Ten-fold serial dilutions were used to develop standard curves for each virus in unit of viral copy number. Plasmid standard curves were then replaced by more stable standards based on extracted viral nucleic acids.

3.4.6.4 qPCR equipment

At CSIRO qPCR assays were run on a BioRad Light cycler (BioRAd while that UNE they were run on either a Rotorgene 3000 (Corbett Robotics, Australia) or Rotorgene 6000 (Corbett Life Sciences, Qiagen, USA). At UNE samples prepared for qPCR assay using a CAS 1200 automated pipetting station and liquid handling system (Corbett Robotics, Aust) to ensure precision.

3.4.7 Serological analysis using ELISA

Sera were assayed for antibody directed against the 5 viruses, using the following methods.

- Infectious laryngotracheitis virus (ILTV). Commercial kit. ProFlok® Fowl Laryngotracheitis Virus Antibody Test Kit. Sybiotics Corporation, Mo. USA (now Zoetis)
- Chicken Anaemia Virus (CAV). Commercial kit. ProFlok® Chicken Anaemia Virus Antibody Test Kit. Sybiotics Corporation, Mo. USA (now Zoetis)
- Infectious bursal disease (IBDV). Commercial kit. ProFlok® Infectious Bursal Disease Virus Antibody Test Kit. Sybiotics Corporation, Mo. USA (now Zoetis).
- Fowl adenovirus (FAdV). Commercial kit. Trop-ELISA IBH kit for detection of Fowl Adenovirus Serotype 8 (Cellabs, Brookvale, NSW).
- Marek's disease virus (MDV). UNE developed ELISA adapted from the method of (Zelnik *et al.* 2004). Description below is taken from (Ralapanawe *et al.* 2015). It does not distinguish between MDV serotypes.

3.4.7.1 Preparation of antigen for Marek's disease ELISA

Rispens CVI988 vaccine (Bioproperties Vaxsafe RIS®) was used to prepare the ELISA antigen. A vaccine vial was thawed, diluted in 2.5 ml of vaccine diluent, centrifuged at 748 xg for 5 minutes at 4 ˚C and the supernatant discarded. The remaining pellet was frozen at -20 ˚C and subjected to four freeze-thaw cycles. After the last thawing the pellet was broken up and dispersed in PBS using a sonicator (MSE Soniprep 150) for 2 minutes at 12 amperes. The homogenized antigen was centrifuged at 4 ˚C for at 1455 x g for 10 minutes and the supernatant retained. The concentration of the antigen was determined by a spectrophotometer (M7 Bio-Rad SmartSpec^{TM} 3000) using bovine albumin serum standards (Sigma, A-3803) and Bradford reagent (Sigma-Aldrich). Antigen was stored in aliquots at -20

˚C until required. The optimum antigen concentration to coat the plates was determined by serial dilution of the antigen against serial dilutions of known positive samples and conjugate.

3.4.7.2 ELISA procedure

The test serum samples were diluted 1:100 with PBST (0.5ml/litre Tween 20 added for 1litre of PBS). The ELISA plates (Immulon ® 2 flat bottom microtitre plates, Cat. No. 011-010-3455) were coated with Marek's antigen (1: 100 dilution, diluted with carbonate buffer 0.05M, pH 9.6). 100 μl of the diluted antigen was added to each well and incubated at 4 ˚C for 16 hours followed by washing twice with PBST. 100 μl of PBST containing 1% skim milk was added to each well to block the plates. The plates were covered and left for 1 hour at room temperature. The contents were removed by inverting the plates and 100 μl of the diluted samples, standards, negative control samples and blanks (PBST+1% skim milk) added, followed by incubation for one hour at 37 ˚C. Positive control sera were from experiments in which specific pathogen free (SPF) chickens were challenged with MDV. Negative control sera were from unchallenged SPF chickens. After incubation the plates were washed twice with PBST followed by addition of 100 μl of rabbit anti-chicken antibody (2^{nd} antibody) conjugated with horseradish peroxidase enzyme (Sigma cat no. A9046, diluted 1:5000 with PBST). The plate was covered and incubated for 1 hour at 37 ˚C. The plates were then washed with PBST three times and 100 μl of substrate (34 mg of o-Phenylenediamine and hydrogen peroxide 30% w/v [Univar/Chem supply] with 100ml of citrate phosphate buffer [pH 5.0]) added to all wells. Plates were covered with aluminium foil and incubated for 10 minutes at room temperature. The chemical reaction was stopped by addition of 50 μl of 98% sulphuric acid per each well. The plate was mixed for 5 seconds and read by microplate reader at 490 nm (Bio-Rad, Benchmark), and the optical density values were obtained, averaged over duplicate samples. The antibody titre was derived from the optical density values of the standards of known dilution in the standard curve. The standard curve consisted of 10 standards in duplicate comprising a 2-fold serial dilution. A cut off value of 500 was used to differentiate positive from negative samples.

4 Strand 1. Optimisation of detection and quantification of viral nucleic acids from litter chicken litter of different types

The work in this strand was designed and carried by Jody McNally and Peter Hunt at CSIRO and Sue Burgess, Fakhrul Islam, Katrin Renz and Steve Walkden-Brown at UNE. Robin Achari (UNE) made a significant contribution to the development of the final FAdV qPCR assay while Michael Cressman (UNE/CRC) contributed significantly to the execution of optimisation experiment 3. Visiting scientist Prof Parimal Roy assisted with refinement of litter and faecal extraction methods for ILTV and other DNA viruses while Kanchana Jayasundara (UNE) significantly refined the litter and faecal extraction methods for IBDV, the only RNA virus we worked with.

4.1 Development and validation of Taqman® qPCR assays for FAdV, CAV, IBDV and ILTV (CSIRO/UNE)

At CSIRO four Taqman® fully quantitative real-time PCR assays were developed or tested and validated to detect infectious bursal disease virus (IBDV), infectious laryngotracheitis virus (ILTV), Fowl adenovirus (all serotypes) (FadV) and chicken infectious anaemia virus (CAV). Subsequently at UNE a FAdV serotype 8 specific assay was developed. Plasmid standard curves were developed for each assay to enable absolute quantification in terms of virus copy number per reaction. These assays complemented the existing Marek's disease virus assays (MDV, various serotypes). Methods were either adapted from published assays or developed by the project as follows: CAV, adapted from (Zhang 2009); FAdV adapted from (Günes *et al.* 2012); ILTV adapted from (Callison *et al.* 2007), IBDV developed by project from GenBank sequence data of the VP2 gene (Ignjatovic and Sapats 2002); FAdV8 targeting the ORF33a gene unique for FAdV-8 (Grgić *et al.* 2011).

4.2 Preparation of test litters containing target virus (UNE)

Virus contaminated litter from 3 sources was used in method development.

4.2.1 Virus free litters (Expt. LT11-C-LP1)

Uninfected SPF chickens were placed on pine shavings, hardwood shavings and rice hulls for 42 days to produce faecally contaminated but virus free negative control litter. Litter of

each type was then stored at -20˚C until required or dried for 7 days at 37˚C and stored until required.

4.2.2 Virus contaminated litter (Expts. LT11-C-LP2 and LT09-C-CB9)

Virus contaminated litter was generated by raising broilers vaccinated with the following live vaccines on pine shavings for 42 days (Expt. LT11-C-LP2).

- MDV Rispens CVI988 Strain (VaxSafe RIS®, Bioproperties Aust);
- ILTV A20 Strain (Poulvac Laryngo A20® Fort Dodge Australia);
- IBDV Strain V877 (Vaxsafe IBD® Bioproperties Aust);
- CAV Strain 26P4 (Intervet Nobilis® CAV Vaccine)
- FAdV Strain E surient (Intervet Nobilis® FAV Vaccine)

Litter was then stored at -20˚C until required.

Stored infective pine shavings from the study of (Islam *et al.* 2013a) were also used. This contained vaccine or pathogenic strains of all of the viruses above except (Expt ILTV LT09-C-CB9).

4.3 Tests for PCR inhibitors in litter (UNE)

The presence of PCR inhibitors in litter of different types was tested at UNE by addition of fixed amounts of viral DNA to serial dilutions of extracted litter material and then investigating the effects of polyvinylpolypyrrolidone (PVPP) in overcoming any inhibition observed.

In the first spiking test, virus free litter samples from Expt. LT11-C-LP1 (4.2.1 above) were put through a DNA extraction process using magnetic bead technology (MagMax™ Total RNA isolation kit, Ambion) which had been shown to effectively extracts both DNA and RNA from litter samples. The extracted material was then subject to a 7 x 10-fold serial dilution and spiked with a small amount of MDV viral DNA and recovery of the material carried out using qPCR (Figure 4.1).

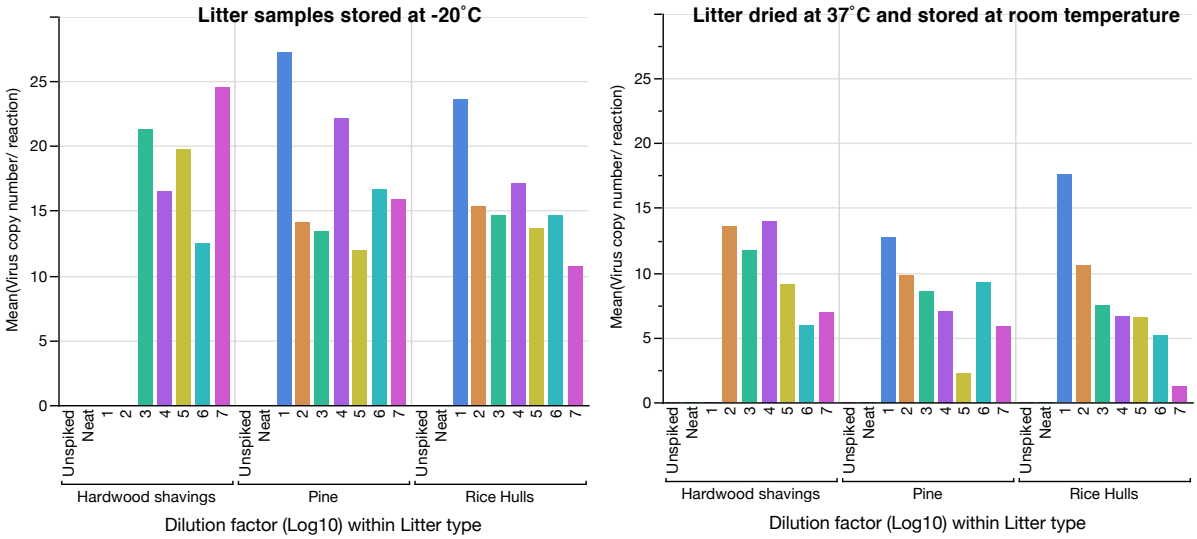

Figure 4.1 Litter spike test 1. Mean virus recovery from DNA extracted from litters of different types and storage backgrounds (-20˚C Left, Dried at 37˚C then room temperature Right), dilutied in a 7 x 10-fold dilution series and "spiked" with a fixed small amount of Marek's disease virus. If there is no inhibition all recovery values would be the same.

The qPCR reaction for MDV was inhibited in undiluted samples (Neat) for all litter types stored at -20˚C, with amplification of the target first occurring at dilutions of 1:10 for pine shavings and rice hulls and 1:1000 in hardwood shavings. Thus hardwood shavings contained a significant inhibitory component (100 fold). When DNA was extracted from infective litter dried at 37˚C rather than stored at -20˚C, similar results were obtained except that amplification occurred at a dilution of 1:100 in the hardwood shavings indicating a lessening of the inhibitory effect of hardwood shavings by approximately one log.

The second spike test repeated the first but included a spiked water treatment to confirm that no overall inhibition was occurring (Figure 4.2). The inhibition results were repeated showing a significant inhibitory effect of hardwood shavings, a reduction in this effect in dried litter and no overall inhibition in other samples relative to water.

Spike test 3 used only hardwood shavings stored at -20˚C and tested the effect of adding 6.6% w/v PVPP added to the extracted DNA sample and mixed for 5 minutes, 1 hour or overnight at room temperature. This was tested on a 6 x 2-fold serial dilution of DNA extracted from the hardwood shavings. Amplification of the target product occurred at dilutions of 1:4, 1:2 and neat respectively (Figure 4.3). In all of the tests, once amplification occurred, there was no effect of further dilution on the amount of target recovered when adjusted for the dilution factor. This demonstrated that the PVPP overcame the inhibitory effects of the hardwood shavings with as little as 5 minutes incubation with PVPP reducing the dilution at which amplification occurred from 1:1000 to 1:4.

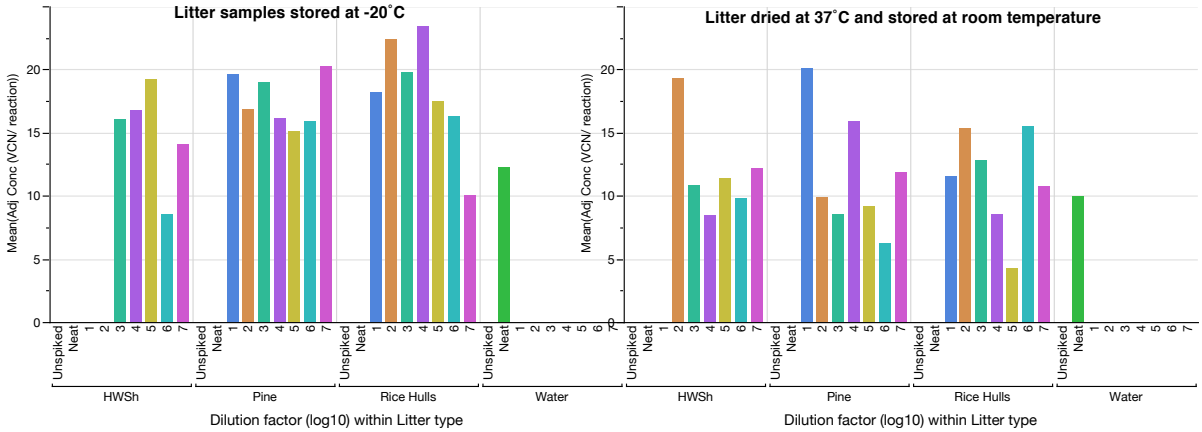

Figure 4.2 Litter spike test 2. A repeat of Spike test 1 with addition of a water positive control to determine if there was inhibition over the entire range of litter dilutions. If there is no inhibition all recovery values would be the same. Ignore dilution value other than neat for water.

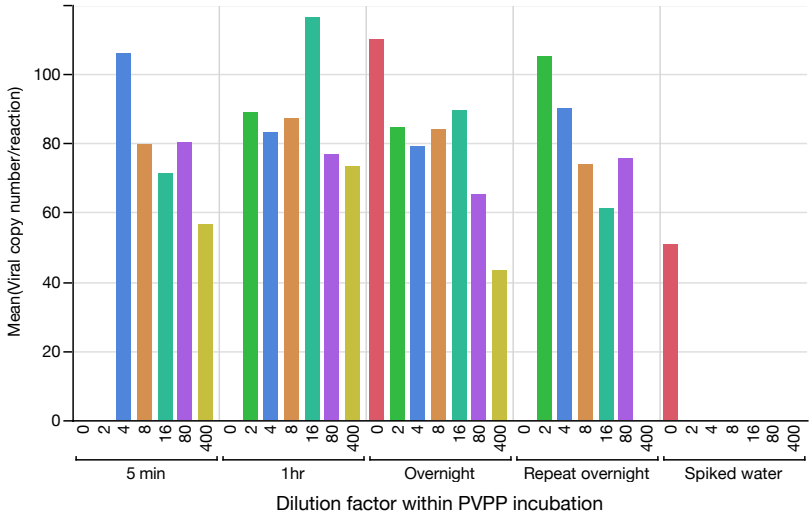

Figure 4.3 Litter spike test 3. Mean virus recovery from DNA extracted from hardwood litter stored at -20˚C, diluted in a 6x2 fold dilution series, “spiked” with a fixed small amount of Marek’s disease virus with the addition of PVPP for various incubation periods at room temperature. If there is no inhibition all recovery values would be the same.

These results demonstrated that there is no significant inhibition of the PCR reaction in softwood shavings or rice hulls, but that hardwood shavings have a significant inhibitory effect. This effect can be overcome by the addition of 6.6% w/v PVPP following the extraction step and incubation at room temperature for 5 minutes.

4.4 Optimisation of qPCR detection of viral DNA/RNA in litter

The most common bedding material for chickens in Australia is softwood shavings and the work described above had revealed no significant inhibition of the PCR reaction by this material. Therefore work on optimising the detection of the 5 target viruses in litter concentrated on this material although some of the studies below include other materials. Our objective was to devise a single method of litter preparation and nucleic extraction that would enable enumeration of all of the target viruses from the same extracted sample.

4.4.1 Optimisation Studies 1 (CSIRO)

This comprised a number of small-scale tests to demonstrate isolation of a DNA (MDV) and an RNA virus (IBDV) from naturally contaminated softwood shavings (LT09-C-CB9) and testing of various preparation steps prior to nucleic acid extraction. These treatments included washing the litter with MilliQ water, or TE buffer containing 0.15% Tween-80 to release the virus from the litter (shaking or blending); washing the litter with 10% beef extract then precipitation of virus with 8% PEG 6000 (Guan *et al.* 2008). Total nucleic acids were extracted from various fractions of the prepared litter materials using various methods including magnetic beads (MagMax® Total RNA isolation kit Ambion), GeneJET® viral DNA and RNA purification kit (Thermo Fisher Scientific) and the Qiagen QIAmp DNA stool extraction kit. The final eluate was diluted 1:10 and used as the template in the qPCR reaction for all 5 viral assays. The fractions tested included the retentate (the materials remaining in the filter after coarse filtration of the washed litter), the filtrate and the pellet remaining after centrifugation of the filtrate.

Nucleic acid targets of both MDV (DNA virus) and IBDV (RNA virus) were successfully recovered from pine shaving litter material naturally contaminated by infected chickens. Significant amounts of both were detected in the retentate, filtrate and pellet fractions following litter preparation. Use of Tween-80 to wash the litter provided better overall results for both viruses than water or the beef extract. Water failed to release IBDV from the litter sample with the majority of the viral RNA detected in the initial litter strainings, however it was successful in releasing MDV. Conversely IBDV was satisfactorily detected using beef extract whilst MDV was not. Blending and shaking samples in Tween 80 produced high recoveries for both viral targets so this was explored further in Optimisation Study 2.

4.4.2 Optimisation Study 2 (UNE/CSIRO)

Optimisation Study 2 comprised a formal factorial experiment testing the effects of litter washing buffer (TE buffer plus 0.15% Tween-80 or 10% beef extract), wash times (2 hr and 16 hr at 4˚C), blending (blend, no blend for 1 min in a commercial blender) and bead beating

(bead beat, no bead beat) on detection of MDV (DNA virus) and IBDV (RNA virus) in DNA/RNA extracted from naturally contaminated softwood shavings litter. Detection was attempted on the unstrained slurry (crude mixture of material), or the retentate (solids) or filtrate (liquids) following coarse straining of the slurry through a 1mm^2 sieve). Two extraction kits were used, MagMax® and Qiagen QIAmp DNA stool extraction kit.

MDV, but not IBDV target sequence amplified successfully in this experiment. Amplification was successful using the MagMax® kit only, with no viral nucleic acid detected following extraction using the Qiagen stool kit (100% negative samples). Thus statistical analysis was performed on MDV recovery data following extraction using the MagMax® kit. Viral copy number data were $\log_{10}(y+1)$ transformed prior to analysis to equalise the variances and better meet the assumptions of analysis of variance. ANOVA was performed on Log_{10} (Viral copy number/g/litter +1) testing the effects of washing method, wash time, blending, bead beating and the fraction assayed, together with 2-way interactions. The proportion of positive samples was also analysed by Chi Square contingency table analysis. All analyses were performed using JMP 11 (SAS Systems).

The results of the ANOVA are presented in Table 4-1

Table 4-1. Optimisation study 2. ANOVA table for analysis for Log_{10} (VCN/g/litter +1)

Source	DF	Sum of Squares	F Ratio	Prob > F
Wash Method	1	0.613498	0.3270	0.5722
Wash Time (hr)	1	12.630626	6.7318	0.0151*
Blend	1	0.608831	0.3245	0.5736
Bead Beat (BB)	1	0.902301	0.4809	0.4939
Fraction	2	5.230452	1.3938	0.2654
Wash Method*Wash Time (hr)	1	4.271851	2.2768	0.1429
Wash Method*Blend	1	0.000463	0.0002	0.9876
Wash Method*Bead Beat (BB)	1	9.076537	4.8376	0.0366*
Wash Method*Fraction	2	9.626024	2.5652	0.0955
Wash Time (hr)*Blend	1	0.131621	0.0702	0.7931
Wash Time (hr)*Bead Beat (BB)	1	1.243378	0.6627	0.4227
Wash Time (hr)*Fraction	2	2.710865	0.7224	0.4947
Blend*Bead Beat (BB)	1	0.000113	0.0001	0.9939
Blend*Fraction	2	11.025734	2.9382	0.0701
Bead Beat (BB)*Fraction	2	2.726578	0.7266	0.4928

For MDV the overall untransformed mean value for viral load recovered was 8417±1813 VC/g litter for all preparation methods and fractions. The overall mean of the transformed Log_{10} viral recovery data was 3.00±0.20. Increasing wash time from 2 hr to 16 hr at 4˚C significantly reduced the number of positive samples from 96% to 67% (P = 0.006) and reduced mean Log_{10} viral recovery from 3.53±0.28 to 2.49±0.28 (P=0.015). There was a non-significant trend for this effect to be greater for samples washed with TE+Tween than Beef extract buffer (P=0.14).

There was significant interaction between the effects of bead beating and washing buffer (P=0.036) with bead beating tending to reduce MDV recovery when beef extract buffer was used, but increase it when TE+ Tween was used (Figure 4.4, left). There was a trend towards interaction between the effects of washing buffer and recovery from the different fractions (P=0.1) with the strainings containing the highest amount of MDV when BE buffer was used, but the lowest when TE+Tween was used (Figure 4.4, centre). There was also trend towards interaction between the effects of blending and recovery from the different fractions (P=0.07) with the filtrate containing the highest amount of MDV following blending, but the lowest when samples were not blended (Figure 4.4, right).

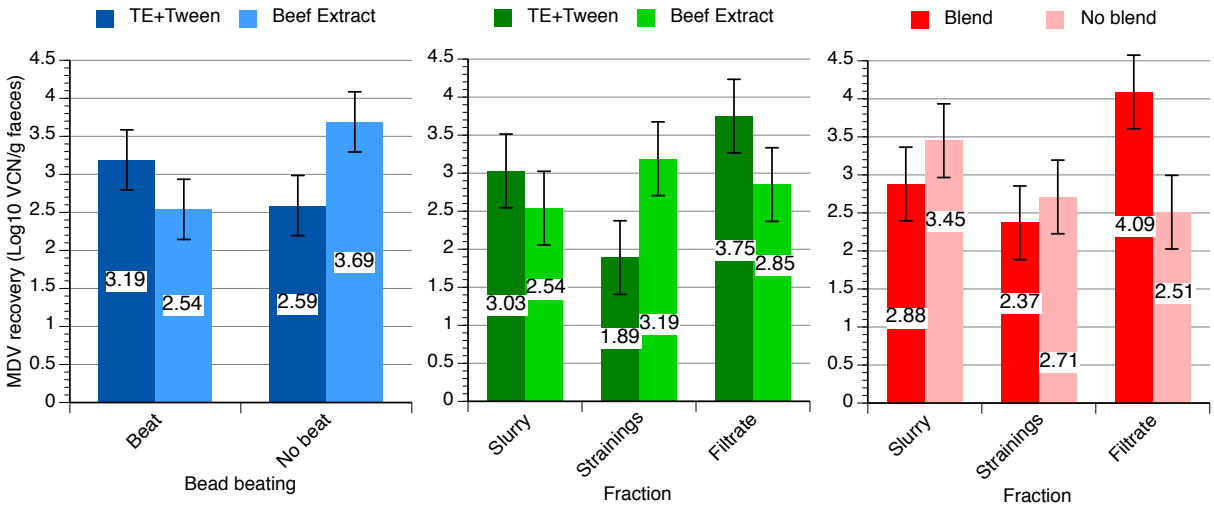

Figure 4.4 Optimisation study 2. Interaction plots showing interaction between effects of bead beating and washing buffer (left), fraction and washing buffer (centre) and fraction and blending (right) on recovery of MDV virus from softwood shavings following various treatments.

The highest Log_{10} viral recovery/g litter was from the filtrate (3.03±0.34) than the total slurry (3.16±0.34) or the solid retentate after straining (2.54±0.34) but this effect was not significant overall (P = 0.27).

These results demonstrated that the Qiagen stool kit was unsuitable for our purposes and that recovery of IBDV proved more difficult than MDV under these conditions. Long washing periods were detrimental to recovery and that virus was recovered in all fractions of material following a range of pre-treatments, none of which provided unequivocal benefits.

4.4.3 Optimisation Study 3 (UNE)

Optimisation Study 3 was a large factorial experiment in which fixed amounts of commercially available vaccine for 4 of the viruses and infective dust for MDV were added to 100 g of SPF litters of different types and recovery in different fractions determined by qPCR following a range of preparatory steps. Samples of the pine shavings, hardwood shavings

and rice hulls artificially contaminated litters were incubated in TE buffer plus 0. 15% Tween-80 on ice with shaking for two hours (blending twice during this time) then filtered through a fine nylon mesh (approximately 1mm^2). The retentate (R) was retained and the filtrate centrifuged at 17,500g for 30 mins at 4C, to provide a supernatant (S) and pellet (P). The R, S and P fractions were then subjected to the following treatments in a complete factorial design: Bead beating (BB) for 5 minutes or not; and treatment with PVPP or not. Total nucleic acids were extracted using magnetic bead technology (MagMax™ Total RNA isolation kit Ambion). The final eluate was diluted 1:10 and used as the template in the qPCR reaction for all 5 viral assays.

The dataset comprised 288 qPCR results comprising 72 samples for each of CAV, IBDV, ILTV, and MDV, but not FAdV which was not detected readily due to the FAdV assay that was in use at the time. The assay has since been replaced by the one reported in Section 4.1. The proportion of positive samples was analysed by Chi Square contingency table analysis. Quantitative qPCR data were $\log_{10}(y+1)$ transformed prior to analysis and ANOVA was performed on Log_{10} (VCN/g/litter +1) testing the effects of litter type (pine shavings, hardwood shavings, rice hulls) , bead beat (yes/no), PVPP (yes/no), Virus CAV, IBDV, ILTV, and MDV and their 3-way interactions. All analyses were performed using JMP 11 (SAS Systems). Results are generally presented as least squares means ± SEM.

Virus was detected in all three litter types with overall percentages of positive qPCR results being 69%, 43%, 22% and 26% for CAV, IBDV, ILTV, and MDV, respectively ($P < 0.0001$). When no PVPP was used the percentage of positive samples was significantly lower from hardwood (6%) than rice hulls (50%) or softwood, (48%) ($P < 0.0001$) but this effect was completely removed by inclusion of PVPP with values of 48%, 48% and 42% respectively, indicating a selective effect of PVPP in overcoming the PCR inhibition observed with hardwood shavings. The effect of PVPP was evident for all viruses but was greatest for CAV and least for IBDV. For the three DNA viruses no amplification occurred at all on hardwood shavings in the absence of PVPP. The pellet had the highest ratio of positive samples (47%) followed by the supernatant (41%), then the retentate (33%) but the difference was not significant ($P = 0.16$). PVPP increased the percentage of positive samples in the pellet from 33 to 60% ($P = 0.008$) without an effect for the other fractions. There was a trend for bead beating to increase the proportion of positive samples from 35% to 45% ($P = 0.09$). This was observed for all viruses except ILTV for which there was no difference with or without bead beating.

The mean measured recovery rates of added virus were 18.0, 10.4, 0.065 and 0.052 % for IBDV, CAV, ILTV and MDV respectively ($P<0.001$). The virus recovery rate was not significantly affected by any of the other factors in the experiment.

The results of the ANOVA of Log_{10} (VCN/g/litter +1) are presented in Table 4-2. The overall effects of all of the main effects in the model were significant apart from the effect of Fraction which approached significance (P=0.06). While the main effects were heavily qualified by interaction with other effects their overall effects are summarised here. Viral recovery was significantly lower from hardwood shavings (1.75±0.24) than either softwood shavings (2.83±0.24) or rice hulls (3.14±0.24) (P<0.0002). There was a tendency towards lower virus recovery from the retentate (2.10±0.24) than the supernatant (2.76±0.24) or pellet (2.86±0.24) (P=0.06). Bead beating significantly increased the recovery from 2.24±0.20 to 2.89±0.20 (P=0.02). Similarly addition of PVPP significantly increased viral recovery from 2.25±0.20 to 2.90±0.20 (P=0.02). Recovery of CAV (4.40±0.28) was significantly higher than for IBDV (2.75±0.28) with both having higher recovery rates than ILTV (1.79±0.28) or MDV (1.34±0.28) which did not differ from each other.

Table 4-2. Optimisation study 3. ANOVA table for analysis for Log_{10} (VCN/g/litter +1)

Source	DF	Sum of Squares	F Ratio	Prob > F
Litter type	2	102.61119	8.9448	0.0002*
Fraction	2	32.89385	2.8674	0.0592
Bead beat	1	29.62170	5.1644	0.0241*
PVPP	1	31.95352	5.5709	0.0192*
Virus	3	395.20117	22.9670	<.0001*
Litter type*PVPP	2	152.53396	13.2967	<.0001*
Litter type*Virus	6	103.91309	3.0194	0.0076*
Fraction*Bead beat	2	40.56756	3.5364	0.0310*
Fraction*PVPP	2	69.51784	6.0600	0.0028*
Litter type*Fraction*PVPP	4	98.33643	4.2861	0.0024*
Litter type*Fraction	4	21.55236	0.9394	0.4422
Fraction*Virus	6	21.62500	0.6284	0.7075
Litter type*Fraction*Virus	12	110.69587	1.6083	0.0917
PVPP*Virus	3	14.61163	0.8492	0.4686
Fraction*PVPP*Virus	6	172.96320	5.0259	<.0001*
Litter type*Bead beat	2	10.90330	0.9505	0.3883
Bead beat*PVPP	1	0.66512	0.1160	0.7338
Bead beat*Virus	3	10.97010	0.6375	0.5917
Litter type*Fraction*Bead beat	4	58.29109	2.5407	0.0411*
Litter type*Bead beat*PVPP	2	69.68747	6.0748	0.0028*
Litter type*Bead beat*Virus	6	89.93884	2.6134	0.0185*
Litter type*PVPP*Virus	6	61.07630	1.7747	0.1061
Fraction*Bead beat*PVPP	2	0.06318	0.0055	0.9945
Fraction*Bead beat*Virus	6	81.00453	2.3538	0.0323*
Bead beat*PVPP*Virus	3	16.31362	0.9481	0.4184

Significant 2-way interactions are shown in Figure 4.5. These clearly show that the effect of PVPP was seen only in hardwood shavings (Figure 4.5, left) and that its action was to increase the amount of virus in the pellet and retentate (ie. solids) rather than in the supernatant (Figure 4.5, centre). The inhibitory effect of hardwood shavings on virus recovery was much greater for CAV than the other viruses (Figure 4.5, Right). Among the

significant 3-way interactions the most important is that between Fraction, PVPP and Virus which further qualifies the two way interactions shown in Figure 4.5. This interaction basically reveals that PVPP addition to hardwood shavings increased virus recovery rate in all of the fractions while in rice hulls it increased it only in the pellet fraction and in softwood shavings it increased it only in the retentate.

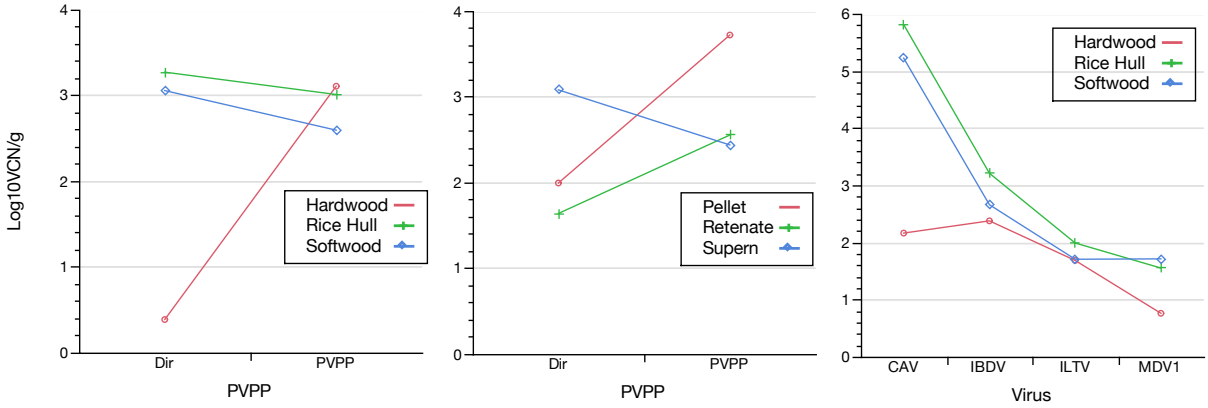

Figure 4.5 Optimisation study 3 Interaction plots showing significant 2-way interactions between effects of PVPP and litter type (left), PVPP and fraction (centre) and virus and litter type (right) on recovery of virus from poultry litter contaminated with virus following various treatments.

This work confirms other published work (Guan *et al.* 2008) demonstrating that viral nucleic acids can be reliably amplified from poultry litter. Clearly the type of litter is important, with hardwood based litter material containing PCR inhibitors. Fortunately these were able to be neutralised with PVPP as demonstrated earlier. For all viruses significant amounts were found in the retentate after filtration, and in both the pellet and supernatant fractions following centrifugation of the filtrate. The results indicate that a method based on washing samples with TE containing 0.15% Tween-80 followed by bead beating and PVPP treatment would enable detection of both DNA and RNA viruses from litter with the greatest concentration of virus found in the pellet fraction after centrifugation. Detectable virus recovery was good for IBDV and CAV, but low for the dsDNA viruses ILTV and MDV. The initial qPCR primers for FAdV detection were based on the publication of Steer *et al.* (2009), and this assay was moderately sensitive when adapted to a probe-based assay but failed to recover and FAdV in this experiment. Another publication (Günes *et al.* 2012) provided an alternative primer set for FAdV detection, and this was tested for virus detection in litter samples. The Gunes primers consistently out-perform the Steer primers when both primer sets are used in qPCR with SYBR green detection. We have now adopted the Gunes primers as the standard for detecting FAdV in litter samples using a Taqman® probe-based version.

4.4.4 Optimisation Study 4 (UNE/CSIRO)

This experiment was designed to see if it was preferable to dry and grind infected litter samples prior to nucleic acid extraction, rather than was them and have the resultant fractions to deal with. It also compared different extraction methods with or without the inclusion of PVPP.

The experiment used the litter samples prepared for Optimisation study 3 on 26/7/12 by adding CAV, IBDV, FAdV8 and ILTV vaccine virus and MDV infected dust to 3 litter types viz Hardwood shavings, Softwood shavings and Rice Hulls. Samples were stored at -20˚C until used in this study in Dec 2012.

A 40 g subsample from each of the 3 litter samples was dried in a tray at 60°C for 48hrs to dry. Dry matter content was 68%, 65% and 65% for hardwood, softwood and rice hulls respectively. Samples were then ground ground using a 1mm grinder. 40 mg of each ground litter grindings was then subjected to nucleic acid extraction testing one or more of the following

- Addition (or not) of PVPP (0.05 g) in 1 ml of lysis buffer for 1hr at room temperature before removal of PVPP by centrifugation (13,000 rpm for 15 min).
- Nucleic extraction of the supernatant by one of 3 DNA extraction kits: ISOLATE II Genomic DNA and RNA kits (Bioline), (MagMax® Total RNA isolation kit Ambion), GeneJET® viral DNA and RNA purification kit (Thermo Fisher Scientific)
- An additional treatment evaluated the SoilMaster® DNA Extraction Kit (Illumina) using 100mg or 50 mg of ground litter material without PVPP addition.
- Positive control samples comprised a combined vaccine sample representing 20ul each vaccine (CAV, FAdV, ILTV, IBDV and MDV dust soln 1mg/ml) to produce a total of 100ul.

Samples were subjected qPCR assay for all 5 viruses as described earlier. The FAdV assay used was the earlier less sensitive assay based upon the primers derived from Steer *et al.* (2009).

Results are presented in Table 4-3. The proportion of positive samples varied between kits and viruses and was generally lower than observed following nucleic acid extraction following wet washing of undried samples in Optimisation study 3. The work involved in sample preparation was much greater for the drying/grinding process in addition, making it a less favourable option. The SoilMaster kit produced very low recoveries from a greater amount of

initial material than the other kits while the other 3 kits produced similar proportions of positive samples. No kit produced positive samples for FAdV from litter using the old less sensitive assay. Most variation was for the detection of CAV which was only detected in litter samples extracted using the Ambion kit. Unlike the situation seen in earlier studies, addition of PVPP did not improved detection of viruses. This may be a response to the drying and grinding steps as the inhibition studies presented in section 4.3 demonstrated that drying alone reduced the inhibitory effect of hardwood shavings at least 10-fold.

Table 4-3. Optimisation study 4. Percentage of samples positive to qPCR for CAV, MDV, FAdV, IBDV and ILTV following extraction of viral nucleic acids from dried and ground litter samples using a variety of methods.

Kit	Litter type*	PVPP	n	% samples qPCR positive					
				CAV	MDV	FAdV	IBDV	ILTV	All
Bioline	Vaccine mix	No	2	100	100	100	50	100	90
	HWS	No	2	0	0	0	50	0	10
	HWS	Yes	2	0	0	0	0	0	0
	RH	No	2	0	50	0	50	50	30
	RH	Yes	2	0	100	0	50	50	40
	SWS	No	2	0	100	0	0	50	30
	SWS	Yes	2	0	100	0	0	50	30
	All litters	**All**	**12**	**0**	**58**	**0**	**25**	**33**	**23**
GeneJet	Vaccine mix	No	2	100	100	100	100	100	100
	HWS	No	2	0	0	0	50	0	10
	RH	No	2	0	0	0	50	0	10
	SWS	No	2	0	100	0	50	100	50
	All litters	**All**	**6**	**0**	**33**	**0**	**50**	**33**	**23**
Ambion	Vaccine mix	No	2	100	100	100	100	100	100
	HWS	No	2	0	0	0	0	0	0
	RH	No	2	100	0	0	0	0	20
	SWS	No	2	100	50	0	100	50	60
	All litters	**All**	**6**	**67**	**17**	**0**	**33**	**17**	**27**
SoilMaster	HWS	No	4	0	0	0	0	0	0
	RH	No	2	0	0	0	50	0	10
	SWS	No	2	0	0	0	0	0	0
	All	**All**	**8**	**0**	**0**	**0**	**17**	**0**	**3**

* Vaccine mix, Positive control; HWS, hardwood shavings; SWS Softwood shavings; RH, Rice hulls

In summary this experiment has demonstrated that while virus can be recovered from dried ground litter material the work involved and the low recovery rate of virus do not make it promising as a method for routine use.

4.5 Summary of main findings

- Nucleic acids both DNA and RNA viruses can be successfully quantified in poultry litter
- Hardwood shavings contain PCR inhibitors whereas softwood shavings and rice hulls appear not to.

- The inhibitors in hardwood shavings can be neutralised by PVPP addition before or after the DNA extraction step.
- Virus is detectable in significant amount in all fractions of washed litter ie. the retentate after filtration, and in both the pellet and supernatant fractions following centrifugation of the filtrate.
- While recovery of the different viruses varied and was influenced differentially by different factors during sample preparation and nucleic acid extraction virus the results indicate that a method based on washing samples with TE containing 0.15% Tween-80 followed by bead beating and PVPP treatment would enable detection of both DNA and RNA viruses from litter with the greatest concentration of virus found in the pellet fraction after centrifugation.
- An alternative method of drying and grinding litter prior to virus detection proved less useful
- The original FAdV qPCR assay developed proved insufficiently sensitive to routinely detect virus in litter and needed to be replaced by a more sensitive assay. This has been done and FAdV recovery in litter has been successful with the new assay.

4.6 Summary of methods developed for use in the project

4.6.1 Sample pre-treatments before DNA/RNA extractions of chicken materials. Original CSIRO developed protocols

To be used on samples that will be qPCR'd for CAV, ILTV, FADV, IBDV & MDV

Purpose – Each sample type must be treated differently before processing through an extraction kit. The various pre-treatments are outlined below for faeces, soft tissue, dust and litter.

TIPS FOR RNA users: RNA viruses need to be handled very carefully after elution from the column. Elute using sterile filter tips and elution buffer provided by the kit. Put the tube on ice straight after elution and keep cold always! Make a 1/10 dilution in TE buffer pH8 straight away to avoid freeze thawing and place both tubes in the -80°C freezer. When defrosting for use put it straight on ice once defrosted. During setup keep the PCR reaction cold until it is ready to load in the PCR machine. RNA will degrade if it is not kept cold.

All water and buffers (including TE) must be made using MQ that is DNase and RNAse free or has been treated with DEPC. This includes PCR reagents, primers, probes etc. Make small aliquots of all reagents to avoid contamination, including your water (store in 2ml tubes). Do not share your reagents with others, they must all be RNAse free, so only use trustworthy reagents.

Filter tips must be used all the time & maintain a clean bench.

Faeces

1. Weigh out 150mg faeces into a 2ml tube. **NB: If using frozen samples use scalpel or spatula to scrape bits of stool into the tube which should be on ice. It is important that samples do not thaw until the ASL buffer is added.**
2. Add 1.0ml ASL lysis buffer and 200ul Proteinase K (20mg/ml), place at 56°C rotating in a hybridisation oven for 2 hours.
3. Spin down Proteinase K treated samples to remove debris at 10 000rpm 10mins. Remove 500ul lysate into a new 1.5ml tube and proceed with the GeneJET extraction kit protocol at ethanol precipitation step. (750ul ethanol (96%)) i.e step 3.

Soft Tissues

Storage – straight after removal of tissue from animal either:
Place in a labelled cryovial, drop directly into liquid nitrogen and store at -80°C.
Or, Place tissue in a tube containing RNALater (Life Technologies) or Trizol – 5 times the volume of RNALater /Trizol to the size of the tissue. This can be at room temperature in post mortem room, put in 4°C for a few hours when back at the lab then store at -20°C.
Samples can be stored at 4°C for one month, at 25°C for one week, or at –20°C indefinitely.
Archive tissues treated with RNA*later*® solution at –20°C.

Extraction:

Follow Bioline Isolate 11 Genomic DNA kit or Bioline Isolate 11 RNA mini kit instructions exactly.

Dust

For DNA: Collect dust into a zip lock plastic bag or 5 ml tube and store minus 20°C.
For RNA: Collect dust into suitable cryo tube snap freeze in liquid nitrogen, store long term minus 80°C.

Extraction:

Weigh out 5mg of dust into a 1.5ml micro tube.
Follow Bioline Isolate II Genomic DNA kit for DNA instructions exactly or Bioline Isolate II RNA mini kit for RNA or
Extraction of DNA from Dust Samples using GeneJET DNA/RNA viral extraction kit.

1. Weigh 50mg samples into 2ml tubes.
2. Add 3g of SiCar beads.
3. Add 4mls of Lysis Buffer (ASL-Qiagen)
4. Spin for 10 mins at 3000rpm at 10^{o}C
5. Remove 500 µL of lysate to a 2ml tube and add 100 µL of Proteinase K.
6. Place all tubes into the hyb oven. Temperature 56^{o}C, turning for 1 hour.
7. Continue with GeneJET kit.

Poultry Litter (as per method 'Extraction of NA from litter samples – GeneJET'.)

Materials

TE Tween (0.15% Tween 80, 10mM Tris, 1mM Na EDTA, pH 8.0), pre-chilled on ice (allow 400mls per sample)
2L Beakers, pre-chilled on ice. (one per sample)
500mL centrifuge bottles and funnels, nylon mesh square (one per sample)
Qiagen lysis buffer ASL

Method

1. Mix litter sample well then remove a 20g sub sample into a 2L Beaker.
2. Add 300mLs ice cold TE + 0.15% Tween 80.
3. Blend the mixture using a hand blender on high for 2 mins (on ice). Wash residual sample off the blender using a squeeze bottle of TE-Tween.
4. Place beaker of tween-litter on orbital shaker for 1 hour at 4°C. Setting of 8 on 10 speed mixer.
5. Blend the samples again on high for 2mins (on ice), wash off with squeeze bottle of TE Tween.
6. Shake a further 1 hour at 4C.
7. Prepare a piece of nylon mesh, funnel and 500ml centrifuge bottle for each sample.

Weight of empty bottle with Lid:

8. Swirl sample then pour through the nylon mesh. Squeeze the mesh removing most of the liquid from the retentate into centrifuge bottle. Balance bottles for the centrifuge – max volume for buckets is approx 400mls.
9. The filtrate is then centrifuged at 17,500g for 30mins at 4C, to provide a supernatant and pellet. Pour off supernatant.

Weight of Pellet + bottle/lid after spin and pouring off S/N:

Total Weight of Pellets:

10. Prepare 3x 50ml tubes. Mix the pellets with a spatula. Add 2g pellet plus 6mls lysis buffer (Qiagen lysis buffer ASL), mix and resuspend by vortexing.
11. Remove 4mLs of above into a BB tube containing 2mL beads (1mm SiCar).
12. Freeze remaining pellets for later use.
13. Bead beat for 5 minutes.
14. SiCar beads and insoluble material is removed by centrifugation 3,000g 10 mins, 10°C.
15. In 2ml tubes, treat 500ul lysate with 100ul Proteinase K (20mg/ml) at 56°C in turning hybridisation oven for 1 hour.
16. Continue with GeneJET kit protocol.

4.6.2 Extraction of Nucleic acid from Chicken litter (UNE adaptation of CSIRO method)

Materials:

2 L beaker
TE Buffer with 0.15% Tween 80 (10mM Tris, 1mM Na EDTA pH 8.0)
Reduced TE buffer (10mM Tris, 0.1mM Na EDTA pH 8.0)
Hand blender
Wash bottle with TE/tween 80
Orbital shaker
Nylon mesh
Funnel
500ml bottles
Centrifuge tubes
50ml tubes
Qiagen ASL lysis buffer
Vortex mixer
Bead beater + tubes + 1mm SiCar beads
High speed refrigerated centrifuge
Refrigerated centrifuge
GeneJet viral DNA/RNA extraction kit
Microcentrifuge
96% Ethanol
Waterbath or oven set to 65°C
PVPP
Horizontal shaker
1.5ml tubes

Method:

Mix litter sample well then take out a 20gm sub sample and place into 2L beaker.
Add 300mls ice cold TE buffer containing 0.15% Tween 80.

Blend the sample, on ice, using a hand blender on high for 2 mins. Wash residual sample off the blender using a squeeze bottle of TE/Tween 80 buffer.
Place litter sample beaker on orbital shaker for 1 hour at 4°C. Set at speed of approx 8 on a 10 speed shaker.
Prepare nylon mesh, funnel and 500ml collection bottle for the sample.
Blend sample again on high for 2 mins (on ice), wash blender using squeeze bottle with TE/tween.
Shake a further 1 hr at 4°C.
Swirl sample and pour through nylon mesh then squeeze mesh into collection bottle to remove most of the liquid.
Sample then needs to be placed in centrifuge tubes and spun at 4°C for 30 mins at 17,500g.
Pour off supernatant as you want the pellet. Mix the pellet with a spatula.
Into a new 50ml tube add 2 gms of mixed pellet plus 6mls of Qiagen lysis buffer ASL, vortex to thoroughly mix.
Remove 4mls of the above suspension and place into a bead beating tube containing 2 mls beads (1mm SiCar)
Bead beat for 5 minutes.
Remove beads plus insoluble material by centrifuging 3000 g, 10 mins at 10°C.
Collect supernatant (Lysate) for nucleic acid extraction.

GeneJet viral DNA/RNA extraction
NB: Read kit instructions and prepare all reagents before starting.
In 2ml tubes add 500ul of lysate plus 100ul Proteinase K (20mg/ml) place at 56°C in turning hybridisation oven for 1 hr.
Prepare spin columns by adding 50ul column preparation liquid to the centre of the filter. Leave at room temp till needed.
Spin down Proteinase K treated samples to remove debris at 10,000rpm 10 mins. Remove lysate into new 1.5ml tube.
Add 750ul (96%) ethanol vortex. Incubate at room temp 3 mins.
Transfer lysate to prepared spin column. Centrifuge for 1 min at 6000g. Discard flow through. Place column into new 2ml wash tube.
NB: 700ul max volume to be loaded onto spin column at a time so repeat load and spins until all is put through column.

Add 700ul wash buffer 1 to column, spin 1 min 6000g. Discard flow through. Place column into new 2ml wash tube
Add 500ul wash buffer 2, spin 1 min 6000g. Discard flow through. Place column into new 2ml wash tube. Repeat this step.
Spin the column for 3 mins at 16,000g to dry column. Discard wash tube.
Place spin column into a new 1.5ml elution tube.

Add 50ul pre-warmed (65°C) elution buffer.

Let sit for 2 mins at RT, spin for 1 min at 13,000g. Discard spin column. Store at -80°C RNA or
-20°C DNA if there is not enough time to PVPP treat.

Post extraction PVPP treatment
Add 0.01g PVPP to required number of 1.5ml tubes.
Dilute sample 1/10 with reduced TE (40ul DNA + 360ul reduced TE) add to the PVPP tube.
Mix tubes on horizontal shaker 1hr at RT.
Centrifuge to remove PVPP.
Take off supernatant from each sample and store -80°C in a new 1.5ml tube until required for qPCR.

TE + reduced TE buffer recipes:
Make up both Tris-HCL 1M and EDTA 0.5M as follows.
If for DNA make to pH 8.0 for RNA make to pH 7.5

For normal TE

1 M Tris-HCL pH 8.0
Dissolve 121.1gms Tris base in approx 800mls milli Q water
Adjust to desired pH with conc HCL (approx 42mls for pH 8.0)
Adjust volume to 1 litre with water
Sterilize before use
Store up to 6 mths at 4°C or RT

0.5M EDTA pH 8.0

Dissolve 186.1gms disodium EDTA dehydrate in approx 700 mls milli Q water.
Adjust pH to 8.0 with 10M NaOH (approx 50mls for pH 8.0 added slowly)
Adjust volume to 1 litre with water
Sterilize before use

1x TE buffer - 1 litre
10 mls 1M Tris-HCL pH 8.0
2mls 0.5M EDTA pH 8.0
988 mls milli Q water

(1x TE is 10mM Tris-HCL and 1mM EDTA)
Sterilize by autoclaving 20 mins at 15 psi

Reduced TE buffer
NB: **Reduced TE uses 0.5M Tris-HCL pH 8.0 + 0.5M EDTA pH 8.0.**
Reduced TE is used to store DNA at pH 8.0 to reduce depurination, which is acid-catalyzed, and RNA at pH 7.5 to reduce degradation of RNA, which is base-catalyzed.
Make up 0.5M Tris-HCL as follows:
0.5M Tris-HCL pH 8.0
Dissolve 60.6 gms Tris base in approx 800mls milli Q water
Adjust to desired pH with conc HCL (approx 42mls for pH 8.0)
Adjust volume to 1 litre with water
Sterilize before use
Store up to 6 mths at 4°C or RT

1x reduced TE buffer – 1 litre
20 mls 0.5M Tris-HCL pH 8.0
200ul 0.5M EDTA pH 8.0
979.8 mls milli Q water

(1x reduced TE is 10mM Tris-HCL + 0.1 mM EDTA)
Sterilize by autoclaving 20 mins at 15 psi

4.6.3 Simplified method for DNA extraction from litter samples developed by Prof Parimal Roy at UNE.

Digestion buffer consists of:

Make: 100mM NaCl, (0.58 gms/100ml OR 5.8gms/litre)
10mM Tris HCl pH 8.0 (0.1576 gms/ 100ml OR 1.576 gms/litre)
25 mM EDTA (0.9306 gms/100ml OR 9.306 gms/litre)
0.33% SDS (0.33 gms/100ml Milli Q OR 3.3 gms/litre)

- Make NaCl and Tris/HCL buffers separately.
- pH Tris/HCL buffer to 8.0.
- Then combine equal volumes of Tris/HCL with an equal volume of NaCl
- Add appropriate quantity of EDTA -- Mix till dissolved completely.
- Add appropriate quantity of SDS -- Mix till dissolved completely.
- This is completed Digestion buffer ready to be used as follows.

Method:

- Weigh out 10 gms litter into 250ml Schott bottle, cap bottle.
- Litter is then mixed with 100ml of digestion buffer
- Mix well and incubate overnight at 60°C in a shaking incubator.
- 1 ml of the suspended liquid is taken from the Schott bottle and placed into a clean 1.5ml tube. 20µl of proteinase K soln (20mg/ml) is then added to the tube.
- Vortex and incubate at 60°C for a further 30 minutes in a heat block.
- Centrifuge at 6000g for 1 minute.
- Take 400 µl of the supernatant and place into new 1.5ml tube.
- Use either:
  - Thermo Scientific GeneJet viral DNA/RNA kit and follow instructions from step 3 of the method to extract the DNA/RNA. Your final volume of DNA will be 50ul.

  OR,
  - Bioline Faecal DNA kit as per instructions from step 4. Your final volume of DNA will be 100ul.

GeneJet Extraction of Litter Samples

Starting at step 3 of method for larger sample volumes.

1. Add 96-100% Ethanol- Use 150ul for every 100ul initial samples volume.
   Mix by votexing.
   Incubate sample RT 3 mins.
   Centrifuge 3-5 secs full speed to collect drops from inside the lid.

2. Transfer lysate to prepared spin column (Don't load more than 700ul lysate onto column at any one time. If volume greater than 700ul add remaining to column and centrifugr a 2nd time.)
   Centrifuge column 1min x 6000 g
   Discard flow-thru. Place column into new 2 ml wash tube.

3. Add 700ul wash buffer 1
   Centrifuge column 1 min x 6000g
   Discard wash tube containing flow thru.
   Place column into new 2 ml wash tube.

4. Add 500ul wash buffer 2

Centrifuge column 1 min x 6000 g
Discard wash tube containing flow thru.
Place column into new 2 ml wash tube.

5. Add 500ul wash buffer 2
Centrifuge column 1 min x 6000 g
Discard wash tube containing flow thru.
Place column into new 2 ml wash tube.

6. Centrifuge column 3 mins x 16,000 g
Discard wash tube containing flow thru.

7. Place spin column into new 1.5 ml tube.
Add 50ul Elution buffer previously preheated to 56˚C to the center of the spin column.
Incubate 2 mins RT.
Centrifuge column 1 min x 13,000 g
Discard spin column. Store minus 20˚C until ready to use.

BEFORE you start make up Wash buffers by adding Ethanol if not already done.
Prepare spin columns by adding 50ul column preparation liquid into the center each.
Equilibrate samples to room temp.

4.6.4 Simplified DNA Extraction from faecal samples for qPCR developed by Prof Parimal Roy at UNE.

Make digestion buffers as follows:
100mM NaCl, (0.58 gms/100ml)
25 mM EDTA (0.9306 gms/100ml)
10mM Tris HCl pH 8.0 (0.1576 gms/ 100ml)
2% of final volume SDS (2 gms/100ml)
Make NaCl and Tris/HCL buffers separately
pH Tris/HCL buffer to 8.0.
Then combine 50mls of Tris/HCL with 50 mls NaCl
Add 0.9306gms of EDTA -- Mix till dissolved
Add 2 gms/100mls SDS -- Mix till dissolved

Method:

- Weigh out 150mg of Faecal sample into 1.5ml tube
- Add 1 ml digestion buffer to each sample.
- plus 20ul Proteinase K to each 1ml sample.
- Vortex vigorously
- Incubate overnight at 65°C
- Vortex after incubation then centrifuge and take off 400ul of supernatant and proceed from step 4 onwards of the Bioline ISOLATE Faecal DNA kit as per kit instructions.

4.6.5 DNA Extraction methods from litter, dust and faeces samples developed by Robin Achari at UNE for FAdV assay

4.6.5.1 FAdV DNA extraction from Litter Samples

10g of poultry litter was mixed with 100ml of digestion buffer (100nM NaCl, 10nM Tris HCL pH 8, 25 Mm EDTA) and 0.33% SDS (= 99ml of buffer and 1ml of 33% SDS)in a 200ml bottle, mixed vigorously and incubated in a 60^{O}C shaking water bath overnight. 1 ml of the suspended liquid was taken and 20µl of proteinase K was added. This was vortexed and further incubated for 30 mins at 60^{o}C on a heat block. After centrifuging at 6000 x g for 1 min, 400µl of the lysate was transferred to another 1.5ml tube and followed step 3 onwards of the Thermo Scientific GeneJet viral DNA and RNA purification kit manufacturer's recommendation. 50 µl DNA was finally eluted and stored at -20^{o}C.

4.6.5.2 FAdV DNA extraction from dust samples

5mg of dust sample was mixed with 250µl of PBS in a 1.5ml Eppendorf tube, vortexed vigorously and then shaken on the vortex-genie 2 shaker for 15 mins. 200µl of the lysate was then loaded on to a new 1.5ml Eppendorf tube, 50µl of proteinase K added and incubated for 15 mins at 56^{o}C on a heat block. Then, followed step 3 onwards of the Thermo Scientific GeneJet viral DNA and RNA purification kit manufacturer's recommendation. 50 µl DNA was finally eluted and stored at -20^{o}C.

4.6.5.3 FAdV DNA extraction from faecal samples

150mg of faecal sample was mixed with 400µl of PBS in a 1.5ml Eppendorf tube, vortexed vigorously and then shaken on the vortex-genie 2 shaker for 15 mins. 200µl of the lysate was then loaded on to a new 1.5ml Eppendorf tube, 50µl of proteinase K added and incubated for 15 mins at 56oC on a heat block. Then, followed step 3 onwards of the Thermo Scientific GeneJet viral DNA and RNA purification kit manufacturer's recommendation. 50 µl DNA was finally eluted and stored at -20oC.

5 Strand 2. Experiment 2.1 Litter treatment experiment – Sydney. "Effects of moisture addition and covering on temperatures in heaped litter on a commercial farm"

5.1 Introduction

Many factors such as the addition of moisture (Lavergne *et al.* 2006) or covering of heaped litter (Macklin *et al.* 2006) can increase the temperature and alter the temperature profiles observed in pasteurising litter. This was the first on-farm experiment investigating these factors in the project and it addressed the goal of developing methods to achieve rapid and uniform temperature increases in litter heaped for pasteurisation. Specific objectives for this activity were:

- to test the effect of moisture level on temperature profiles at various depths; and
- to test the effect covering the heap on temperature and moisture content of the litter.

The experiment was carried out in three sheds of a meat chicken farm in western Sydney, NSW, by Dr Fakhrul Islam. The experimental code for the experiment was LT12-C-LSOP1. The experiment ran from 21-29 May 2012.

5.2 Materials and methods

5.2.1 Experimental design

The experiment utilized a 3 (moisture) x 2 (cover) factorial design with two replicates of each combination using all 3 sheds on the farm.

- Moisture content (MC) - Normal moisture content without addition of any extra moisture (M), M with estimated 5% water added (M5), MC with estimated 10% water added (M10)
- Cover - Covered (C) or not (NC) with a polyethylene tarpaulin

The physical arrangement of the sheds on the farms is shown in Figure 1. The three sheds differed in size and type, the smaller two being conventional open-sided sheds with curtains (Shed 1 & 2) and the remaining shed (Shed 3), being a modern tunnel ventilated shed.

Litter was originally wood shavings and had been previously used for 2 batches of broilers (one reuse). Twelve litter heaps were made, 3 in Shed 1, 4 in Shed 2 and 5 in Shed 3 placed

centrally along the midline of the sheds. The details of the heap placements, size and initial moisture content are shown in Table 5-1. Heaps were formed and subsampled on 21/5/2012 (day 0) and iButtons removed and heaps again sub-sampled on 29/5/2012 (day 8).

Figure 5.1. Google view of the three sheds

Table 5-1. Expt. 2.1. Summary of arrangement of treatments and starting conditions

Heap	Shed	Added moisture*	Cover	Height (m)	Length (m)	Width (m)	Estimated volume (m^3)	Initial moisture (%)
1	1	M10	No	1.15	3.3	2.8	4.61	32.2
2	1	M5	Yes	1.15	3.3	2.8	4.61	27.5
3	1	M0	No	1.15	3.3	2.8	4.61	20.8
4	2	M0	No	1.25	4.25	3.5	8.20	22.4
5	2	M10	Yes	1.25	4.25	3.5	8.20	38.1
6	2	M5	Yes	1.25	4.25	3.5	8.20	27.9
7	2	M5	No	1.25	4.25	3.5	8.20	26.7
8	3	M0	Yes	1.2	4.1	3.3	7.24	22.1
9	3	M0	Yes	1.2	4.1	3.3	7.24	24.1
10	3	M5	No	1.2	4.1	3.3	7.24	27.8
11	3	M10	Yes	1.2	4.1	3.3	7.24	32.9
12	3	M10	No	1.2	4.1	3.3	7.24	34.5

*M0 = No added moisture, M5 = ~5% by weight added, M10 = ~10% by weight added

5.2.2 Experimental procedures

In Shed 1, the floor was marked equally into three sections. The area of the shed was calculated and the volume of the litter in each section was estimated. The required amount of water was added to the M5 and M10 sections using a water hose after calculating the flow rate. Sheds 2 and 3 were similarly divided into 4 and 5 equal sections marked with flags. The required amount of water was added in the relevant sections in the same way.

Once the heaps were made, 10 iButtons were inserted in each heap at five depths (0, 5, 10 25 and 50 cm) and two sites (replicates). Time of insertion and the location of each iButton

were recorded. Representative litter samples were also collected for determining litter moisture contents. Covered heaps were covered with large sized single polyethylene tarpaulin.

5.2.3 Data analysis

Litter moisture data were analysed using 1 way ANOVA fitting the effects of moisture addition, cover and the interaction between the two.

Temperature data from 5 iButtons were discarded due to malfunction and profiles were trimmed to 168 hours (7 days). Data were analysed in several ways viz:

- Scatter plots of hourly data points from each iButton for different treatments or depths were prepared and a spline smoothed curve produced to illustrate the profile over the measurement period.
- The period of time at 55˚C or over was calculated for each ibutton and analysed by ANOVA testing the effects of Moisture treatment, Cover and Depth and their interactions
- The maximum temperature for each iButton was calculated and analysed by ANOVA as for Time above 55˚C.
- The time to maximum temperature (in hr) was calculated for each iButton and analysed by ANOVA as above.
- To assess the overall effects on temperature, individual iButton hourly readings were averaged by day post treatment and the means analysed in a repeated measures ANOVA (mixed model REML), testing the effects of Moisture treatment, Cover, Depth, Day post heaping and their interactions, with Heap nested within treatment as a random effect. This provides a rich interrogation of the effects. A mixed restricted maximum likelihood model (REML) is an appropriate method for repeated measures type variables as in this experiment.

Data visualisation, exploratory analysis, and final analyses were done using JMP 11 (SAS Systems, NC, USA). Most data are reported as LS means and SEs from the outputs of the analysis. In most cases the means do not differ from the raw means. Significant differences amongst means were determined by Tukey's HSD test.

5.3 Results

5.3.1 Litter moisture

Raw data for initial and final litter moisture content by treatment are presented in Figure 5.2. The mean initial litter moisture level was 22.3 ± 0.92% and this was increased significantly by the addition of water such that all treatments differed from each other significantly (M0 22.3 ± 0.92%, M5 27.5 ± 0.92%, M10 34.4 ± 0.92%, P<0.001). The M5 and M10 treatments increased litter moisture by 5.2 and 12.1 percentage units respectively.

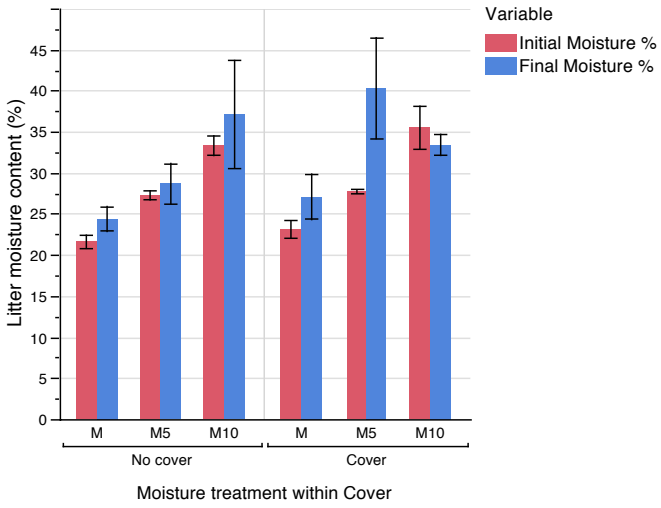

Figure 5.2. Experiment 2.1. Initial and final litter moisture content by treatment (raw data)

Final moisture content was not influenced significantly by moisture treatment (P=0.10), Cover (P=0.33) or interaction between these (P=0.24). As can be seen in Figure 5.2 the values were variable, and contrary to expectations, final moisture content was numerically higher in each treatment than initial moisture content M0 25.7 ± 2.86%, M5 34.5 ± 2.86%, M10 35.3 ± 2.86%).

5.3.2 Litter temperatures

5.3.2.1 Temperature profiles

Smoothed temperature profiles showing the effects of covering, moisture treatment and depth are shown in Figure 5.3, Figure 5.4 and Figure 5.5 respectively. Overall effects of moisture and covering were subtle while those of depth were large.

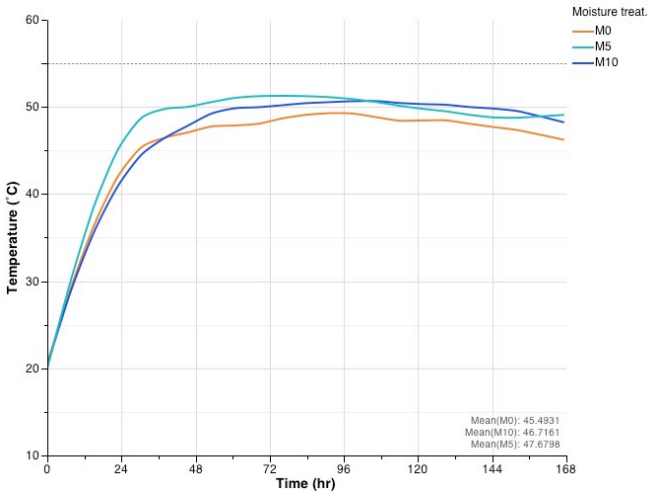

Figure 5.3. Experiment 2.1. Spline smoothed temperature data showing profiles over time for each moisture treatment. Data are from 105 iButtons recording hourly (approx. 35/line)

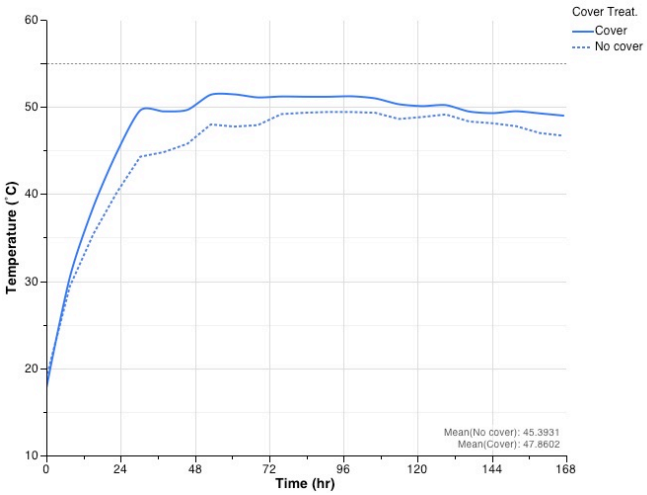

Figure 5.4. Experiment 2.1. Spline smoothed temperature data showing profiles over time for each cover treatment. Data are from 105 iButtons recording hourly (approx. 52/line)

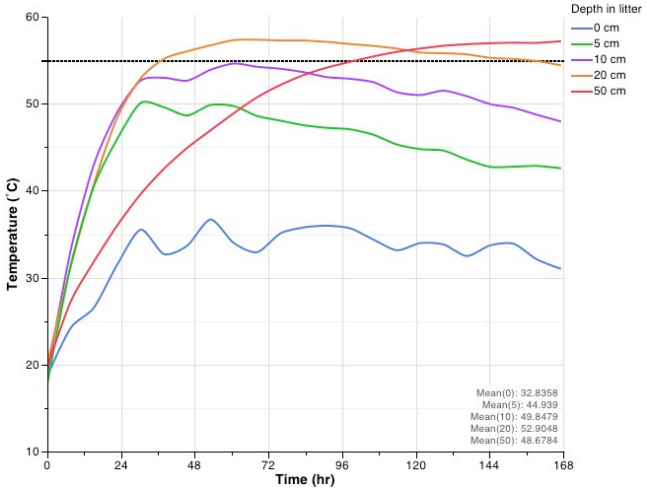

Figure 5.5. Experiment 2.1. Spline smoothed temperature data showing profiles over time for each depth within the heap. Data are from 105 iButtons recording hourly (approx. 21/line)

5.3.2.2 Time above 55˚C

A summary of the analysis of variance of litter temperature is provided in Table 5-2. Only the effect of depth was significant. Main effects are illustrated in Figure 5.6.

Table 5-2. Experiment 2.1. ANOVA table for analysis of time spent above 55˚C (hr)

Source	DF	Sum of Squares	F Ratio	Prob > F
Moisture treatment	2	1098.955	0.2968	0.7442
Cover	1	195.069	0.1054	0.7465
Moisture treatment*Cover	2	2948.026	0.7961	0.4551
Depth	4	91656.549	12.3754	<.0001*
Moisture treatment*Depth	8	15846.764	1.0698	0.3942
Cover*Depth	4	4497.518	0.6073	0.6587
Moisture treatment*Cover*Depth	8	9882.037	0.6671	0.7185

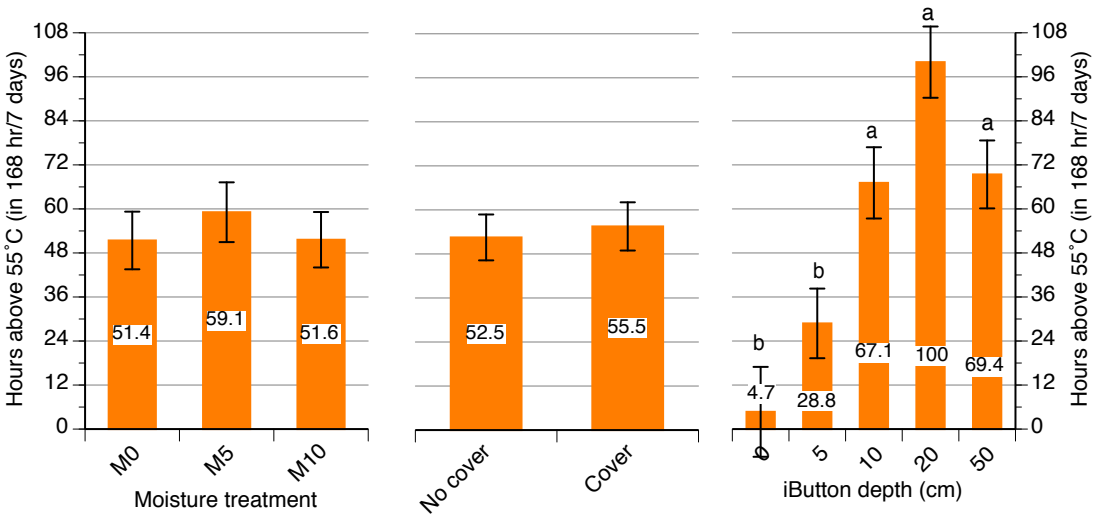

Figure 5.6. Experiment 2.1. Overall effects of moisture treatment, covering and depth within the heap on the number of hours temperatures were above 55˚C out of a total period of 168 hrs (7 days). (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

The highest sustained temperatures were found at 20 cm below the surface where they exceeded 55˚C for 59.5% of the experimental period. This was followed by 50˚C for which temperatures were still increasing at the end of the experiment, whereas they were declining for all other treatments (Figure 5.5). If weighted for proportion of heap affected and assuming 50 cm data for depths greater than 50cm, these data suggest that over the entire heap and week, temperatures above 55˚C were achieved 43% of the time (Table 5-3).

Table 5-3. Experiment 2.1. Estimate of the proportion of the heap over the 7 days that experienced temperatures of 55˚C or above. Based on a weighted mean heap size of 6.8 m^3 and that values measured at a given depth uniformly to the stratum below them. Data in column 6 is the product of the data in columns 2 and 5.

Depth strata	% of heap volume	Hrs>55˚C	Total hrs	% hrs >55˚C	% heap >55˚C
0-5 cm	6.7%	4.70	168	3%	0.2%
5-10cm	6.4%	28.80	168	17%	1.1%
10-20cm	12.1%	67.10	168	40%	4.8%
20-50cm	30.8%	100.00	168	60%	18.4%
>50 cm	43.9%	69.40	168	41%	18.1%
Sum	100.0%				42.6%

5.3.2.3 Maximum temperature

A summary of the analysis of variance of maximum temperature is provided in Table 5-4.

Only the effect of depth was significant. Main effects are illustrated in Figure 5.7

Table 5-4. Experiment 2.1. ANOVA table for analysis maximum temperature (˚C)

Source	DF	Sum of Squares	F Ratio	Prob > F
Moisture treatment	2	3.2730	0.0536	0.9478
Cover	1	88.0111	2.8843	0.0939
Moisture treatment*Cover	2	67.0841	1.0992	0.3388
Depth	4	2101.9518	17.2210	<.0001*
Moisture treatment*Depth	8	143.9133	0.5895	0.7833
Cover*Depth	4	207.8612	1.7030	0.1591
Moisture treatment*Cover*Depth	8	287.4464	1.1775	0.3250

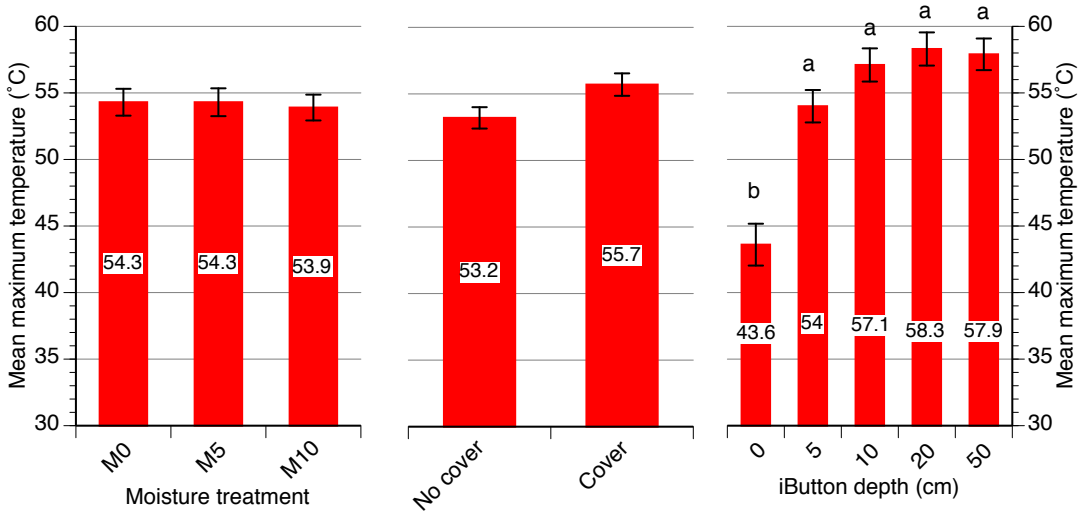

Figure 5.7. Experiment 2.1. Overall effects of moisture treatment, covering and depth within the heap on the mean maximum temperature achieved. (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

Maximum temperatures of 57-58.3˚C were obtained at depths of 10, 20 and 50 cm but these did not differ statistically from the 54 ˚C observed at 5 cm (Figure 5.7). Maximum temperatures attained at the surface were significantly cooler at 43.6˚C.

5.3.2.4 Time to reach maximum temperature

A summary of the analysis of variance of time to reach maximum temperature is provided in Table 5-5. Only the effects of cover and depth were significant. Main effects are illustrated in Figure 5.8.

Table 5-5. Experiment 2.1. ANOVA table for analysis of time to reach maximum temperature (hr)

Source	DF	Sum of Squares	F Ratio	Prob > F
Moisture treatment	2	3852.08	2.2952	0.1083
Cover	1	4766.94	5.6805	0.0199*
Moisture treatment*Cover	2	2054.52	1.2241	0.3002
Depth	4	152542.95	45.4445	<.0001*
Moisture treatment*Depth	8	4709.89	0.7016	0.6890
Cover*Depth	4	2080.62	0.6198	0.6498
Moisture treatment*Cover*Depth	8	13239.81	1.9722	0.0627

Moisture level did not affect time taken to reach peak temperature although there was a trend towards longer time with the M10 treatment. Covered heaps reached maximum temperatures 14.6 hr earlier than uncovered heaps (P=0.02) (Figure 5.8).

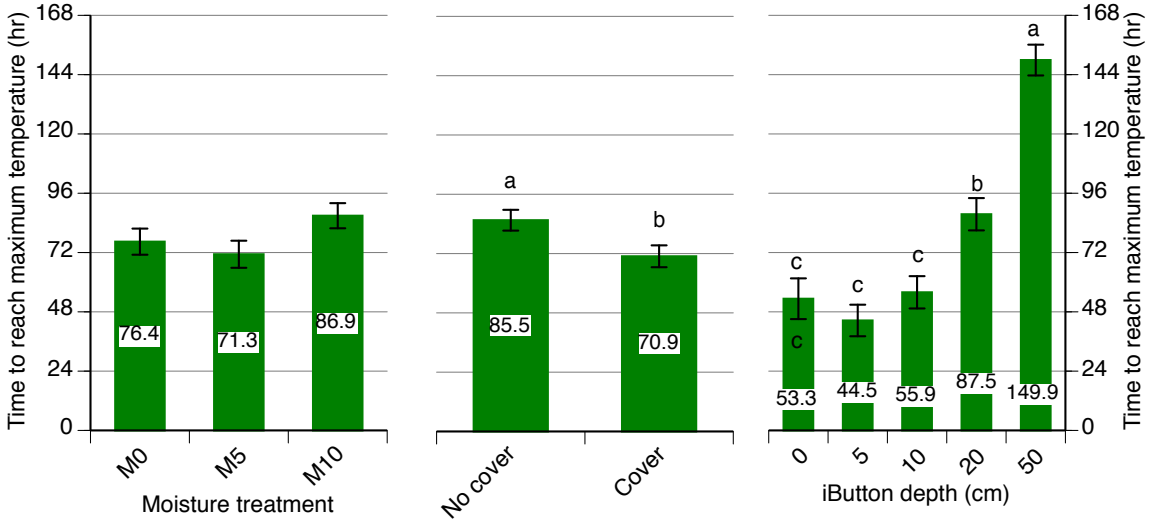

Figure 5.8. Experiment 2.1. Overall effects of moisture treatment, covering and depth within the heap on the time taken to reach maximum temperature (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

Peak temperatures were achieved much earlier at 0-10cm depths (~2 days) than 20 cm (3.6 days) or 50 cm (6.25 days). However the latter value is an underestimate as temperatures

were continuing to increase at the end of the experiment for many iButtons at this depth so a true peak had not been reached yet.

5.3.2.5 Overall temperature analysis (repeated measures)

A summary of the repeated measures ANOVA (mixed model REML), testing the effects of Moisture treatment, Cover, Depth, Day post heaping and their 3-way interactions with iButton as a random effect is presented in Table 5-6

Table 5-6. Experiment 2.1. ANOVA table for repeated measures analysis of litter temperature

Source	DF	F Ratio	Prob > F
Moisture treatment	2	0.1353	0.8760
Cover	1	0.3788	0.5607
Depth	4	137.1005	<.0001*
Day	6	73.3459	<.0001*
Moisture treatment*Cover	2	0.7189	0.5248
Moisture treatment*Depth	8	4.4509	<.0001*
Moisture treatment*Day	12	0.5431	0.8866
Cover*Depth	4	1.8385	0.1200
Cover*Day	6	1.1547	0.3292
Depth*Day	24	6.5695	<.0001*
Moisture treatment*Cover*Depth	8	8.2672	<.0001*
Moisture treatment*Cover*Day	12	0.3694	0.9738
Moisture treatment*Depth*Day	48	0.5966	0.9860
Cover*Depth*Day	24	0.3816	0.9970
Cover*Depth*Day*moisture	48	0.2938	1.0000

While the overall effects of Day and Depth were highly significant, the effects of moisture treatment and cover were not significant overall. However there were highly significant interactions with the other variables indicating significant, but conditional effects.

Overall means temperatures for each of the main effects is presented in Figure 5.9. There was little overall effect of moisture or cover with slight trends towards higher temperatures in the M10 group and in covered heaps.

There was significant interaction between the effects of moisture addition and depth as shown in Figure 5.10(left). This shows that the M10 treatment (12% additional moisture) heated the outer layers of the heap but cooled the inner layers relative to the M0 and M5 treatments. This resulted in a more uniform temperature distribution in the heap. There was also significant interaction between the effects of depth in the heap and day post heaping and cover as shown in Figure 5.10 (right). This is very similar to the smoothed profiles shown in Figure 5.5 and clearly shows that increasing depth in the heap is associated with slower increase in temperature but higher peaks and slower declines than shallower depths.

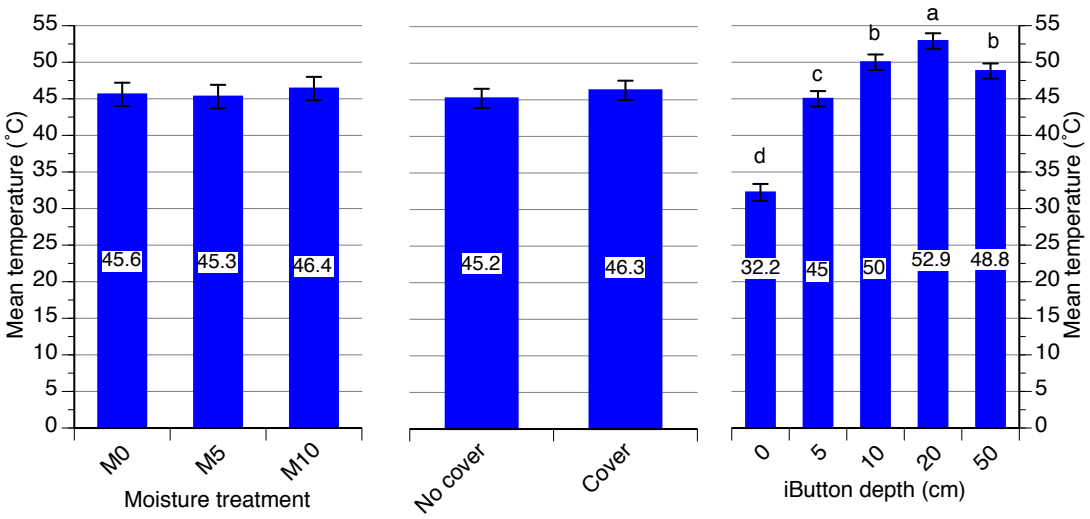

Figure 5.9. Experiment 2.1. Overall effects of moisture treatment, covering and depth within the heap on temperature (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

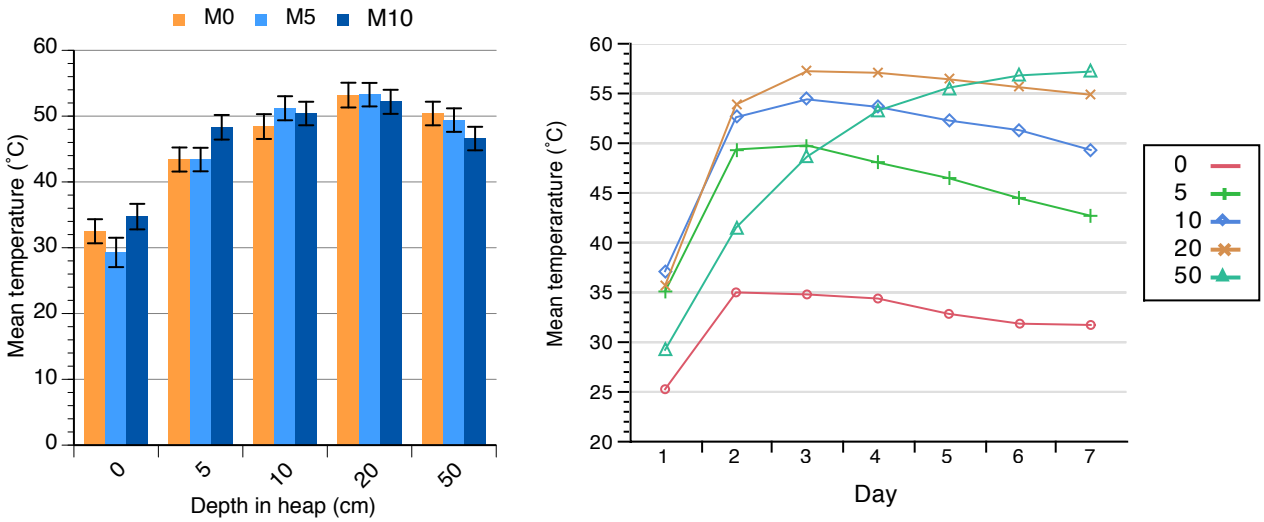

Figure 5.10. Experiment 2.1. Plots illustrating interaction between the effects of Moisture content and depth (Left) (P<0.001) and Depth and Day (Right) (P<0.001).

There was significant 3-way interaction between the effects of Moisture content, Day and Cover (Figure 5.11) shows that the temperature increasing effects of covering were largely restricted to the M0 treatment except at the surface and that they were greatest at 5 and 10cm depths.

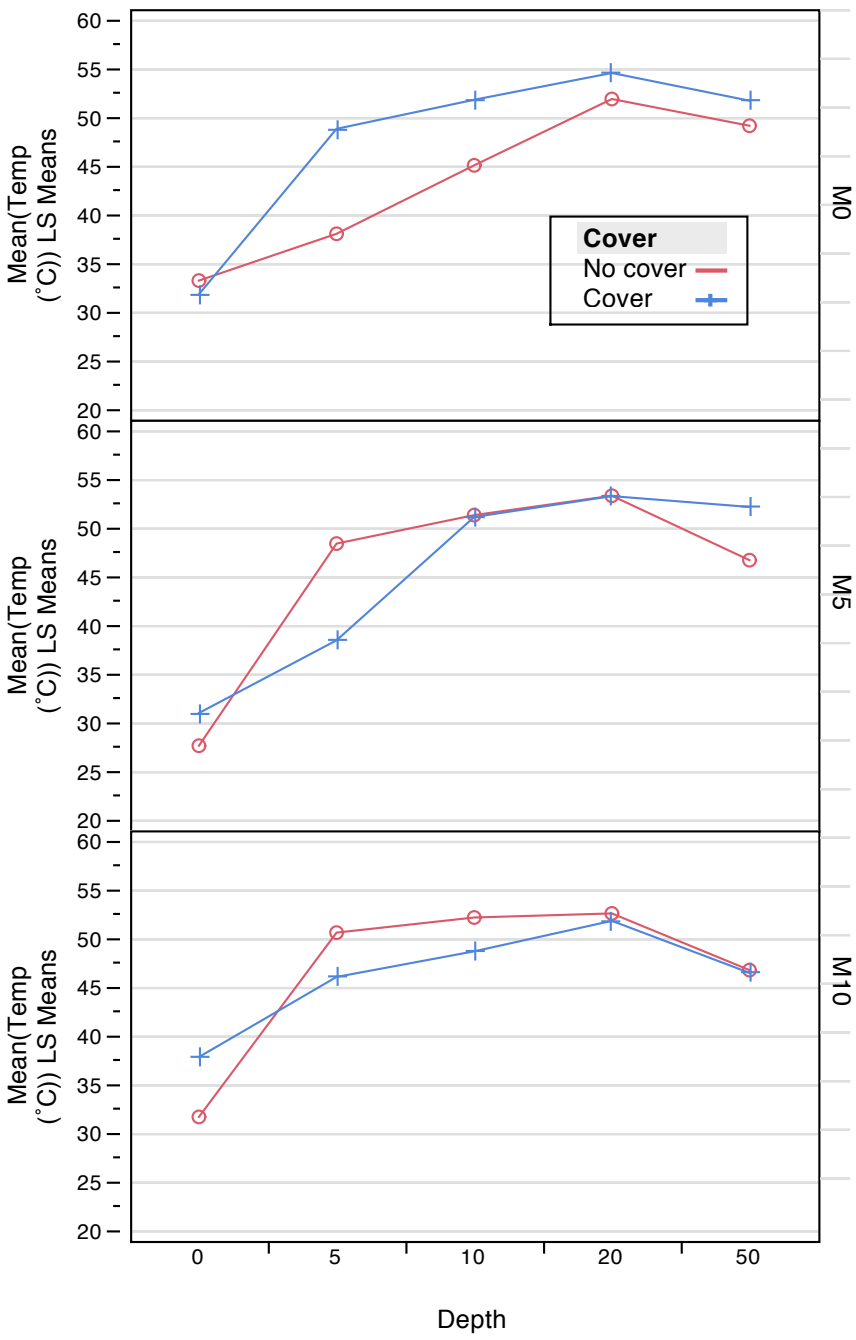

Figure 5.11. Experiment 2.1. Plot illustrating 3-way interaction between the effects of Moisture content, Day and Cover (P<0.001).

5.3.2.6 Association between variables

Pairwise correlations for the major variables analysed are shown in Table 5-7. Temperature variables were all significantly associated as might be expected. However none of the temperature variables was significantly associated with initial moisture content, despite the wide variation in this variable (range 20.8–34.5%).

Table 5-7. Table of pairwise correlations coefficients for major variables based on 100 data points from individual dataloggers. Associations are sorted from most to least significant.

Variable	by Variable	Correlation	Count	Signif Prob
Mean Temp (˚C)	Max Temp (˚C)	0.9498	100	<.0001*
Mean Temp (˚C)	Hours above 55˚C	0.7844	100	<.0001*
Hours above 55˚C	Max Temp (˚C)	0.7078	100	<.0001*
Hours above 55˚C	Time to max temp (hr)	0.3095	100	0.0017*
Max Temp (˚C)	Time to max temp (hr)	0.3093	100	0.0017*
Mean Temp (˚C)	Time to max temp (hr)	0.2385	100	0.0168*
Time to max temp (hr)	Initial Moisture %	0.1063	100	0.2927
Mean Temp (˚C)	Initial Moisture %	0.0482	100	0.6341
Max Temp (˚C)	Initial Moisture %	-0.0248	100	0.8063
Hours above 55˚C	Initial Moisture %	0.0080	100	0.9370

5.4 Brief discussion and conclusions

5.4.1 Effect of covering heaps

At the depths measured in this experiment there was no overall effect of covering heaps on hours above 35˚C, peak temperature, or mean temperature, although there was a strong trend towards higher peak temperatures in covered heaps (Figure 5.7). However covers did significantly reduce the time taken to reach peak temperature by 14.6 hr (Figure 5.8).

The lack of overall significant effects however masked significant interaction between the effects of cover, moisture and day indicating that covers increased temperatures by 6–10˚C at 5 and 10 cm depths in M0 heaps with much smaller effects on the surface and 20–50 cm. With addition of moisture covering generally led to cooling at 5-10cm depths but some slight heating at the surface (Figure 5.11).

Moisture transfers within the heap could be responsible for these results. In drier heaps, trapping of moisture near the surface could be beneficial for microbial proliferation whereas in wetter heaps moisture may not be as limiting near the surface and excessive precipitation under the covers (“sweating”) may have a cooling effect. What is clear from these findings is that covering of heaps in this moisture content range did not produce large beneficial effects on litter temperature although they significantly advanced heating in the early stages and improved the distribution of heat within the heap in heaps in which moisture was not added.

In a USA study using much smaller heaps and wetter litter (37–40% moisture), and with temperature measurements made only at 30cm into the heap, almost a reverse interaction was observed with tarp covering only being effective with the addition of moisture (Macklin *et al.* 2006). In that trial much lower amounts of water were added to the litter (7.6 L to a 0.9m high pile) and this induced a non-significant increase in moisture content of only 0.64 percentage points.

5.4.2 Effect of additional moisture

The litter moisture content at the start of the experiment was comparatively low at 22.3%. Moisture addition increased moisture content to 27.5% in M5 (5.2 % points increase) and 34.4% in M10 (12.1 % points increase). Despite this, at the depths measured in this experiment there was no overall effect of moisture addition on hours above 35˚C, peak temperature, time to reach peak temperature or mean temperature. However the lack of an overall effect did mask significant moisture-influenced events in the heap. These can be summarised as follows:

- Addition of 12% additional moisture (M10) resulted in heated the outer layers of the heap but cooling the inner layers relative to the M0 and M5 treatments and a more uniform distribution of temperatures in the heap (Figure 5.10 Left).
- Addition of moisture (M5 and M10) removed the beneficial effect of covering at 5 and 10 cm depths, leading to cooling at these layers. Conversely moisture addition increased the surface temperature in uncovered heaps (Figure 5.11).

The effects above would be consistent with moisture addition overcoming an inhibitory drying effect in the outer surfaces but deep in the heap, where moisture appears not to limiting under these conditions, the limiting factor is probably aeration and addition of moisture reduces rather than enhances this. This is consistent with the speculation of Lavergne *et al.* (2006) that at high moisture levels, porosity and air penetration deep into the heap are adversely affected and that this reduces thermophile bacterial activity. When moisture addition and covering were combined however, there was cooling at 5–10cm depths, suggestive of excessive moisture and possibly restricted aeration. At the very surface where aeration is likely to be least compromised, moisture addition increased temperatures slightly.

Macklin *et al.* (2006) reported quite large responses to minor additions of water to heaped litter with a moisture content close to 40%, but measurements were very limited in that experiment and there was no statistical analysis. Lavergne *et al.* (2006) in a trial in dewar bottles found increases in temperatures generated with litter moisture contents up to 38%. However on-farm trials gave conflicting results regarding temperature outcomes, with application of water to the surface of windrows delaying peak temperatures and producing unfavourable results with the final product being wetter than optimum for the farmers. The authors counselled against using this approach to increase litter temperatures. Indeed, the batch of chickens that was placed on the litter from the present experiment suffered from ammonia toxicity quite severely giving support to this. Barker *et al.* (2011) found no increase in temperature with water addition at 4.073 L/m^3 which increased litter moisture from 21.4 to 23.5%.

5.4.3 Temperature distribution within the heaps

This experiment once again demonstrated the great spatial and temporal diversity of temperatures within the heaped litter as observed by Walkden-Brown *et al.* (2010a). The profiles shown in Figure 5.5 revealed that generally speaking, the deeper in the heap, the slower the increase in temperature, but the higher the maximum temperature achieved and the slower the decline. Temperatures were still increasing at 50cm depth at the end of the experiment. For this reason the hours at 55˚C or above was greatest at 20 cm depth, although temperatures at that depth had commenced a slow decline overall by the end of the experiment. For much of the experiment, the temperature differential between the surface and hottest layer was 20˚C. Temperatures 5cm deeper than the surface tended to be closer to those at 20cm than those at the surface, and those at 10cm below were much closer to 20cm than the surface, indicating that the decline in temperature with increasing depth from the surface is not linear.

5.4.4 Maximum temperature, time to maximum and time at 55˚C or above

Maximum temperatures at the different depths ranged from 43.6˚C at the surface to 58˚C at 20cm, and peak temperatures were achieved at 45–56 hrs at 0–10cm, 88 hr at 20cm and 150 hr at 50 cm (probably an underestimate as temperatures were still increasing for many dataloggers at this depth. Time taken to achieve high temperatures is important as there is pressure on turnaround times and long pasteurising treatments of 10 days or so are increasingly impractical. The slow increase in temperature in the bulk of the heap (around 50cm deep) is therefore potentially undesirable.

Mean temperatures ranged from 33.3˚C at the surface to 53.1˚C at 20cm. In the USA working on 4 different farms pasteurising litter between broiler batches with different levels of litter reuse and measuring depth at 6 and 12 inches (15.2 and 30.5 cm respectively) Lavergne *et al.* (2006) reported maximum temperatures in the range 50–58˚C and means of 38–51 ˚C. Temperatures at equivalent depths in the present experiment, also using reused litter, were at the high end of these ranges. The same authors reported declining temperatures with increased level of reuse, and as our litter was only 2nd reuse, this would be consistent with their findings.

Time spent at 55˚C or above and the proportion of the heap achieving this are important considerations given the USA EPA (Class B) requirements of 3 days (72 hrs) at 55˚C in static piles to achieve a “process to further reduce pathogens” (PFRP). Time spent at 55˚C or above varied widely with depth as might be expected, ranging from 4.7 hr at the surface to 100 hr at 20cm and 69 hr at 50 cm (Figure 5.6). Multiplying the proportion of time spent above 55˚C during the experiment, by the proportion of the heap represented by the

temperature bands as shown in Table 5-3 indicated that overall temperatures exceeded 55˚C in approximately 43% of the heap over the duration of the experiment. Clearly the corollary of this is that much of the heap (57%) does not achieve this temperature at all. However, it should be noted that while the 55˚C is an important target temperature, particularly for bacterial inactivation, temperature-time relationships to inactivate viruses are much less well understood and are unlikely to be uniform.

5.4.5 Summary of major findings

1. Neither covering heaps nor addition of moisture had large effects on temperature within the heap. Covering did however significantly increase the speed at which maximum temperatures were achieved.
2. The small increases in temperature that these treatments induced were not additive with covers being most effective on unwatered heaps.
3. Chickens reared on litter pasteurised during this experiment suffered excessive ammonia.
4. Overall an estimated 43% of the heap was at temperatures of 55˚C or above during the experiment.
5. On the basis of these results, neither addition of moisture nor use of covers could be recommended for good used litter with a starting moisture content of around 22%.

6 Strand 2. Experiments 2.2.1-2.2.3. Litter treatment experiments at UNE. “Effects of moisture addition, covering, aeration and litter amendments on temperatures in heaped litter”

6.1 Introduction

Following the first farm experiment a number of smaller experiments were carried out at UNE to test various factors that might influence litter temperature. This chapter describes 3 experiments conducted at Kirby Research Station at UNE testing the effects of different factors on temperature profiles in heaped litter. The experiments were:

1. Expt 2.2.1 Effects of covering and adding 10% additional moisture (LT12-C-LSOP2a)
2. Expt 2.2.2 Effect aeration on heap temperature (LT12-C-LSOP2b)
3. Expt 2.2.3 Effect of chemical amendments on litter temperature during heaping (Broccoli box expt, LT12-C-LSOP3)

The experiments were carried out by Dr Fakhrul Islam with assistance from Mr Aaron Van Den Heuvel using pine shavings litter generated by broilers in Kirby Shed 1 Poultry shed. Due to limitations on available volume of litter, the experiments were small and indeed, experiment 2.2.3 tested the concept of measuring temperature dynamics in a polystyrene “broccoli box”. Experiment 2.2.1 took place in early June 2012 while the other two experiments ran concurrently in late July 2012.

6.2 Materials and Methods

6.2.1 Expt 2.2.1 Effects of covering and adding 10% additional moisture (LT12-C-LSOP1)

This experiment tested the effects of moisture addition and covering on litter temperature profiles over seven days in heaped litter following a broiler growout experiment in the UNE. In contrast to earlier projects, temperatures at depths between 0 and 25 cm were included to define temperature profiles in the sub-surface zone. The general hypotheses under test were:

1. Provision of 10% additional moisture would significantly increase heap temperatures;

2. Covering of heaps with a tarpaulin would significantly increase heap temperatures, particularly close to the surface of the heap; and
3. There is a roughly linear temperature gradient effect between the surface and 20cm depth.

The experiment used spent pine shavings litter from a previous broiler growout experiment at the UNE Kirby Poultry Research Shed 1 in a 2 x 2 factorial design with the following factors:

- Two levels of Moisture (0 and 10% added water. M0 and M10 respectively)
- Two levels of Cover with a standard blue polyethylene tarpaulin (Cover or No Cover)
- Five levels of Depth within the heap (0, 5, 10, 20 and 50 cm)

Due to the limited amount of litter available there was no replication at the heap level. The spent litter was accumulated into four equal size heaps of approximately 1.5 m wide x 1.5 m long x 1.3 m high (~1.15 m^3). In two of the heaps (selected randomly) water was added to increase the moisture content by approximately 10% while the heap was being formed and thoroughly mixed. The litter was heaped on a concrete floor in the shed in early winter in a cold climate (Armidale). Two heaps, one with added water and one without, were covered with a standard blue polyethylene tarpaulin (purchased from Bunnings Warehouse).

Ten iButtons were inserted in each heap at depths of 0, 5, 10, 20 and 50cm, at two sites (replicate sites for each depth). iButtons were collected a little after 7 days (up to 190 hrs) and the temperature data were retrieved. At the time of iButton insertion and collection, representative litter samples were collected from each heap to measure moisture content. All iButtons were set to record temperature at 60-minute intervals. Two iButtons were placed in the shed to measure ambient temperature.

The experiment was conducted from 29 May to 05 June 2012.

6.2.1.1 Data analysis

Litter moisture data were analysed using a mixed restricted maximum likelihood model (REML) fitting Heap (1-4) as a random effect and Moisture and Cover as fixed effects with their interaction also fitted.

Temperature data were tabulated and the following derived variables added

- Day post heaping. The 24hr period from the iButton insertion
- Period time (hr) at 55˚C or above calculated for each iButton
- Maximum temperature for each iButton

- Time post heaping (hr) at which maximum temperature was achieved, for each iButton (Time to maximum temperature)

Temperature data were analysed in several ways:

- Scatter plots of hourly data points from each iButton for different treatments or depths were prepared and a spline smoothed curve produced to illustrate the profile over the measurement period.
- The period of time at 55˚C or over, maximum temperature and time to maximum temperature were analysed by ANOVA testing the effects of Moisture treatment, Cover and Depth and their interactions
- Mean daily temperatures were calculated for individual iButtons using hourly data and analysed using a mixed (REML) fitting iButton as a random effect and Moisture, Cover, Depth and Day as fixed effects and the interactions between them fitted.

Significance of differences between effects were determined by analysis of variance (ANOVA) and differences between levels within an effect were determined using Tukey's HSD test. Data were analysed with JMP11 (SAS Institute Cary, NC, USA). Least squares means and standard errors arising from the analyses above are reported.

6.2.2 Expt 2.2.2 Effect aeration on heap temperature (LT12-C-LSOP2)

This was a small experiment involving only two litter heaps of approx.1.25m diameter x 0.80m high (~0.5 m^2) made from a mixture of second reused and first reuse pine shavings litters. One heap (Aerated) was formed over a coiled garden weeper hose through which air was pumped 15 minutes every six hours at a rate of 40L per minute by an electric air pump connected to a timer. The other heap was not aerated.

A single iButton was placed on the surface and at 5, 10, 20 and 50cm depths to record temperatures over the 7 days following heaping. One of the iButtons at 20cm depth malfunctioned and data for that depth is excluded. As there was no replication in the experiment, data was not statistically analysed and the descriptive data are presented.

6.2.3 Expt 2.2.3 Effect of chemical amendments on litter temperature during heaping (Broccoli box expt, LT12-C-LSOP3)

This experiment used spent pine shavings litter from a previous experiment in which groups of broiler chickens were reared on litter various litter amendments at various concentrations.

The previous experiment and results have been reported (Walkden-Brown *et al.* 2013). The objectives of the study were:

1. To investigate the effect of available chemical amendments in litter pasteurisation at two moisture levels; and
2. To study the feasibility of assessing litter pasteurisation at a laboratory scale by pasteurising litter in polystyrene vegetable boxes (broccoli boxes).

The experimental design had a 6x2 factorial design, with five different litter amendments and a negative control with two levels of moisture. The experimental litter was collected from a previous broiler experiment using chemical amendments in second batch litter before reusing (Walkden-Brown *et al.* 2013). The list of amendments and their concentration is presented in Table **6**-**1**. Each amendment had been placed on the surface of the litter in two replicate pens in which broiler chickens were reared to 42 days. The litter collected from each pen was placed into two 34-liter polystyrene broccoli boxes with approximately 28 L of litter in each box. The approximate weight of litter in each filled broccoli box was 20 kg. Extra water (2 L) was added to one of the broccoli boxes in each treatment pair to provide extra ~10% moisture (M10) while the other was unwatered (M0). A single iButton was placed in the middle of the broccoli box at about half depth of the litter, ie in the centre of the material and temperatures recorded for 7 days at hourly intervals. All broccoli boxes were kept open for the whole experimental period. Litter samples were collected to measure moisture content and pH at the end of the experiment (day 7). The experiment ran from 24/7/2012 (day 0) to 31/7/2012 (day 7).

Table 6-1. Expt 2.2.3. Chemical amendments used in the litter and the application rate. Application of the amendments was prior to chick placement, 42 days before the litter pasteurising experiment.

Amendment	**Rate (kg/m^2)**	**Rate (%, w/w)**	**Treatment name**
Alum	0.42	3.2	Alum
Bentonite	1.56	13	Bentonite
Sodium Bisulfate	0.42	3.2	Na-bisuph
NaturClean®	0.10	0.9	NaturClean 0.9
Zeolite	1.56	13	Zeolite
No amendment	0	0	Nil

Ambient ammonia inside each box was measured using MultiRAE ammonia meters (RAE Systems Inc. San Jose, CA, USA) at days 3 and 7. The air above the surface of litter in the broccoli box was cleared using a fan and the box was covered with a close fitting lid with two small holes in it. The sampling nozzle of two ammonia meters was inserted inside the broccoli box through the holes and the ambient ammonia level was recorded at 15, 30, 45

and 60 seconds following the closure of the box. The mean value recorded by the two machines was then analysed.

6.2.3.1 Data analysis

Litter moisture and pH data were analysed by ANVOA fitting the effects moisture treatment (M0, M10), amendment and their interaction. Mean and maximum temperatures for each ibutton were calculated and analysed using ANOVA testing the same effects as above, but with Day 7 moisture content fitted as a covariate.

Temperature profiles for each ibutton were also analysed in a repeated measures ANOVA (mixed model REML), testing the effects of Amendment, added Moisture, Day post heaping and their 2-way interactions with iButton as a random effect and final litter moisture content as a covariate. Temperature data were meaned by day prior to analysis to remove pseudoreplication.

Ammonia concentrations at 15, 30, 45 and 60 seconds following the closure of the box were analysed in a repeated measures ANOVA (mixed model REML), testing the effects of Amendment, added Moisture, Day of sampling, final litter moisture and their 2-way interactions with Broccoli box as a random effect.

6.3 Results

6.3.1 Expt 2.2.1 Effects of covering and adding 10% additional moisture

6.3.1.1 Ambient temperature

Mean daily minimum temperature recorded in the shed was 10.6 ˚C (range 7–12 ˚C), the maximum was 16.3˚C (range 14.5–19.5 ˚C) and the overall mean was 12.6 ˚C (range 10.3–14.0 ˚C).

6.3.1.2 Litter moisture content

The litter moisture content of the heaps immediately on heaping and 7 days later is shown in

Table 6-2 and summary of the analysis of variance table in Table 6-3. The initial moisture content was low (15.0 ± 0.6%) and addition of water increased it by 14.2 percentage points to 29.2 ± 0.62% ($P<0.04$). Water loss during heaping was not statistically significant overall with the uncovered heaps reducing in moisture content by 1.99 percentage units in moisture over the 7-day period of heaping while the moisture content of the covered heaps actually increased by 1.69 percentage units in moisture over the same period, presumably through translocation of moisture from deep in the heap to the sampling depths.

Table 6-2. Expt 2.2.1. Litter moisture content (%) by treatment and day of heaping

Moisture treat	Cover treat	Day 0 moisture %	Day 7 moisture %	Change (% units)
M0	Cover	17.15	17.90	0.75
M0	No cover	14.89	10.23	-4.66
M10	Cover	29.28	31.90	2.62
M10	No cover	27.56	28.25	0.69

Table 6-3. Expt 2.2.1. Summary of ANOVA table for analysis of litter moisture content (%)

Source	DF	F Ratio	Prob > F
Day	1	0.0297	0.8913
Moisture	1	266.5901	0.0389*
Cover	1	19.3297	0.1424
Day*Moisture	1	4.3044	0.2859
Day*Cover	1	4.4487	0.2818
Moisture*Cover	1	1.7170	0.4150

6.3.1.3 Temperature profiles

Profiles of mean litter temperature over time by treatment and by depth in the heap are shown in Figure 6.1.

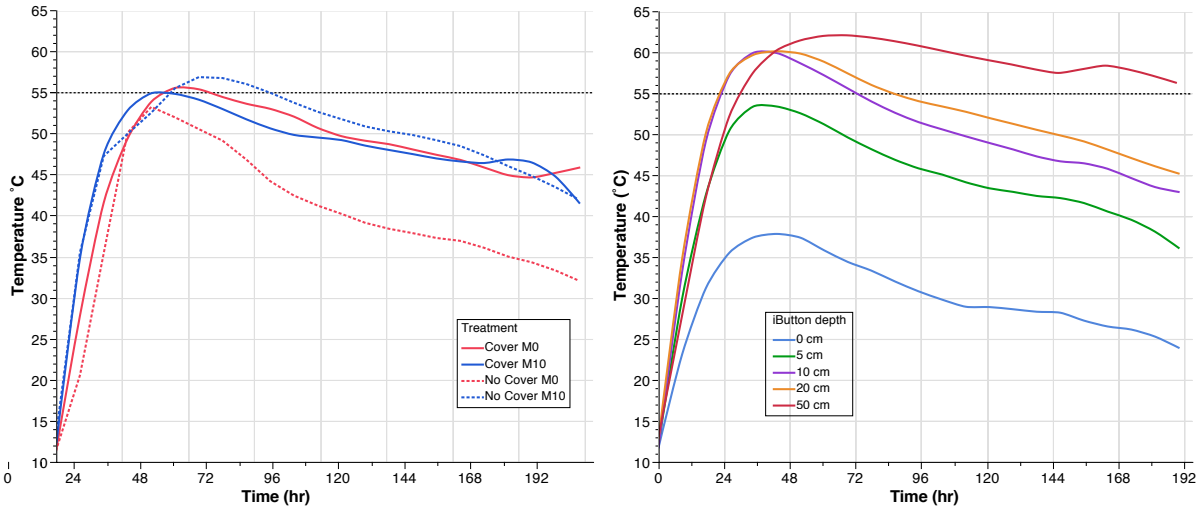

Figure 6.1. Expt 2.2.1. Smoothed mean temperature profiles by litter treatment (Left) and by depth in the heap (Right)

Litter temperatures for all treatments peaked between 24 and 48 hr after heaping at between 53 and 57˚C (Figure 6.1). However these means include heap surface temperatures, which were up to 30˚C cooler than core temperatures. Three of the treatments exhibited very similar temperature profiles overall, but the No Cover/No additional moisture treatment was substantially cooler, peaking earlier at a lower temperature and then exhibiting a more rapid decline in temperature to be ~10˚C cooler at the end of the heaping period (Figure 6.1).

Depth in the heap had a profound effect on temperature. The surface remained comparatively cool but as little as 5 cm into the heap resulted in peak temperatures above 50˚C. In general, the deeper the iButton, the higher the peak temperature and the slower the decay in temperature after the peak.

6.3.1.4 Hours above 55˚C

The summary of the analysis of variance table for hours spent above 55˚C is shown in Table 6-4.

Table 6-4. Expt 2.2.1. Summary of ANOVA table for analysis time spent above 55 ˚C

Source	DF	Sum of Squares	F Ratio	Prob > F
Depth	4	56232.981	18.5933	<.0001*
Cover	1	13300.021	17.5904	0.0007*
Depth*Cover	4	6500.581	2.1494	0.1217
Moisture treatment	1	4700.521	6.2168	0.0240*
Depth*Moisture treatment	4	12336.714	4.0791	0.0182*
Cover*Moisture treatment	1	3622.688	4.7913	0.0438*
Depth*Cover*Moisture treatment	4	3779.857	1.2498	0.3299

The overall effects of depth, cover and moisture treatment were highly significant with significant 2-way interaction observed between all of the main effects (Figure 6.2).

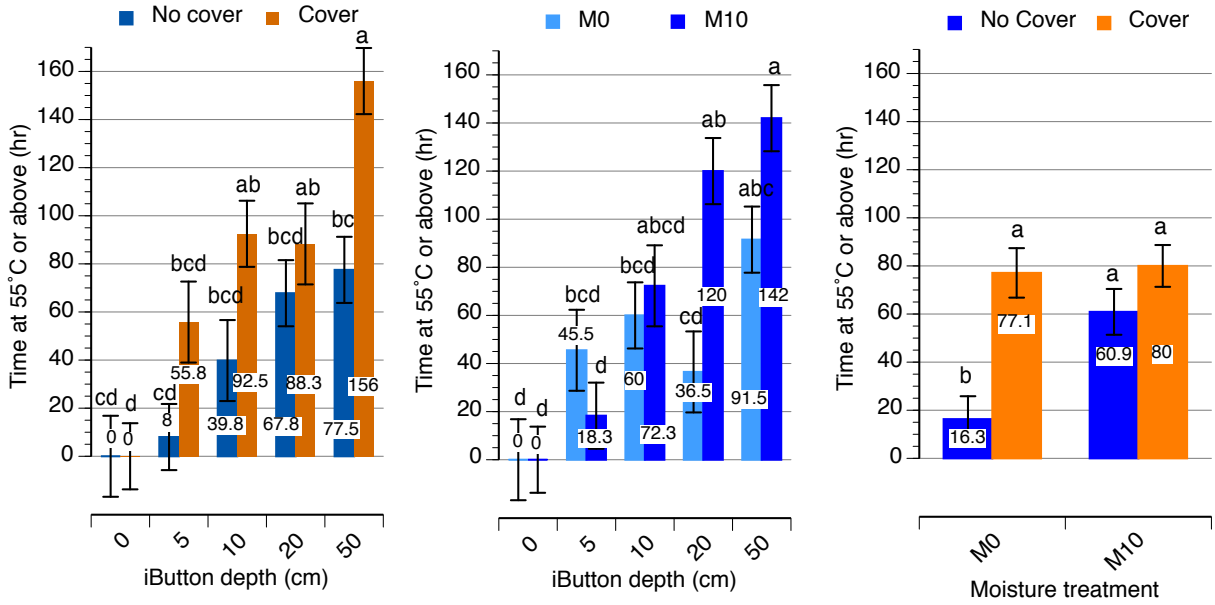

Figure 6.2. Expt 2.2.1. Plots of time at 55˚C or higher (LS means ± SE) illustrating interaction between the effects of Cover and Depth (Left) (P=0.12), Moisture and Depth (Centre) (P=0.018) and Cover and Moisture (P=0.04) (Right). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05). Note for Left panel means separation by Tukey's HSD does not require a significant overall interaction effect.

Covering doubled time above 55˚C overall (No Cover 38.6 ± 6.7 hr, Cover 78 ± 6.7 hr, P<0.001). The effect of covering was greatest at the greatest depth, and in the low moisture treatment with no significant effect in the high moisture treatment (Figure 6.2). Addition of

moisture increased time above 55˚C overall (M0, 46.7 ± 7.0 hr; M10, 78 ± 6.5 hr; P=0.02) with the effect only evident in uncovered treatments and at the deeper parts of the heap.

The proportion of time spent above 55˚C at different depths ranged from 0% at the surface to 70% at 50cm (Table 6-5) and the estimated 43% of the heap experienced temperatures over 55˚C during the experimental period.

Table 6-5. Expt 2.2.1. Table estimating the proportion of the heap over the experimental period that experienced temperatures of 55˚C or above. Based on a mean heap size of 1.15 m^3 and that values measured at a given depth represent the stratum below them. Data in column 6 is the product of the data in columns 2 and 5.

Depth strata	% of heap volume	Hrs>55˚C	Total hrs (mean)	% hrs >55˚C	% heap >55˚C
0-5 cm	10.1%	0.0	183	0%	0.0%
5-10cm	9.4%	31.8	170	19%	1.8%
10-20cm	16.9%	66.1	180	37%	6.2%
20-50cm	36.2%	78.0	171	46%	16.5%
>50 cm	27.4%	117.0	168	70%	19.0%
Total	100.0%				43.5%

6.3.1.5 Maximum temperature

A summary of the analysis of variance of maximum temperature is provided in Table 6-11.

Table 6-6. Expt 2.2.1. ANOVA table for analysis maximum temperature (˚C)

Source	DF	Sum of Squares	F Ratio	Prob > F
Depth	4	2692.5071	62.7076	<.0001*
Cover	1	18.7500	1.7467	0.2049
Depth*Cover	4	140.8071	3.2794	0.0383*
Moisture treatment	1	11.0208	1.0267	0.3260
Depth*Moisture treatment	4	50.4310	1.1745	0.3590
Cover*Moisture treatment	1	133.3333	12.4212	0.0028*
Depth*Cover*Moisture treatment	4	29.9786	0.6982	0.6043

The overall effect of depth was significant as was the 2-way interaction between depth and cover and the cover and moisture (Figure 6.3). Mean maximum temperatures ranged from 39.5˚C at the surface to 64.3˚C at 50cm depth. Covers reduced the maximum temperature at the surface but increased it at other depths (Figure 6.3 Centre). Covers increased maximum temperatures by 5.5˚C in heaps with no added moisture but not significantly in those with covers (2.5 ˚C, Figure 6.3 Right).

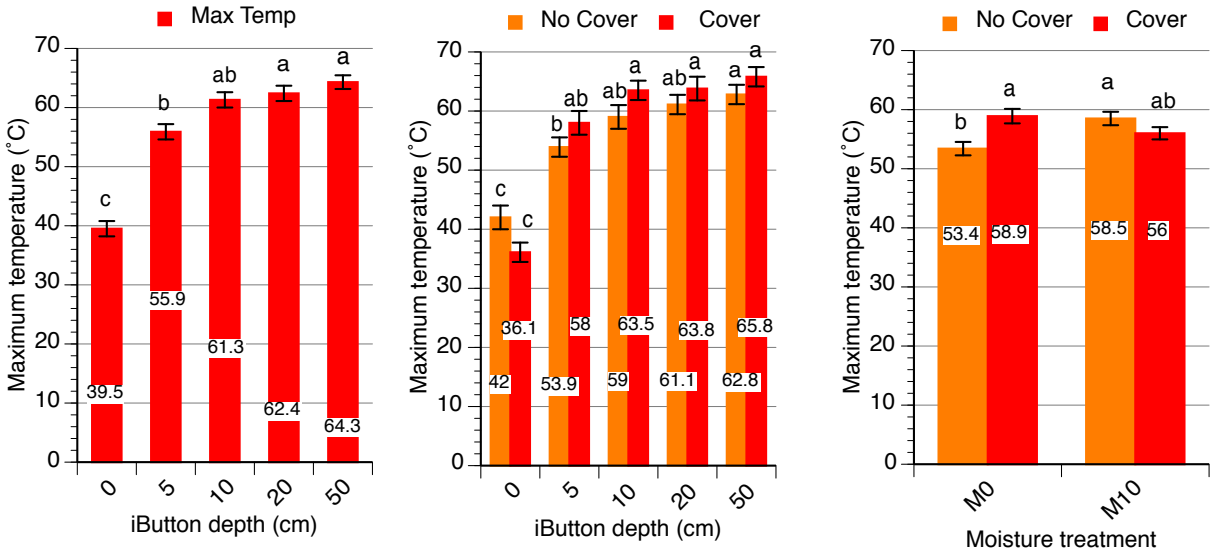

Figure 6.3. Expt 2.2.1. Plots of maximum temperature (LS means ± SE) illustrating the overall effect of Depth (Left) (P<0001), and interaction between Depth and Cover (Centre) (P=0.04) and Cover and Moisture (P=0.003) (Right). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

6.3.1.6 Time to reach maximum temperature

A summary of the analysis of variance of time to reach maximum temperature is provided in Table 6-7. Only the effects of depth and cover x moisture treatment were significant.

Table 6-7. Expt 2.2.1. ANOVA table for analysis of time to reach maximum temperature (hr)

Source	DF	Sum of Squares	F Ratio	Prob > F
Depth	4	7916.8952	7.8893	0.0010*
Cover	1	645.3333	2.5723	0.1283
Depth*Cover	4	1236.7810	1.2325	0.3364
Moisture treatment	1	234.0833	0.9331	0.3484
Depth*Moisture treatment	4	1120.4952	1.1166	0.3831
Cover*Moisture treatment	1	2914.0833	11.6157	0.0036*
Depth*Cover*Moisture treatment	4	875.6952	0.8726	0.5017

The overall effects of Moisture level and Cover did not affect time taken to reach peak temperature although there was a trend towards longer time with the Covered treatment. On the other hand depth had a profound effect with the 50cm layer taking approximately twice as long as the other depths to reach peak temperature (79 hr vs 37-47 hr, P=0.001, Figure 6.4 Left). The significant interaction between the effects of moisture treatment and cover reflected the fact that covers significantly reduced the time taken to reach peak temperature in the unwatered heap (M0) whereas this effect was not significant in the M10 treatment Figure 6.4 Right).

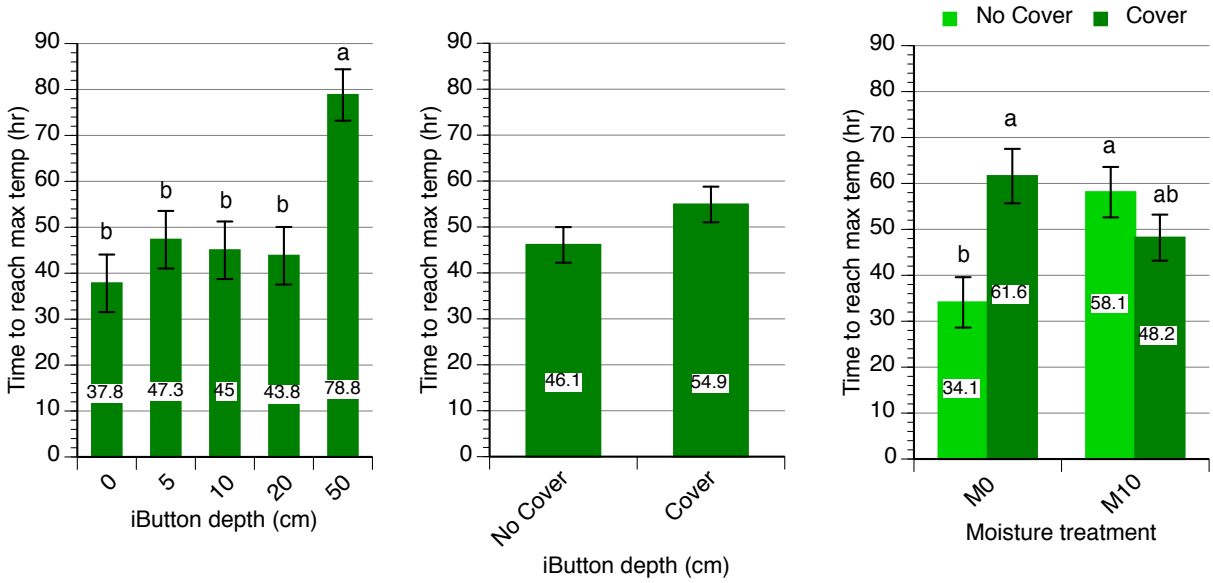

Figure 6.4. Expt 2.2.1. Plots of time taken to reach maximum temperature (hr, LS means ± SE) illustrating the overall effect of Depth (Left) (P=001), Cover (Centre) (P=0.13) and Cover and Moisture (P=0.004) (Right). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

6.3.1.7 Overall temperature analysis (repeated measures)

The summary of the analysis of variance table for litter temperatures is shown in Table 6-8.

Table 6-8. Expt 2.2.1. Summary of ANOVA table for analysis of litter temperature (˚C)

Source	DF	F Ratio	Prob > F
Depth	4	48.5567	<.0001*
Cover	1	10.1099	0.0055*
Moisture treatment	1	7.3005	0.0152*
Day	7	152.4571	<.0001*
Depth*Cover	4	4.2417	0.0148*
Depth*Moisture treatment	4	0.3326	0.8522
Depth*Day	28	6.1654	<.0001*
Cover*Moisture treatment	1	17.8689	0.0006*
Cover*Day	7	1.3997	0.2115
Moisture treatment*Day	7	3.6475	0.0013*
Depth*Cover*Moisture treatment	4	0.3747	0.8234
Depth*Cover*Day	28	1.2913	0.1729
Depth*Moisture treatment*Day	28	0.8121	0.7330
Cover*Moisture treatment*Day	7	3.4921	0.0019*

All main effects, and many 2-way and 3-way interactions were highly significant indicating the complexity of the effects. Addition of moisture increased temperatures overall by 4.2˚C (8.1%) Figure 6.5 Left) while covering heaps also increased temperatures by 4.2˚C.

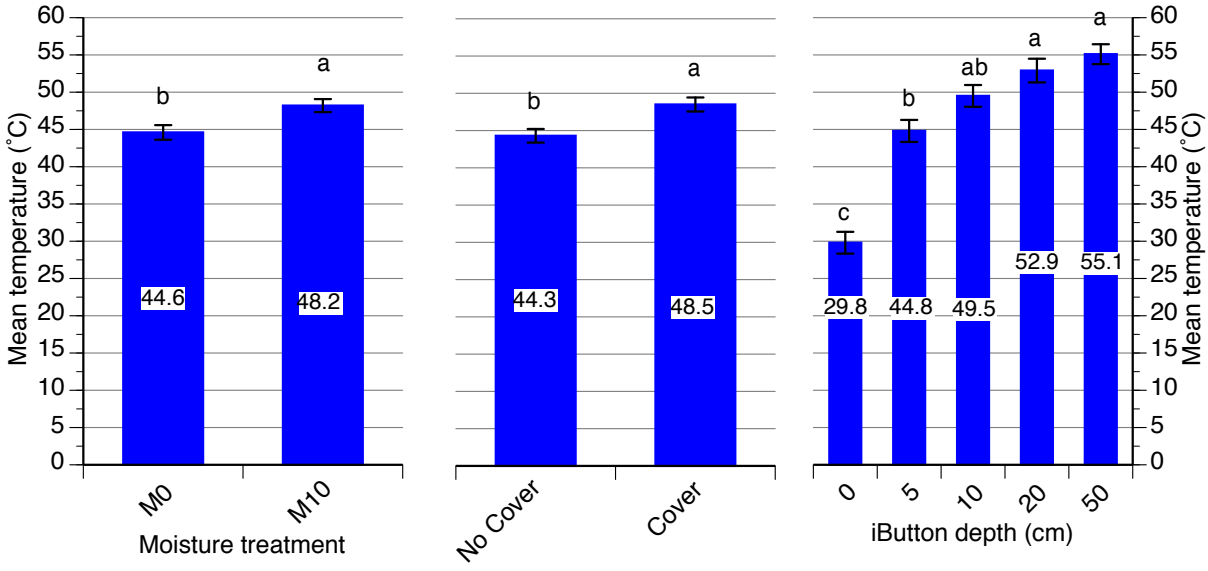

Figure 6.5. Expt 2.2.1. Overall effect of moisture treatment (Left) Cover (centre) and Depth (Right) on litter temperatures in heaps of litter over 7-8 days. Data are LS means ± SEM. Means not sharing a common letter differ significantly (P<0.05).

However the main effects were heavily qualified by interaction with other factors. The most important of these is the interaction between cover and moisture treatment. Covers increased mean temperatures by 9.7˚C (24%) in unwatered heaps but did not significantly increase temperatures in the M10 treatment, even decreasing them slightly numerically (Figure 6.6).

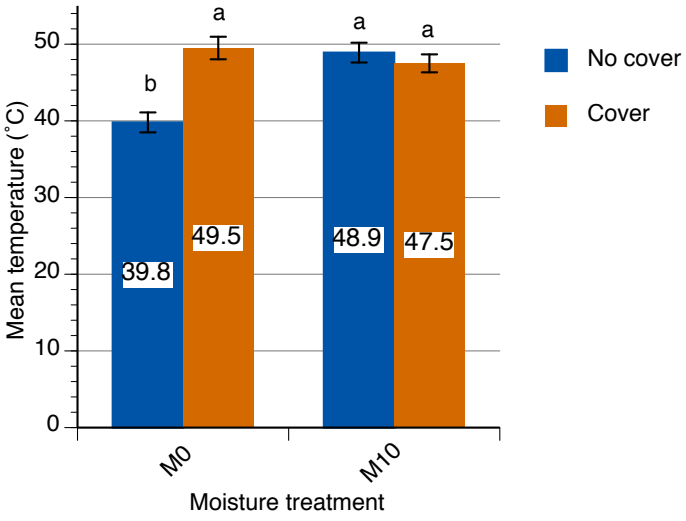

Figure 6.6. Expt 2.2.1. Plot illustrating 2-way interaction between the effects of Cover and Moisture (P<0.001) on litter temperatures in heaps of litter over 7-8 days. Data are LS means ± SEM. Means not sharing a common letter differ significantly (P<0.05).

Covers led to cooler temperatures at the surface of the heap, but warmer temperatures at other depths (Figure 6.7, Left) probably due to “sweating”. The effect of depth was affected

by day with depths 0-20cm associated with earlier peaks at lower temperatures than 50cm, and with a more rapid decline in temperatures thereafter (Figure 6.7, Centre). The effect of moisture treatments varied with day with a large positive effect of adding water on day 1 (~8˚C), minimal effect on day 2 and more modest effects (2-4˚C) thereafter (Figure 6.7, Right)

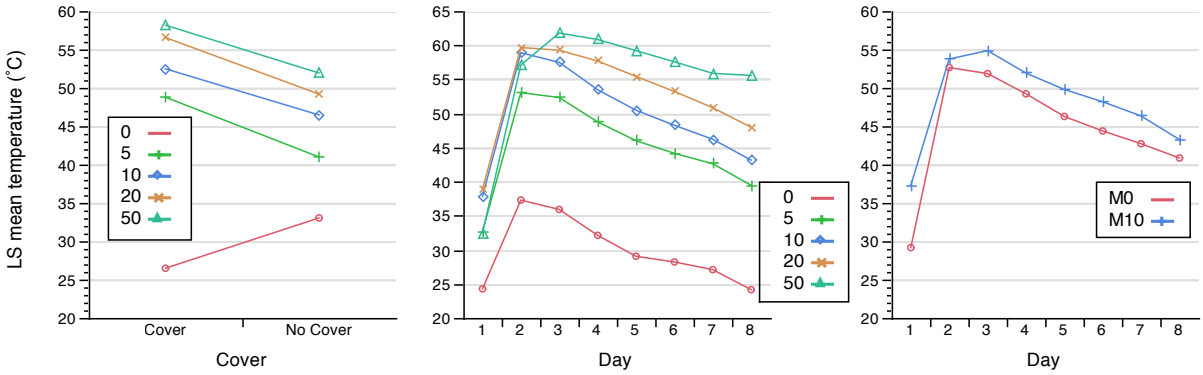

Figure 6.7. Expt 2.2.1. Plots illustrating 2-way interaction between the effects of Depth and Cover (Left) (P=0.015), Depth and Day (Centre) (P<0.0001) and Moisture treatment and Day (P=0.001) on litter temperatures in heaps of litter over 7-8 days. Data are LS means.

The latter effect was heavily qualified by the 3-way interaction shown in Figure 6.8 revealing that after day 2, covering led to cooling of the heap in watered heaps (M10) whereas in uncovered heaps a substantial and persistent heating effect was evident on all but day 2.

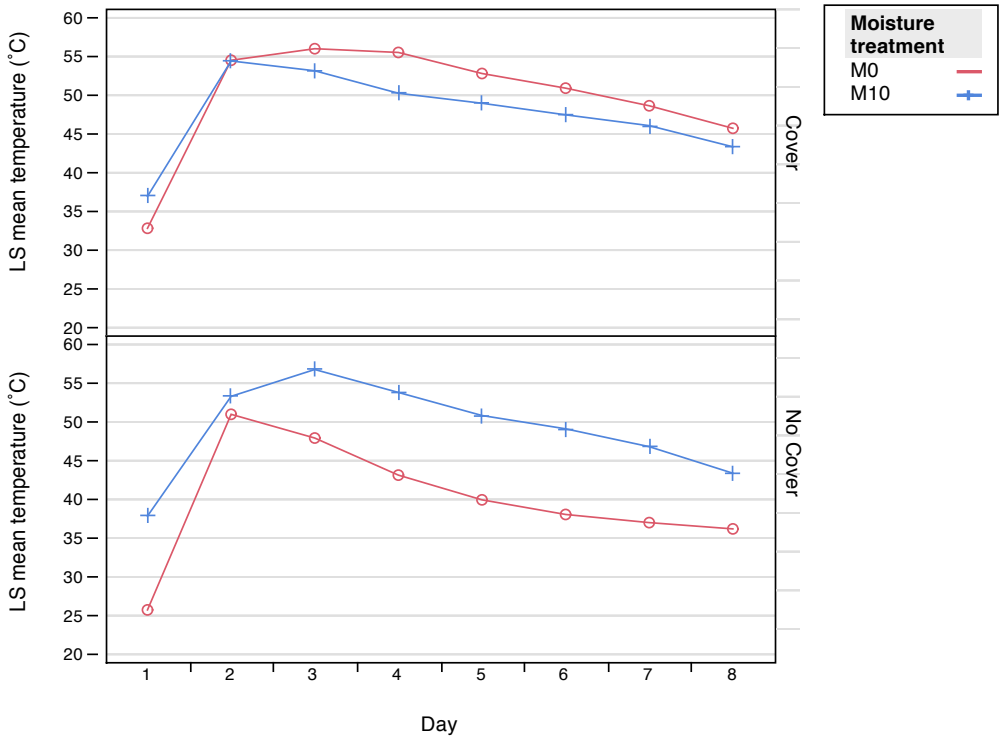

Figure 6.8. Expt 2.2.1. Plot illustrating 3-way interaction between the effects Cover, Moisture and Day (Right) (P=0.002) on litter temperatures in heaps of litter over 7-8 days. Data are LS means.

6.3.2 Expt 2.2.2 Effect aeration on heap temperature

The temperature profiles at different depths in aerated and non- aerated heaps are shown in Figure 6.9 and means of depths 5-50 cm in Figure 6.10. Aeration consistently elevated temperatures at 0 and 50 cm, variably at 5cm and not at all (consistently cooler) at 10cm, making overall conclusions difficult to draw. Overall effects were minor.

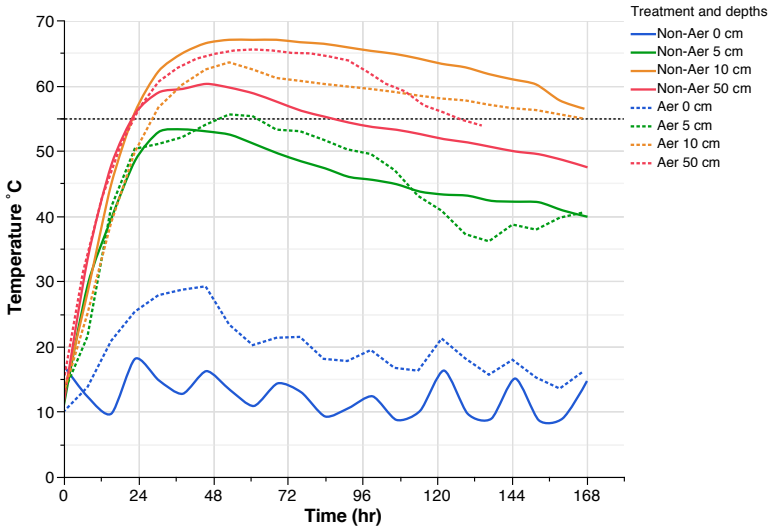

Figure 6.9. Expt 2.2.2. Temperature profiles over 7 days at 4 depths in litter heaps that were aerated (dotted lines) or non-aerated (solid lines).

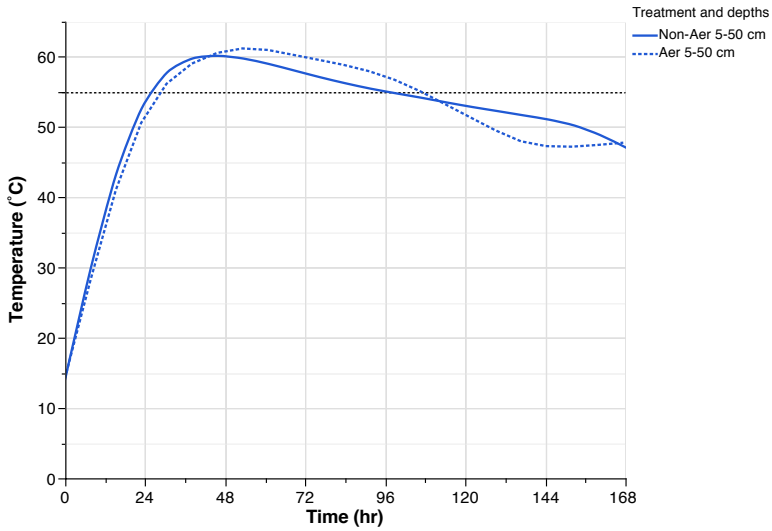

Figure 6.10. Expt 2.2.2. Mean temperature profiles for depths 5–50 cm over 7 days in litter heaps that were aerated (dotted lines) or non-aerated (solid lines).

Aeration tended to increase maximum temperatures and the time taken to achieve them at all depths except 10 cm (Table 6-9). Effects of aeration on time above 55˚C were minimal except at 50cm depth where a large increase (26%) was observed (Table 6-9).

Table 6-9. Expt 2.2.2. Maximum temperature, time to maximum temperature and hours above 55˚C.

Aeration	Depth	Max temp. (˚C)	Time to max. (hr)	Day	Hrs at ≥55˚C	Total hours recorded
No	0	20.5	26	2	0	168
Yes	0	29.5	46	2	0	168
No	5	53.4	38	2	0	168
Yes	5	56.5	56	3	14	168
No	10	67	74	4	146	168
Yes	10	63.5	57	3	140	168
No	50	60.4	46	2	64	168
Yes	50	65.5	68	3	105	168

6.3.3 Expt 2.2.3 Effect of chemical amendments on litter temperature during heaping (Broccoli box expt)

6.3.3.1 Litter moisture and pH

The moisture content of the litter after 7 days in the broccoli box was not influenced by water addition (P=0.23) but was significantly different between amendment treatments (P<0.001, Figure 6.11, Left). There was no interaction between these effects. Litter pH at day 7 was similarly influenced by amendment (P<0.0001, Figure 6.11, Right) but not water addition (P=0.54) or interaction between the two. As can be seen the litter moisture content was substantial (>50%) in some treatments and the reasons for this are not clear, but cannot be simply ascribed to the amendment used. Addition of water increased mean water content from 44.7% to 48.3% a non-significant increase of 3.6%.

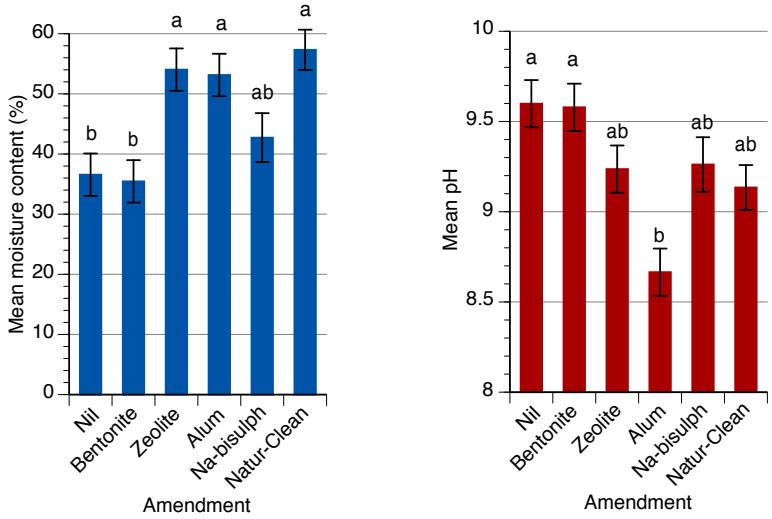

Figure 6.11. Expt 2.2.3. Differences between litter amendment treatments on moisture content (Left) and pH (Right) of the litter after 7 days in the broccoli box (LS means ± SEM). Means not sharing a common letter differ significantly.

6.3.3.2 Litter temperature

Litter temperature profiles over the 7-day duration of the experiment showing the effects of amendment/litter type and moisture addition are shown in Figure 6.12 and Figure 6.13 respectively. The small litter boxes generated significant heat, peaking at between 45 and 60˚C on day 2, but temperatures fell away sharply relative to what is observed in large heaps on farm. Addition of moisture appeared to hinder the early temperature increase, but enhance temperatures slightly after day 3. Closer examination of the effects of actual moisture level in the litter (as determined at day 7) and temperatures achieved revealed significant curvilinear associations between moisture content, maximum temperature achieved and mean temperature achieved

Figure 6.14).

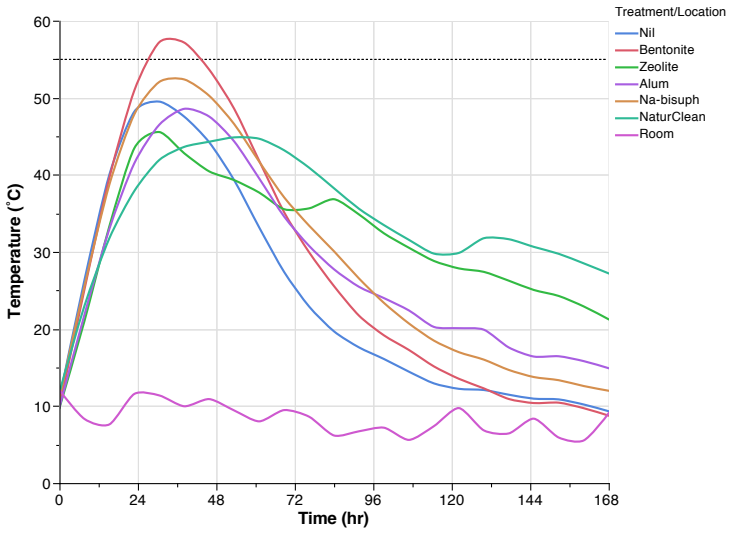

Figure 6.12. Expt 2.2.3. Temperature profiles showing the overall profiles for the different amendments and room temperature

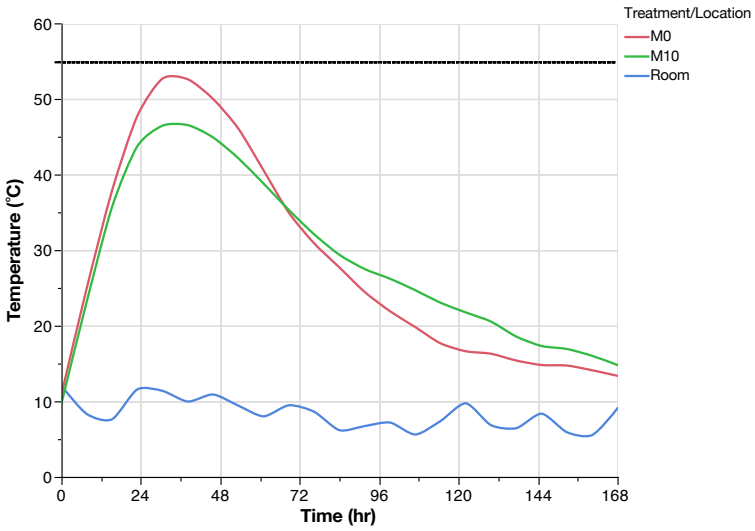

Figure 6.13. Expt 2.2.3. Temperature profiles showing the overall profiles for the moisture addition treatments (none or 2L added water) and room temperature

The 2nd order polynomial associations between day 7 litter moisture content and mean and maximum temperatures recorded are described below.

- Mean Temp (˚C) = 19.264331 + 0.2997603*D7 moisture (%) - 0.0130629*(D7 moisture (%)-43.8831)^2, P<0.0001, R^2 = 0.36
- Maximum Temp (˚C) = 72.353441 - 0.2968683*D7 moisture (%) - 0.0249984*(D7 moisture (%)-43.8831)^2, P<0.0001, R^2=0.69

These indicate that mean temperatures achieved increase with moisture content up to 50%, plateau, then decline above 60%. Maximum temperatures increase with moisture content up to around 35% moisture content plateau, then decline above 45%, falling to very low temperatures, over 20% below peak values at very high moisture contents around 60-70% (

Figure 6.14).

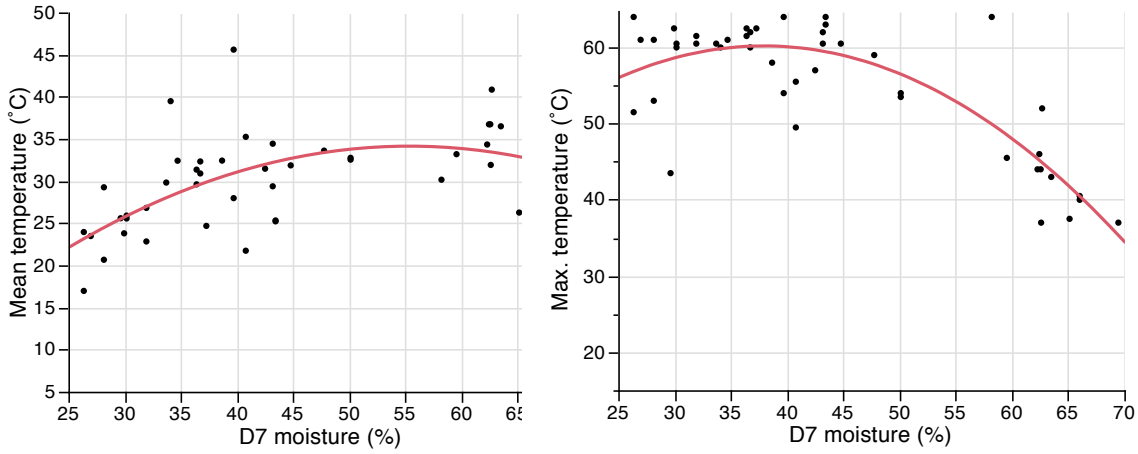

Figure 6.14. Expt 2.2.3. Association between day 7 moisture content and mean (Left) and maximum (Right) temperatures achieved by individual dataloggers in Broccoli boxes (n=45). Lines represent 2nd order polynomial curves. Quadratic associations are highly significant (P<0.001) and equations and R^2 values are presented in the text.

Formal ANOVA of mean and max temperatures testing the effects of moisture addition, amendment and their interaction with day 7 moisture fitted as a covariate revealed no significant effects of treatment of moisture addition or interaction between them, but a highly significant effect of day 7 moisture content (P<0.0023 and 0.0159 respectively) as shown in the association analysis reported above.

Repeated measures analysis of temperature data for each iButton testing the effects of Amendment, added Moisture, Day post heaping, Day 7 Moisture content and their 2-way interactions produced the ANOVA table shown in

Table 6-10.

Table 6-10 Summary of ANOVA table for analysis of litter temperature (˚C) in the broccoli box experiment.

Source	DF	F Ratio	Prob > F
Amendment	5	1.4249	0.2440
Moisture	1	1.6828	0.2046
Day	6	48.5944	<.0001*
Amendment*Moisture	5	1.2429	0.3148
Amendment*Day	30	1.8543	0.0071*
Moisture*Day	6	1.0756	0.3786
D7 moisture (%)	1	7.5031	0.0099*

There was no significant overall effect of amendment (Figure 6.15, Left) and the moisture treatment (Figure 6.15, Centre) but a highly significant effect of the day (Figure 6.15, Right).

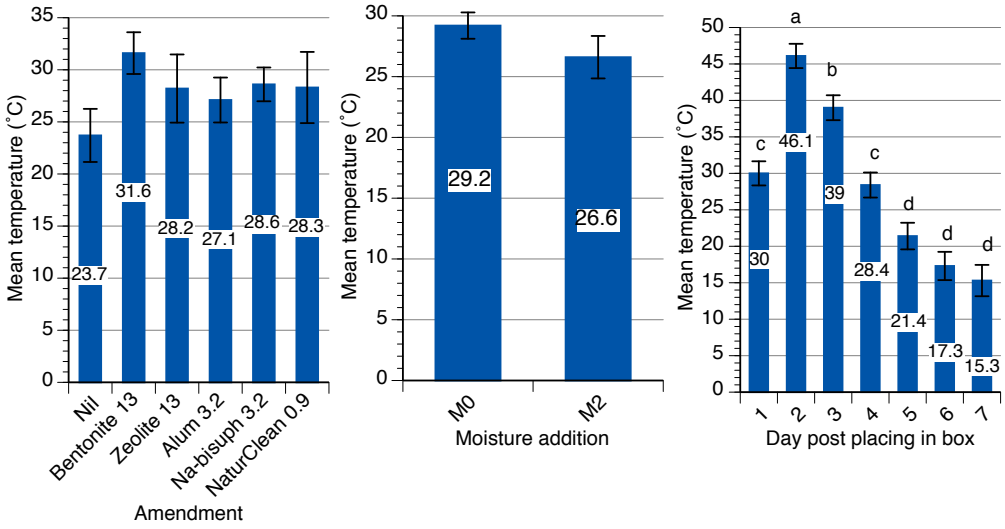

Figure 6.15. Expt 2.2.3. Overall effects of amendment (Left) Moisture addition (Centre) and day (Right) in the broccoli box experiment. Results are LS means ± SEM. Means not sharing a common letter differ significantly.

There was significant interaction between the effects of Amendment and Day associated with variation in temperature profiles over time (

Figure 6.16). The nil amendment treatment showed a low peak and rapid decline in temperature, Bentonite and NaBisulp high peaks and rapid declines, while zeolite and NaturClean® exhibited low peaks but a slow decline in temperature, providing much more sustained elevated temperatures.

.

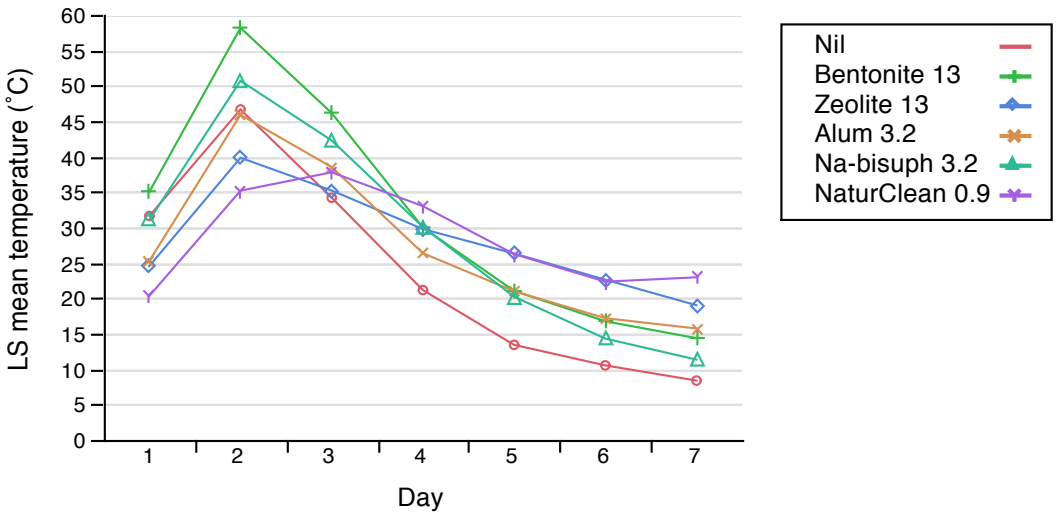

Figure 6.16. Expt 2.2.3. Interaction plot showing significant interaction between the effects of amendment and Day (P<0.001) in the broccoli box experiment. Results are LS means ± SEM.

6.3.3.3 Litter ammonia production

Ammonia production was much higher on day 3 than day 7 and on each of these days concentrations increased with sampling time from 15 seconds to 60 seconds after box closure (Figure 6.17).

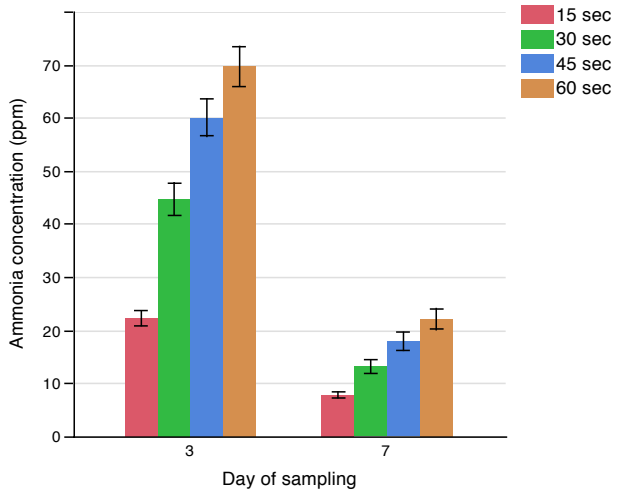

Figure 6.17. Expt 2.2.3. Ammonia concentrations in the broccoli box experiment showing the effects of day of sampling and time after box lid closure. Data are means ± SEM.

Repeated measures analysis of mean ammonia concentrations (average of 15, 30, 45 and 60 second samplings) testing the effects of Amendment, added Moisture, Day of sampling,

day 7 moisture content and their 2-way interactions produced the ANOVA table shown at Table 6-11

Table 6-11. Expt 2.2.3. Summary of ANOVA table for analysis of ammonia concentration (ppm) in the broccoli box experiment.

Source	DF	F Ratio	Prob > F
Amendment	5	0.7801	0.5777
Moisture	1	0.2596	0.6122
Day	1	222.7230	<.0001*
Litter final moisture content	1	0.5234	0.4776
Amendment*Moisture	5	2.6941	0.0290*
Amendment*Day	5	0.3601	0.8734
Amendment*Litter final moisture content	5	1.9151	0.1253
Moisture*Day	1	1.2326	0.2720
Moisture*Litter final moisture content	1	3.2492	0.0763
Day*Litter final moisture content	1	6.7936	0.0119*

There was highly significant effect of day with higher concentrations on sampling day 3 (54.1 ± 3.8 ppm) than day 7 (20.2 ± 3.8 pmm). There was significant interaction between the effects of Amendment and Moisture as shown in Figure 6.18. Addition of moisture reduced ammonia production in the Nil amendment treatment, increased it in the Zeolite treatment and had little effect on the other treatments.

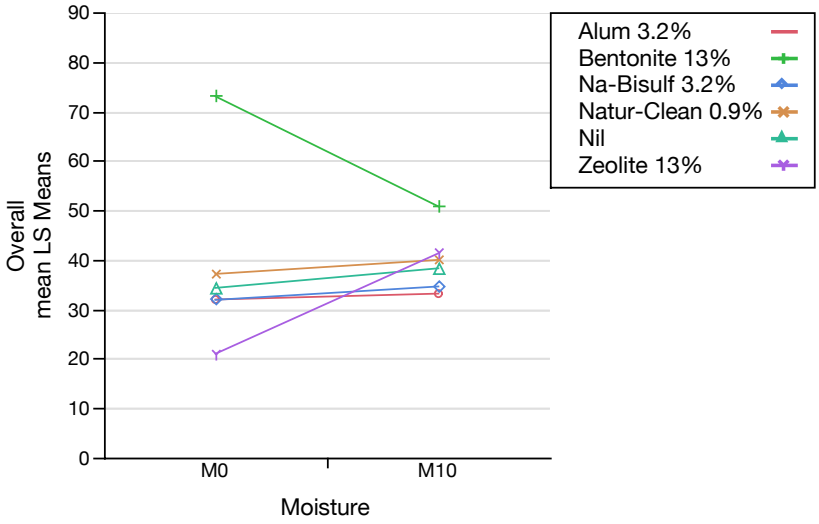

Figure 6.18. Expt 2.2.3. Interaction plot showing significant interaction between the effects of added Moisture level and Amendment (P<0.001) in the broccoli box experiment. Results are LS means ± SEM.

There was significant interaction between the effects of Day of sampling and final litter moisture content as illustrated in Figure 6.19. Litter moisture affected ammonia production at day 3, but not 7 and in fact the effect was quadratic rather than linear. Ammonia production was greatest at moisture contents of 40-50%, falling away on either side of this.

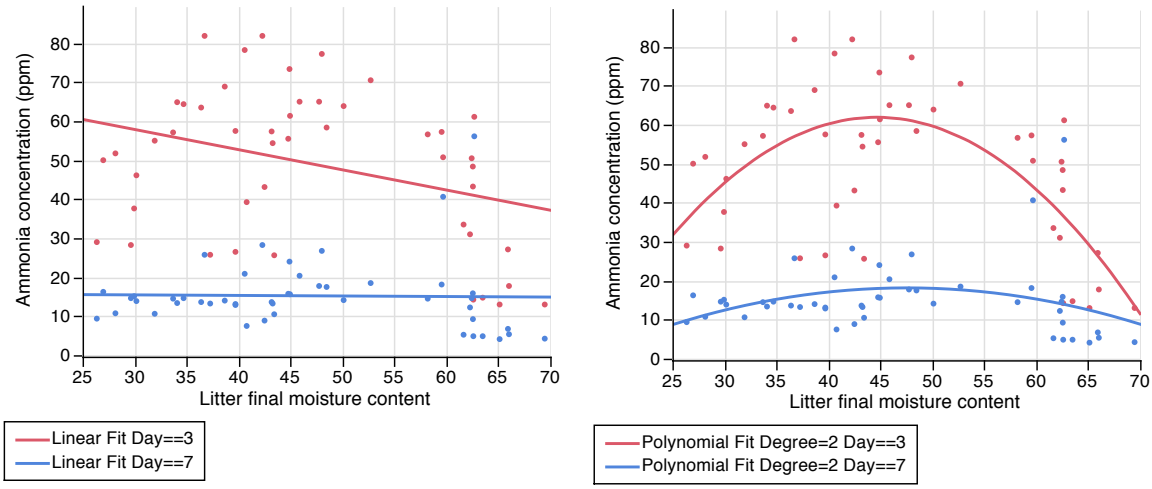

Figure 6.19. Plot showing significant interaction between the effects of Final litter moisture content and Day on ammonia concentration in the broccoli box experiment. The linear fits (Left) illustrate the interaction in the repeated measures ANOVA while the quadratic fits (Right) are much better fits (Day 3 P<0.0001, R^2 0.42). In both cases the association is significant at day 3 but not day 7.

There was a strong linear association between final litter pH (measured on day 7) and measured ammonia production across all treatments at day 7 but not day 7 (Figure 6.20).

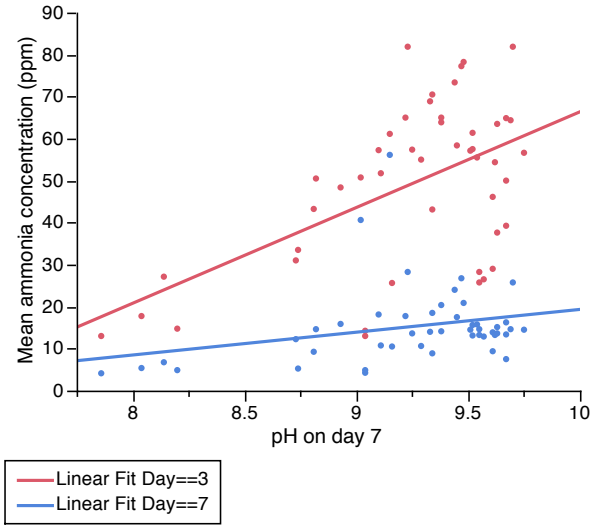

Figure 6.20. Expt 2.2.3. Plot showing the linear association between mean ammonia concentrations at days 3 and 7 and final litter pH (measured at day 7). The fit at day 3 is highly significant (P<0.0001, R^2 0.09) but at day 7 it is not (P=0.07, R^2 0.07).

6.4 Discussion and conclusions

6.4.1 Effect of covering

Covering increased overall heap temperatures in Experiment 2.2.1 but only in litter to which water was not added, ie litter with initial moisture content of 15-17%. Increasing moisture

content to 27–29% increased overall temperatures, but there was no additional benefit of covering in the higher moisture group. In the high moisture group there was considerable "sweating" under the tarpaulin cover and a significant cooling of the surface layer below that of the uncovered heaps. Covers had a heating effect throughout the period in unwatered litter but in watered litter covers had an overall cooling effect after day 3.

The net result was an increase in mean temperature of approximately 10 ˚C due to covers in drier heaps and a slight decrease (non-significant) in wetter ones. Possibly the use of a more "breathable" covering material would enable benefits at higher moisture contents although moisture translocation is likely to be an important aspect of the increased temperatures observed below the surface in covered heaps.

In a USA study using slightly smaller heaps and wetter litter (37–40% moisture), and with temperature measurements made at 30cm into the heap, almost a reverse interaction was observed with tarp covering only being effective with the addition of moisture (Macklin *et al.* 2006). In this trial much lower amounts of water were added to the litter (7.6 L to a 0.9m high pile) and this induced a non-significant increase in moisture content of only 0.64 percentage points. The ambient temperatures were very similar in the two studies, but "sweating" under the covers was not mentioned by Macklin *et al.* (2006).

Covering the heap also significantly increased the period of time with temperatures above 55˚C, but again, only in the uncovered treatment (77 hrs vs 16.3 hrs, Figure 6.2). It also increased the maximum temperature achieved, again only in the uncovered heap (58.9˚C vs 23.4 ˚C, Figure 6.3). On the other hand covering slowed the time of maximum temperature considerably, again only in uncovered heaps (61.6 hr vs 34.1 hr ˚C, Figure 6.4). The latter is largely because temperatures were being driven to a higher maximum (Figure 6.4, Right).

The effect of covers in Experiment 2.2.1 was much greater than that observed in Experiment 2.1 in Sydney in which effects were more subtle. Again however the effects were greatest in the unwatered heaps and results skewed by the negative effect on surface temperatures, particularly at high moisture contents. The moisture contents in the Sydney experiment were higher at the start of the experiment than in 2.2.1 (22.3% vs 16%) and addition of moisture took moisture contents up to a mean of 27.5% (M5) and 34.4% (M10). This contrasts with a moisture content of 28.4% in the M10 treatment in the present experiment. Based on the lack of major response to covering in the Sydney experiment it seems that covering requires moisture contents of around 20% or less to have a beneficial effect.

6.4.2 Effect of additional moisture

The addition of moisture in Experiment 2.2.1, raising fairly dry litter (16% moisture) to moderately damp litter (28.4% moisture) resulted in an increase in mean temperatures of

8.9˚C mainly by inducing a higher peak temperature (5.5˚C higher, Figure 6.3, Right) and much more sustained temperatures over the 7 days (Figure 6.8, Right). As a consequence addition of moisture significantly increased the time spent at 55˚C Figure 6.2, Right) but slowed the time to achieve peak temperature by 24 hr (Figure 6.4, Right). However, when litter was covered, there was no benefit of additional moisture, as discussed above. This suggests that part of the mechanism for the effectiveness of covering is trapping of moisture and prevention of drying out of the outer layers of the heap. However it seems that if moisture levels are too high, and external temperatures are low, the "sweating" effect under the covers has a depressing effect on temperatures in the outer layers.

Macklin *et al.* (2006) reported quite large responses to minor additions of water to heaped litter with a moisture content close to 40%, but measurements were very limited in that experiment and there was no statistical analysis. Lavergne *et al.* (2006) in a trial in dewar bottles found increases in temperatures generated with litter moisture contents up to 38%. However on-farm trials with litter moistures in the range 22-28% gave conflicting results regarding temperature outcomes. Application of water to the surface of windrows delayed peak temperatures and additional moisture produced unfavourable results with the final product being wetter than optimum for the farmers. The authors counselled against using this approach to increase litter temperatures. (Barker *et al.* 2011) found no increase in temperature with water addition at 4.1 L/m^3 which increased litter moisture from 21.4 to 23.5 %.

In experiment 2.1.3 (Broccoli boxes) addition of moisture (3.6 percentage units) to litter averaging 44.7% moisture content did not increase overall mean temperatures, in line with the finding of Lavergne *et al.* (2006) that moisture content above 38% was not associated with increases in temperature. Lavergne *et al.* (2006) speculate that at high moisture levels, porosity and air penetration deep into the heap are adversely affected and that this reduces thermophile bacterial activity.

6.4.3 Temperature distribution within the heaps

These experiments once again demonstrated the great spatial and temporal diversity of temperatures within the heaped litter as observed by Walkden-Brown *et al.* (2010a). In experiment 2.2.1 deep within the heap (50cm) temperatures above 55˚C were reached during day 2 and were sustained throughout the 7 days (Figure 6.1) in covered heaps resulting in periods above 55˚C of 156 h whereas in uncovered heaps the period above 55˚C was 77.5 hr at this depth. There was little difference in the profiles at 10 and 20 cm deep, with both reaching 55˚C within 24 hrs, and maintaining temperatures above it for 2 days before declining to temperatures of around 45˚C by day 7. At 5cm depth temperatures

peaked on day 2 just below 55˚C and declined to around 35˚C by day 7. Broadly similar findings were observed in experiment 2.1.2 (Figure 6.9). Thus much of the heap falls outside the USA EPA (Class B) requirements of 3 days at 55˚C in static piles with an estimated 43.5% being exposed to that temperature during the experimental period of a little over 7 days (Table 6-5). With regards the hypothesis of linear temperature profiles between 10 and 20 cm depths, the evidence from these experiments suggest that it is non linear, with temperatures changing more rapidly closer to the surface. This effect is illustrated nicely in Figure 6.1 (Right) where the 5 cm profile is closer to the 20 cm profile than the 0 cm profile.

Maximum temperatures of the heaps in experiment 2.2.1 with smaller, drier heaps of litter exceeded those of seen in Experiment 2.1 in Sydney with much larger heaps of damper litter. In the former maximum temperatures at 10, 20 and 50cm depths exceeded 60˚C whereas in the latter they fell between 55˚C and 60˚C. Time to achieve maximum temperature was also shorter but overall mean temperatures were only slightly lower due to the slower decline in temperatures in the larger heaps in Sydney. The even smaller heaps used in experiment 2.2.2 also generated high maximum and average temperatures. These results suggest that smaller heaps generate heat more quickly and may be preferable when a short pasteurisation is all that is possible. The results as a whole also indicate that relatively dry litter (15% moisture) can generate substantial heat as in Expt 2.2.1, but that maximum temperatures and retention of heat in the heap were improved by either covering or water addition.

6.4.4 Effect of aeration

Aeration can be used to facilitate full composting in very large piles of litter or other materials and both passive (Brodie *et al.* 2000) and forced (Tiquia and Tam 2002) aeration methods have been reported. It is possible that the very slow heating reported deep in large heaps of litter during pasteurisation reported by Walkden-Brown *et al.* (2010a) could be due to low availability of oxygen for aerobic thermophiles. In experiment 2.2.2 there was no evidence of a beneficial effect of aeration, possibly because the heap was relatively small. Even with very large heaps Brodie *et al.* (2000) found few temperature differences with passive aeration of long composted mixed material including poultry litter. Additional aeration is unlikely to be a practical means of increasing temperatures in pasteurising litter.

6.4.5 Use of broccoli boxes

Using a smaller experimental model than full on-farm heaps has potential for screening multiple treatments and examples of doing this have been previously reported eg. (Lavergne *et al.* 2006). Using broccoli polystyrene boxes holding 20 L of litter with an open lid resulted in peak temperatures between 40 and 60 ˚C indicating the capacity for quite high

temperatures to be achieved (Figure 6.16). The peaks were also achieved on day 2, as in the larger heaps, but temperatures tailed off rapidly. It can be concluded that the method has application for screening large numbers of treatments in replicated experiments but findings would need to be verified in full sized heaps.

6.4.6 Effect of litter amendments

One likely consequence of increase litter reuse and pasteurisation will be increased use of litter amendments to reduce ammonia production from reused litter. Experiment 2.2.3 took advantage of a trial at UNE testing a wide range of litter amendments on broiler performance, to see if the amendments influence litter temperature kinetics during subsequent pasteurisation using the broccoli box model. Although confounded by widely different moisture contents, analysis taking this into account showed that while overall temperatures were not significantly affected by amendment type, there was a significant interaction with Day of measurement such that the treatments produced 3 types of profiles (Figure 6.16) viz:

- Rapid rise in temperature to modest peak then rapid decline (Nil, Alum)
- Rapid rise to higher peak then rapid decline (Bentonite, NaBisulph)
- Slower rise to a modest peak then slow decline (Zeolite, NaturClean)

These results do suggest a capacity for amendments, applied at industry rates, to influence the temperature kinetics of heaped litter. It is clear that these effects are not mediated only by changes in litter pH, since the significant differences in pH between treatments (Figure 6.11, Right) were not clearly associated with any particular temperature profile. It can be concluded that despite potential differences, adding amendments to litter is unlikely to greatly influence the ability to undertake litter pasteurisation by heaping.

6.4.7 Production of ammonia from pasteurising litter

In experiment 2.1.3 ammonia production from litter during composting following chicken rearing on amended litter was not significantly affected overall by amendment, despite significant effects on ammonia production during the growout phase on the litter (Walkden-Brown *et al.* 2013). What did affect ammonia production significantly was day of sampling, moisture content of the litter and its pH. The latter are both factors well known to influence ammonia production and the strong associations produced in this experiment suggest that the broccoli box method does produce sensible results.

Ammonia production declined sharply between days 3 and 7 in line with the rapid decline in temperatures. This suggests some limiting factor on the thermophilic aerobic composting process. Water is unlikely to be the limiting factor given the adequate moisture content at the end of the experiment (46.5%). It is more likely depletion of oxygen or substrate, or lack of

insulation that reduced microbial activity. Successive re-use of litter has been shown to reduce the pasteurising potential when heaped (Lavergne *et al.* 2006) presumably due to a less favourable C:N ratios over time. However the fact that the litter used in this experiment was 2nd use litter is unlikely to explain the very rapid fall in temperature as sustained pasteurising temperatures in heaps were observed in litter up to the 6th use by (Lavergne *et al.* 2006). Oxygen depletion in these comparatively wet litters in a box open only on one side is a possibility, as is lack of sufficient mass to retain temperatures during Armidale winter temperatures. The insulated nature of the polystyrene box should mitigate against this.

The influence of moisture was non-linear with ammonia production increasing in concert with moisture content to 40%, maintaining high levels at 40-50% moisture content, then declining as moisture levels increase beyond that (Figure 6.18). Presumably, favourable moisture conditions provide moisture and nutrients to the microbial population while excessive moisture reduces aeration and possibly absorbs ammonia in solution. The positive association between moisture content and ammonia production is another reason why addition of water to the heaps may not be a good strategy unless litter is very dry and moisture is limiting as in Experiment 2.1.1 (15% moisture in uncovered control). However, in this case additional heating, equivalent to adding 14% moisture, can be achieved by simply covering the heaps.

pH is well known to influence ammonia production in litter and is the basis for use of acidifying chemical amendments in litter such as Alum. Very little ammonia is released from litter at a pH lower than 7. However, the release of ammonia increased rapidly when pH increases above 8 (Reece *et al.* 1979) and ammonia concentration immediately above the litter increased linearly with increasing pH at a constant ventilation rate (Carr *et al.* 1990). Our results are in agreement, showing a linear increase in ammonia production with pH over the range 7.75 to 9.75.

6.4.8 Summary of major findings

1. Covering heaps with low moisture content (16%) significantly increased maximum temperature, mean temperature, amount of time above 55˚C and time to maximum temperature. This effect was not observed heaps with 28% initial moisture content for which covering reduced overall maximum temperature, mean temperatures and time to maximum temperature, but increased time above 55˚C (all non significant).
2. Increasing initial moisture content of heaps from 15 to 29% significantly increased mean temperatures, time above 55˚C and time to maximum temperature in uncovered heaps, with a non-significant increase in maximum temperature. In covered heaps, increasing

the moisture content brought little additional benefit over that obtained by covering. In high moisture heaps covering slightly increased time above 55˚C, decreased mean and maximum temperatures and decreased time to maximum temperature, all non significantly.

3. Thus the effects of moisture addition and covering are not additive in this moisture range.
4. Temperatures in heaps increase sharply below the surface in a non-linear manner. Outer layers heat up and cool down more quickly than deeper layers and do not achieve as high maximum temperatures.
5. Aeration of a small heap failed to have a major effect and aeration is unlikely to be a practical method for improving litter pasteurisation.
6. Placing used litter in broccoli boxes resulted in generation of significant temperatures and has potential to be used as a screening method for intra-litter factors that may influence temperature generation.
7. There is preliminary evidence in such a screening trial that litter amendments can affect heat generation in piled litter and that this is not mediated by changes in pH. This requires confirmation.
8. Ammonia production from piled litter in a screening trial declined sharply over time in line with declining temperatures.
9. The influence of moisture on ammonia production was non-linear with ammonia production increasing in concert with moisture content to 40%, maintaining high levels at 40-50% moisture content, then declining as moisture levels increase beyond that.
10. Regarding the effect of pH on ammonia production our results are in agreement with the general literature showing a linear association between pH and ammonia production in the pH range 7.75 to 9.75.

7 Strand 2. Experiment 2.3 –Tamworth. "Effects of covering, turning and heap size on temperatures in heaped litter on a commercial farm"

7.1 Introduction

Following on from the initial experiments in Sydney and UNE this large on-farm experiment was undertaken in Tamworth. The earlier studies had not produced encouraging results regarding water addition so in this experiment the effects of covering were coupled with turning and heap size in a large factorial experiment. Turning of pasteurising litter is advocated by some to incorporate the cooler outer surfaces deeper into the pile, and to aerate the core of the heap. In an earlier project we found that turning could increase temperatures after turning, but that this did not affect inactivation rate of the viruses tested (Walkden-Brown *et al.* 2010a).

In line with the overall objective of developing methods to achieve rapid and uniform temperature increases in litter heaped for pasteurisation the specific objectives were:

- To test the effects of covering, turning and heap size on the distribution of temperatures within heaped broiler litter
- To test effects of these treatments on the distribution of moisture within the heaps.

The experiment was carried out in three sheds of a meat chicken farm located west of Tamworth, NSW. The work was carried out by Dr Fakhrul Islam and his Master of Science in Agriculture student Mahmoud Mahmoud with assistance from the Project team. Aspects of the work were reported in the thesis component Mahmoud's degree. The experimental code for the experiment was LT12-C-LSOP4. The experiment ran from 15–22 November 2012.

7.2 Materials and methods

7.2.1 Experimental design

The experiment had a 2 x 2 x 4 factorial design with two replicates thus requiring the construction of 32 litter heaps. The factors and levels were:

- Cover — Covered or not with a blue polyethylene tarpaulin (Polytarp)
- Turn — Heap turned or not turned on day 3
- Heap size — Large heap, medium heap, small heap, small windrow

Temperatures were measured in the heaps at 4 different depths (5, 25, 50 and 100 cm) providing an additional factor in the analysis.

Heaps and windrows were prepared in 3 of the 8 sheds on the farm, using a Bobcat front-end loader. Non de-caked litter from within each shed was used to construct the heaps/windrows. A fully blocked design could not be used as 32 is not divisible by 3, but treatments were stratified to provide as close to full blocking within sheds as possible. Each of the three sheds used in this experiment had 42 equal bays, each approximately 3.5 m long and 12 m wide. Small heaps and windrows used litter from 1 bay, medium heaps 3 bays, and large heaps 9 bays. The depths of the litter on the shed floor were 5-10 cm, with an average of 7.2 cm. Therefore, the estimated volumes of the small, medium and large heaps were ~3 m^3, ~9 m^3 and ~27 m^3 respectively. Details of the arrangements of treatments is shown in Table 7-1 and photographs of the experimental arrangements are shown in Figures 7.1–7.4.

Table 7-1. Expt 2.3. Summary of individual heaps, treatments and initial moisture content.

Heap No	Shed No	Heap size/ shape	Cover	Turn	Height (m)	Length (m)	Width (m)	Initial mean moisture (%)
1	1	Medium	NC	NT	1.65	4.7	4.3	25.4
2	1	Small	C	NT				23.2
3	1	Large	C	T	2.1	8.5	5	24.2
4	1	Medium	NC	T				15.2
5	1	Large	NC	NT				18.7
6	1	Small	NC	NT	0.85	3	3.1	15.2
7	1	Medium	C	T				15.3
8	1	Windrow	NC	T	1.2	6.2	2.3	16.8
9	1	Windrow	C	T				16.4
10	1	Windrow	NC	NT				16.8
11	1	Large	C	NT				20.4
12	2	Medium	C	T				12.7
13	2	Small	NC	T	1	4.6	3.9	
14	2	Medium	NC	NT	1.45	5.4	5	20.3
15	2	Windrow	NC	NT	0.75	7.5	2.5	26.3
16	2	Windrow	C	NT				20.7
17	2	Large	C	T				19.9
18	2	Small	C	T				14.6
19	2	Large	NC	T	1.7	9	6	18.5
20	2	Small	NC	NT				14.1
21	2	Medium	C	NT				18.7
22	4	Large	C	NT				14.9
23	4	Small	NC	T	1.05	3.6	3	18.4
24	4	Medium	C	NT				24.2
25	4	Large	NC	T	1.85	9.7	5.9	19.1
26	4	Small	C	T				14.1
27	4	Large	NC	NT				16.9
28	4	Windrow	C	NT				18.3
29	4	Windrow	NC	T				16.3
30	4	Windrow	C	T	0.7	6.4	2	18.9
31	4	Small	C	NT				16.3
32	4	Medium	NC	T	1.75	6	6	17.3

Figure 7.1 Experiment 2.3. Empty shed prior to litter heaping (Left) and shed with row of heaped litter down the middle (Right)

Figure 7.2 Experiment 2.3. Large uncovered heap (Left), coverered and uncovered windrows and small heaps (Right)

Figure 7.3. Experiment 2.3. Inserting iButtons at the start of the experiment (Left) and sub-sampling litter at the end of the experiment (Right)

Figure 7.4. Experiment 2.3. Examples of “sweating” or condensation on the surface areas of heaps following cover removal.

7.2.2 Procedures and measurements

As soon as heaps were formed on day 0, iButtons were inserted at 4 depths (5, 25, 50 and 100 cm). In the smallest heaps a depth of 100cm was not always possible, so a depth of approximately 80cm was achieved. In addition, one iButton was placed in each shed to record the ambient temperature. All iButtons were set to record the temperature once every hour. Either 4 or 8 ibuttons were placed in each heap resulting in 174 complete ibutton recordings for the entire experiment.

At days 0 and 7, 128 litter samples (four samples from each heap/windrow) were collected for measuring litter moisture content. The samples were collected from the surface 5, 25 and 50 cm depths. For each depth, a litter sample was gathered from four different locations of the heap or windrow, mixed homogeneously, put in ziplock plastic bags and transported to the UNE laboratory. The following day, moisture content of all samples was calculated and expressed as a percentage.

To assess whether cake is broken down as part of the pasteurising process 4 moderate pieces of cake were measured for size and placed in onion bags and inserted some 20cm into heaps on day 0. At the end of the experiment they were removed and re-measured.

On day 3 of the experiment (turning day), iButtons were removed from the relevant twelve heaps and four windrows, the litter mass was turned using a front-end loader. The removed iButtons were then re-inserted into the same heap or windrow at exactly the same depth. Covered heaps and windrows were re-covered. Data for the period when the iButtons were out of the heaps was deleted from the record ie. For the 1-3 hour period involved there are missing temperature records.

7.2.3 Data analysis

Litter moisture data were analysed using repeated measures ANOVA (mixed model REML) testing the effects of Heap size, Cover, Turn, Depth and Day post heaping and their interactions, with Heap nested within treatment as a random effect.

Temperature data from 174 iButtons were trimmed to 168 hours (7 days). Data were analysed in several ways:

- Scatter plots of hourly data points from each iButton for different treatments or depths were prepared and a spline smoothed curve produced to illustrate the profile over the measurement period.
- The period of time at 55˚C or over was calculated for each ibutton and analysed by ANOVA testing the effects of Heap Size, Cover, Turn and Depth and their interactions with heap initial moisture content fitted as a covariate.
- The maximum temperature for each iButton was calculated and analysed by ANOVA as for Time above 55˚C.
- The time to maximum temperature (in hr) was calculated for each iButton and analysed by ANOVA as above.
- To assess the overall effects on temperature individual hourly readings were meaned by day post heaping and the meaned data analysed in a repeated measures ANOVA (mixed model REML), testing the effects of Heap size, Cover, Turn, Depth and Day post heaping and their interactions, with Heap nested within treatment as a random effect.

Data visualisation, exploratory analysis, and final analyses were done using JMP 11 (SAS Systems, NC, USA). Most data are reported as LS means and SEs from the outputs of the analysis. In most cases the means do not differ from the raw means. Significant differences amongst means were determined by Tukey's HSD test.

7.3 Results

7.3.1 Litter moisture content

Analysis of litter moisture content provided the ANOVA table presented at Table 7-2. Although the main effects of Turn, Cover, Day and Depth were all significant these effects were generally qualified by significant interaction with other effects, mostly that of Day. The main effects and interactions are well illustrated in Figure 7.5 and Figure 7.6.

Table 7-2. ANOVA table showing the results of the analysis of litter moisture content.

Source	DF	F Ratio	Prob > F
Heap Size	3	2.7858	0.0768
Turn	1	4.6859	0.0469*
Cover	1	20.4577	0.0004*
Day	1	29.9075	<.0001*
Depth	3	15.5926	<.0001*
Heap Size*Turn	3	2.1441	0.1374
Heap Size*Cover	3	1.1605	0.3574
Turn*Cover	1	1.5111	0.2379
Heap Size*Turn*Cover	3	1.3967	0.2824
Heap Size*Depth	9	1.1698	0.3222
Turn*Depth	3	0.7821	0.5065
Heap Size*Turn*Depth	9	0.8515	0.5709
Cover*Depth	3	47.9242	<.0001*
Heap Size*Cover*Depth	9	1.2547	0.2707
Turn*Cover*Depth	3	0.3872	0.7624
Heap Size*Turn*Cover*Depth	9	1.2367	0.2811
Heap Size*Day	3	3.1362	0.0287*
Turn*Day	1	0.0632	0.8019
Heap Size*Turn*Day	3	1.7362	0.1642
Cover*Day	1	87.2860	<.0001*
Heap Size*Cover*Day	3	0.8339	0.4782
Turn*Cover*Day	1	2.5401	0.1140
Heap Size*Turn*Cover*Day	3	0.2169	0.8845
Depth*Day	3	23.9291	<.0001*
Heap Size*Depth*Day	9	0.5724	0.8170
Turn*Depth*Day	3	1.7495	0.1616
Heap Size*Turn*Depth*Day	9	1.1928	0.3076
Cover*Depth*Day	3	69.7358	<.0001*
Heap Size*Cover*Depth*Day	9	1.5204	0.1505
Turn*Cover*Depth*Day	3	1.1483	0.3333
Heap Size*Turn*Cover*Depth*Day	9	0.8885	0.5383

Moisture content on day 7 (21.1 ± 0.58 %) was significantly higher overall than it was on day 0 (18.3 ± 0.58 %). However this effect was due solely to the effect in covered heaps in which moisture content increased by 7.5 percentage points between days 0 and 7, while it declined by 1.9 percentage points in uncovered heaps (Figure 7.5 Centre). Even further, the effect was evident only at the surface of the heap, with covering resulting in an increase in moisture content from 15.1 to 41.5 % while in uncovered heaps surface moisture declined from 17.3 to 9.1% (Figure 7.6 Right). Effects of covering at other depths were not significant. Turned heaps had lower moisture content than unturned heaps from 18.6 ± 0.75 % compared to 20.8 ± 0.71 %, $P<0.05$) but this cannot be attributed to the turning as the difference did not vary greatly between days 0 (before turning) and day 7 (after turning) (Figure 7.5, Right). Heap Size/shape did not have significant overall effect on litter moisture content but there was a significant interaction with day ($P=0.03$) due to significant increases in moisture content between days 0 and 7 in large and medium heaps but not small heaps and windrows (Figure 7.5, Left)

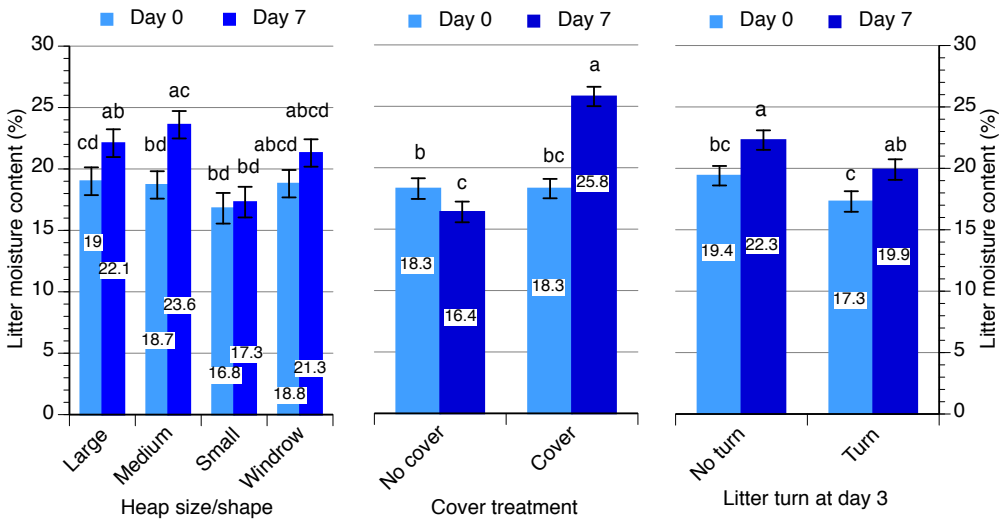

Figure 7.5. Experiment 2.3. Plots of litter moisture content (LS means ± SE) illustrating interaction, or lack of it, between the effects of Heap size and Day (Left) (P=0.32), Cover and Day (Centre) (P<0.0001) and Turn and Day (P=0.80) (Right). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

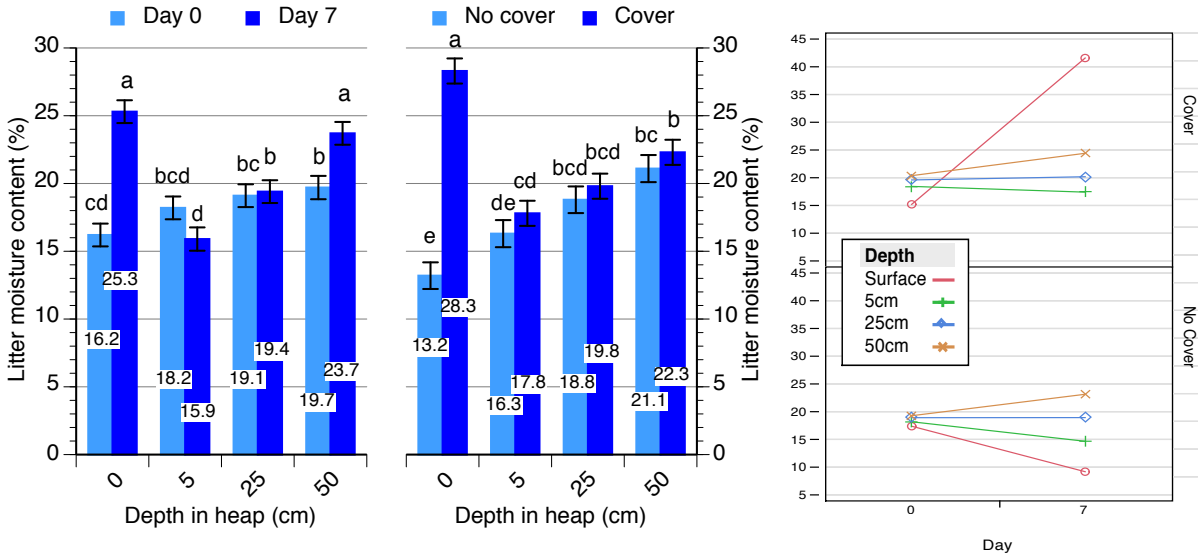

Figure 7.6. Experiment 2.3. Plots of litter moisture content (LS means ± SE) illustrating interaction between the effects of iButton Depth and Day (Left) (P<0.0001), Cover and Depth (Centre) (P<0.0001) and Cover Depth and Day (P=0.80) (Right). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

7.3.2 Litter temperature profiles

Smoothed hourly profiles for the duration of the experiment for the main effects of Turning and Covering and Heap size/shape and depth in the heap are shown in Figure 7.7 and Figure 7.8 respectively.

Turning resulted in a decline in temperatures for about 1.5 days after which temperatures then exceeded those on unturned heaps (Figure 7.7, Left). Covering had little effect on overall temperatures for the first 4 days, with a slight cooling effect thereafter (Figure 7.7, Right).

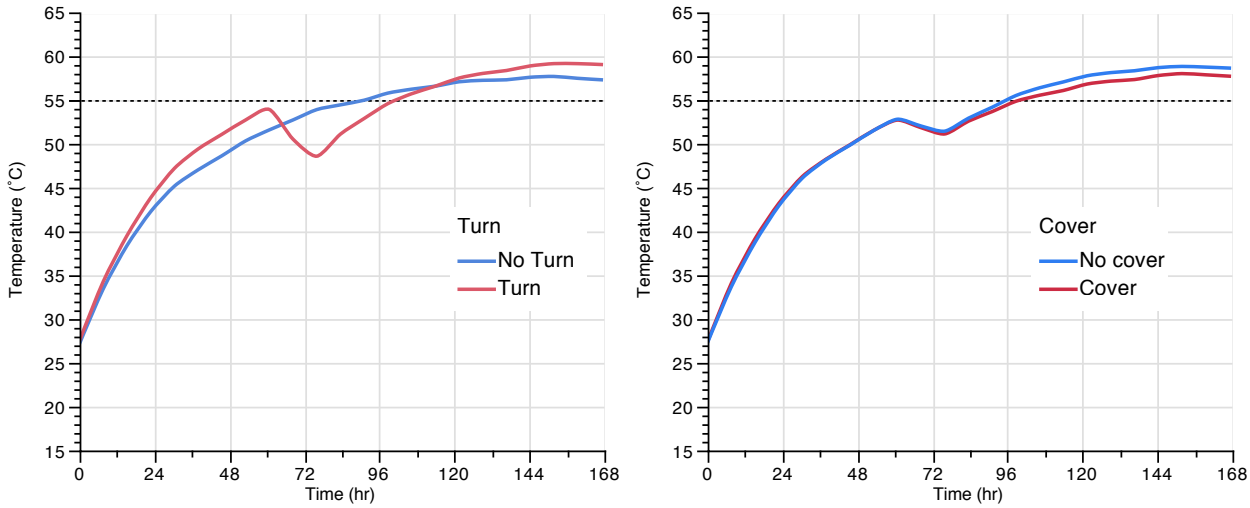

Figure 7.7. Experiment 2.3. Smoothed mean temperature profiles by Turn treatment (Left) and by Cover treatment (Right). Curves are spline smoothed curves from 171 data points per hr.

Smaller heaps heated more quickly than larger heaps but were cooling by the end of the 7 day period whereas temperatures were still increasing in larger heaps (Figure 7.8, Left). As expected, temperatures varied greatly with depth with heating slowed with increasing depth and shallower parts of the heap peaking earlier and at lower temperatures than deeper parts of the heap (Figure 7.8, Right). Temperatures of 50-55˚C were attained from days 2-7 at 5cm depth with a slight remnant of the diurnal variation evident in the shed temperatures.

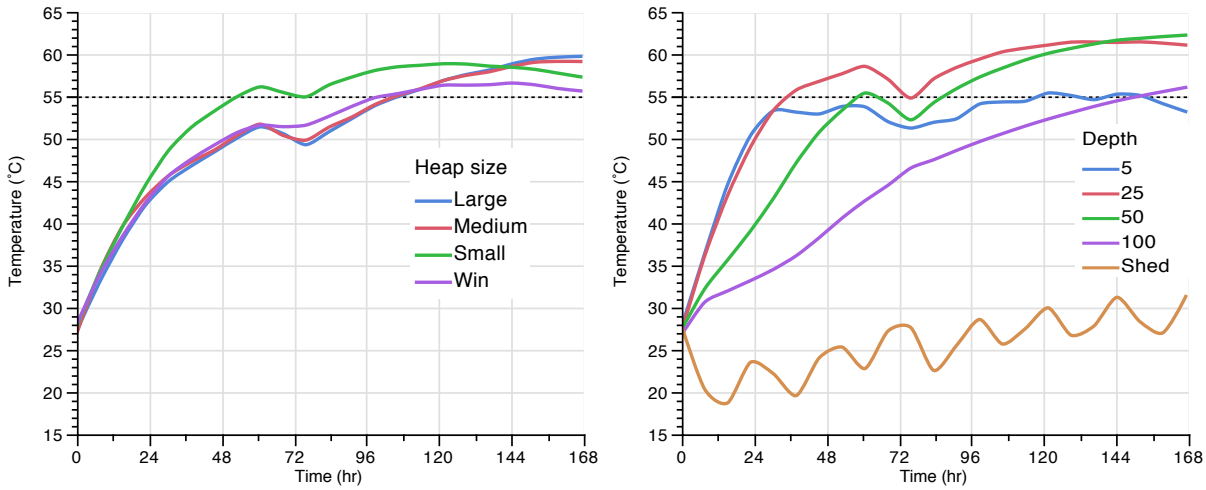

Figure 7.8. Experiment 2.3. Smoothed mean temperature profiles by Heap size/shape (Left) and by Depth in the heap, including ambient shed temperature (Right). Curves are spline smoothed curves from 171 data points per hr (Left) and 174 data points/hr (Right).

7.3.3 Hours above 55˚C

The summary of the analysis of variance table for hours spent above 55˚C is shown in Table 7-3.

Table 7-3. Expt 2.2.1. Summary of ANOVA table for analysis time (hr) spent above 55 (˚C)

Source	DF	Sum of Squares	F Ratio	Prob > F
Heap Initial moisture (%)	1	1161.27	1.1394	0.2884
Turn	1	396.88	0.3894	0.5340
Depth	3	142606.92	46.6407	<.0001*
Heap size	3	15215.85	4.9765	0.0029*
Cover	1	40.43	0.0397	0.8425
Turn*Depth	3	838.14	0.2741	0.8439
Turn*Heap size	3	16332.96	5.3418	0.0019*
Turn*Cover	1	8597.23	8.4354	0.0045*
Depth*Heap size	9	20345.19	2.2180	0.0268*
Depth*Cover	3	3798.09	1.2422	0.2986
Heap size*Cover	3	9051.02	2.9602	0.0360*
Turn*Depth*Heap size	9	7090.79	0.7730	0.6415
Turn*Depth*Cover	3	926.14	0.3029	0.8232
Turn*Heap size*Cover	3	9201.99	3.0096	0.0338*
Depth*Heap size*Cover	9	10804.54	1.1779	0.3175
Turn*Depth*Heap size*Cover	9	15468.22	1.6863	0.1022

The overall effects of Depth, Heap Size/Shape, Covering and Turning on the time spent above 55˚C are shown in Figure 7.9. The most time above 55˚C was seen at 25 cm (121 hr, 72% of the time) and 50 cm (94 hr, 56% of the time) depths, significantly higher than observed at 5 cm (77 hr, 46% of the time) and 100 cm (36 hr, 21% of the time) (Figure 7.9, Left). Heap size also had a significant effect with the small heaps having the highest overall proportion of time above 55˚C (101 hr), significantly higher than either large heaps (76 hr) or windrows (70 hr) (Figure 7.9, Centre Left). There were no significant overall effects of covering and turning (Figure 7.9, Centre Right and Right).

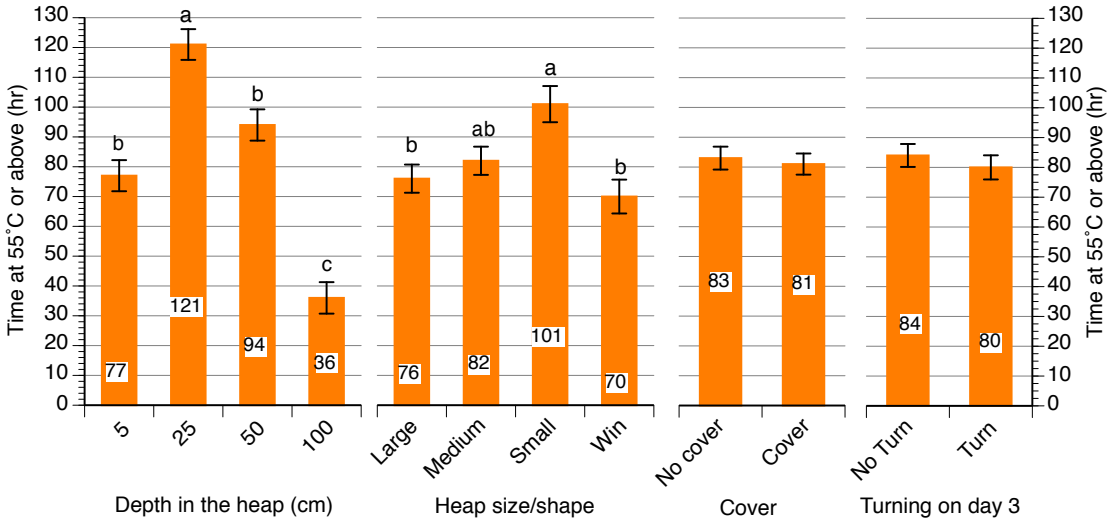

Figure 7.9. Experiment 2.3. Overall effects of Depth in the heap (Left), Heap Size/Shape (Centre Left), Covering (Centre Right) and Turning (Right) on the number of hours temperatures were above 55˚C out of a total period of 168 hrs (7 days). (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

Some of the significant interactions from table Table 7-3 are illustrated in Figure 7.10. Turning influenced the effect of covering such that it decreased the time above 55˚C in covered heaps, but increased it in uncovered heaps (Figure 7.10, Left). The effect of turning was also influenced by heap size/shape with it reducing time above 55˚C in Large and Small heaps, having little effect in Medium heaps and increasing time above 55˚C in Windrows (Figure 7.10, Centre).

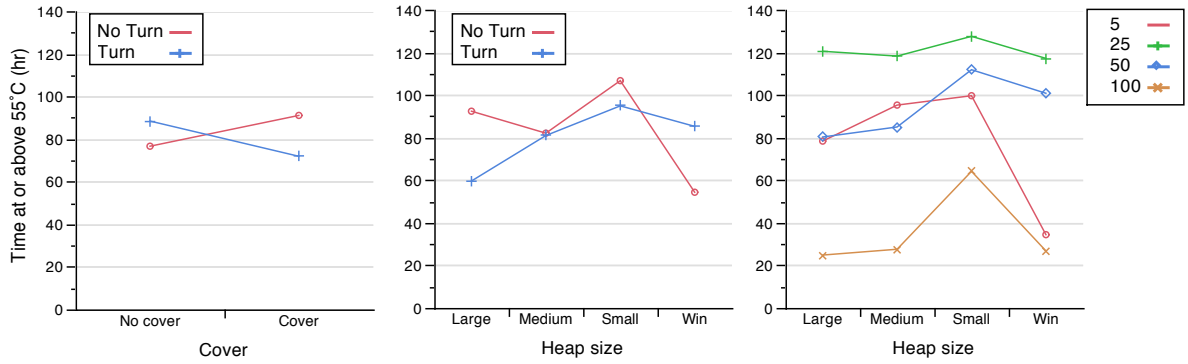

Figure 7.10. Experiment 2.3. Plots of time at 55˚C or higher (LS means ± SE) illustrating interaction between the effects of Cover and Turning (Left) (P=0.03), Heap Size/Shape and Turning (Centre) (P<0.00001) and Heap Size/Shape and Depth (P=0.01) (Right).

There was also interaction between the effects heap Size/Shape and Depth in the heap (Figure 7.10, Right). At 5 cm depth, time above 55˚C was markedly lower in windrows than in the other heaps while at 100 cm depth, the reverse was true. At 25cm depth the small heap and windrows had more time above 55˚C than the larger heaps, while at 25˚C there were few differences between the heaps.

The significant interaction between Heap Shape/Size and Cover was due to covering having little effect on time above 55˚C in Windrows, reducing it somewhat in small and large heaps but increasing it in medium heaps. The significant interaction between the effects of Turning, Heap Size/Shape and Cover reflected the fact that covering generally reduced temperatures overall in turned heaps but had little effect in unturned heaps except for the medium heap size in which covering led to a large increase in mean temperature (from 54 to 110 hrs).

7.3.4 Maximum temperature

The summary of the analysis of variance table for the maximum temperature achieved by each iButton is shown in

Table 7-4. The overall effects of Depth, Heap Size/Shape, Covering and Turning on maximum temperature were all significant and are illustrated in Figure 7.11.

Table 7-4. Expt 2.2.1. Summary of ANOVA table for maximum temperature.

Source	DF	Sum of Squares	F Ratio	Prob > F
Heap Initial moisture (%)	1	0.2287	0.0220	0.8823
Turn	1	125.2793	12.0709	0.0008*
Depth	3	1378.9318	44.2877	<.0001*
Heap size	3	223.3303	7.1728	0.0002*
Cover	1	111.2684	10.7210	0.0015*
Turn*Depth	3	27.0711	0.8695	0.4597
Turn*Heap size	3	144.3253	4.6354	0.0045*
Turn*Cover	1	68.5154	6.6016	0.0117*
Depth*Heap size	9	47.2967	0.5063	0.8668
Depth*Cover	3	26.1391	0.8395	0.4754
Heap size*Cover	3	121.5053	3.9024	0.0111*
Turn*Depth*Heap size	9	86.2305	0.9232	0.5086
Turn*Depth*Cover	3	38.0289	1.2214	0.3060
Turn*Heap size*Cover	3	252.1514	8.0985	<.0001*
Depth*Heap size*Cover	9	179.0505	1.9169	0.0580
Turn*Depth*Heap size*Cover	9	67.8093	0.7260	0.6841

Maximum temperatures were highest at 25 and 50 cm depths (63.2 and 63.0 ˚C respectively), significantly higher than maxima at 5 cm (57.4˚C) and 100 cm (56.7˚C) (Figure 7.11, Left). Windrows had significantly lower maximum temperatures overall than the other heap sizes by approximately 2.5-3 ˚C (Figure 7.11, Centre Left). Covering reduced overall temperatures by 0.8˚C

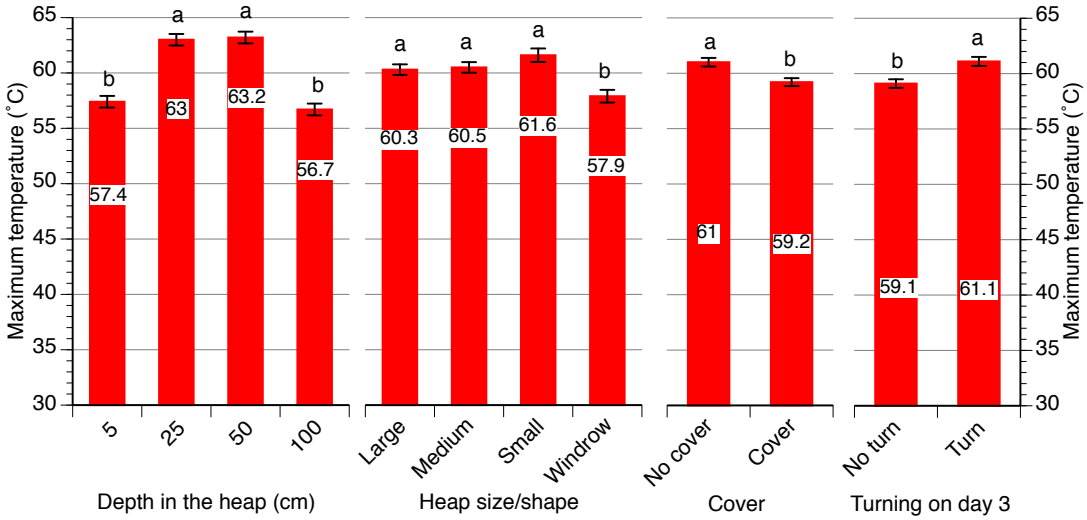

Figure 7.11. Experiment 2.3. Overall effects of Depth in the heap (Left), Heap Size/Shape (Centre Left), Covering (Centre Right) and Turning (Right) on the maximum temperature achieved by each iButton (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

Some of the significant interactions from Table 7-4 are illustrated in Figure 7.12. The effect of turning was influenced by heap size/shape with it increasing maximum temperatures by 1-4 ˚C in all heap types except Large heaps (Figure 7.12, Left). Turning also influenced the effect of covering such that it increased maximum temperatures in covered heaps, but not uncovered heaps (Figure 7.12, Centre). The effect of covering differed between the heap sizes and types with covering reducing temperatures by 0.5-4.5˚C in all heaps other than medium heaps in which it had a small positive effect (Figure 7.12, Right).

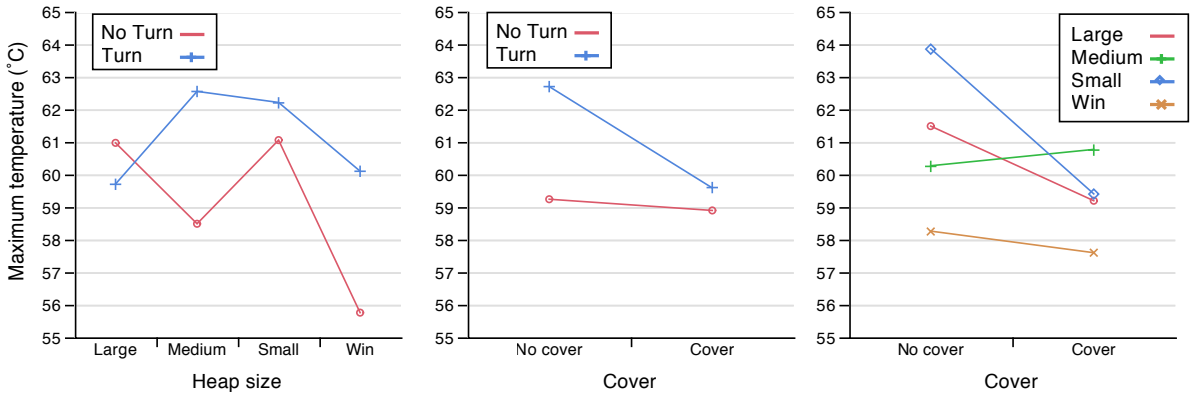

Figure 7.12. Experiment 2.3. Plots of time at maximum temperature (LS means ± SE) illustrating interaction between the effects of Turning and Heap Size/Shape (Left) (P=0.005), Turning and Cover (Centre) (P=0.011) and Heap Size/Shape and Cover (P=0.011) (Right).

The significant 3-way interaction between the effects of Depth, Heap Size/Type and Cover (P<0.0001) extends the 2-way interactions at (Figure 7.12, Centre and Right) by revealing that the increase in temperature with covering observed in the medium heaps was due soley to a large effect (~5˚C) in unturned heaps. In turned medium sized heaps by contrast, a decline of ~4.5˚C was observed.

7.3.5 Time to reach maximum temperature

The summary of the analysis of variance table for the maximum temperature achieved by each iButton is shown in Table 7-5. The overall effects of Depth, Heap Size/Shape, Covering and Turning on maximum temperature are illustrated in Figure 7.13.

Table 7-5. Expt 2.2.1. Summary of ANOVA table for maximum temperature.

Source	DF	Sum of Squares	F Ratio	Prob > F
Heap Initial moisture (%)	1	3580.756	4.5900	0.0346*
Turn	1	1543.550	1.9786	0.1627
Depth	3	55459.462	23.6970	<.0001*
Heap size	3	18817.253	8.0403	<.0001*
Cover	1	493.821	0.6330	0.4282
Turn*Depth	3	4170.868	1.7822	0.1555
Turn*Heap size	3	1444.615	0.6173	0.6054
Turn*Cover	1	543.153	0.6962	0.4061
Depth*Heap size	9	9076.591	1.2928	0.2504
Depth*Cover	3	1792.079	0.7657	0.5159
Heap size*Cover	3	6931.355	2.9617	0.0359*
Turn*Depth*Heap size	9	6505.653	0.9266	0.5057
Turn*Depth*Cover	3	1961.046	0.8379	0.4762
Turn*Heap size*Cover	3	10449.709	4.4650	0.0055*
Depth*Heap size*Cover	9	8072.252	1.1497	0.3358
Turn*Depth*Heap size*Cover	9	4771.920	0.6797	0.7256

Initial heap moisture content had a significant positive linear association with time to maximum temperature such that each unit increase in litter moisture content (%) increased time to maximum temperature by 2.3 ± 1.1 hr (P=0.03). Time to reach maximum temperature increased with increasing depth in the heap (P<0.0001) ranging from 109 hr (4.5 days) at 5cm to 158 hr (6.6 days) at 100 cm (Figure 7.13, Left). However these are underestimates, particularly at the greater depths, as a significant number of iButtons were still recording increasing temperatures at the end of the experiment (168 hr/7 days).

Small heaps and windrows achieved maximum temperatures faster than large heaps (128-130 hrs compared to 156 hrs, P<0.0001) (Figure 7.13, Centre Left). There was no significant

effect of covering (Figure 7.13, Centre Right) or turning of heaps (Figure 7.13, Right) on the time taken to reach maximum temperature.

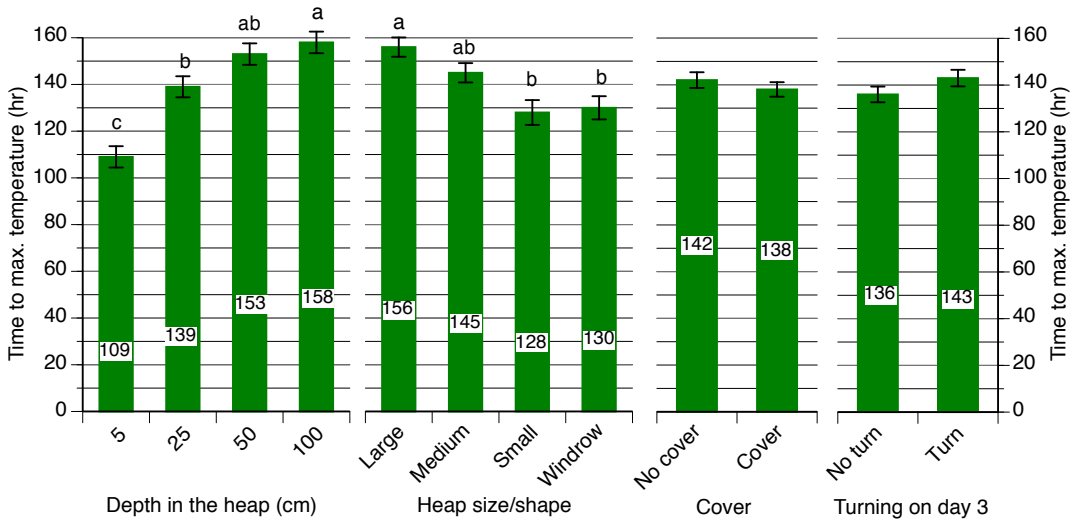

Figure 7.13. Experiment 2.3. Overall effects of Depth in the heap (Left), Heap Size/Shape (Centre Left), Covering (Centre Right) and Turning (Right) on the time taken to reach maximum temperature for each iButton (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

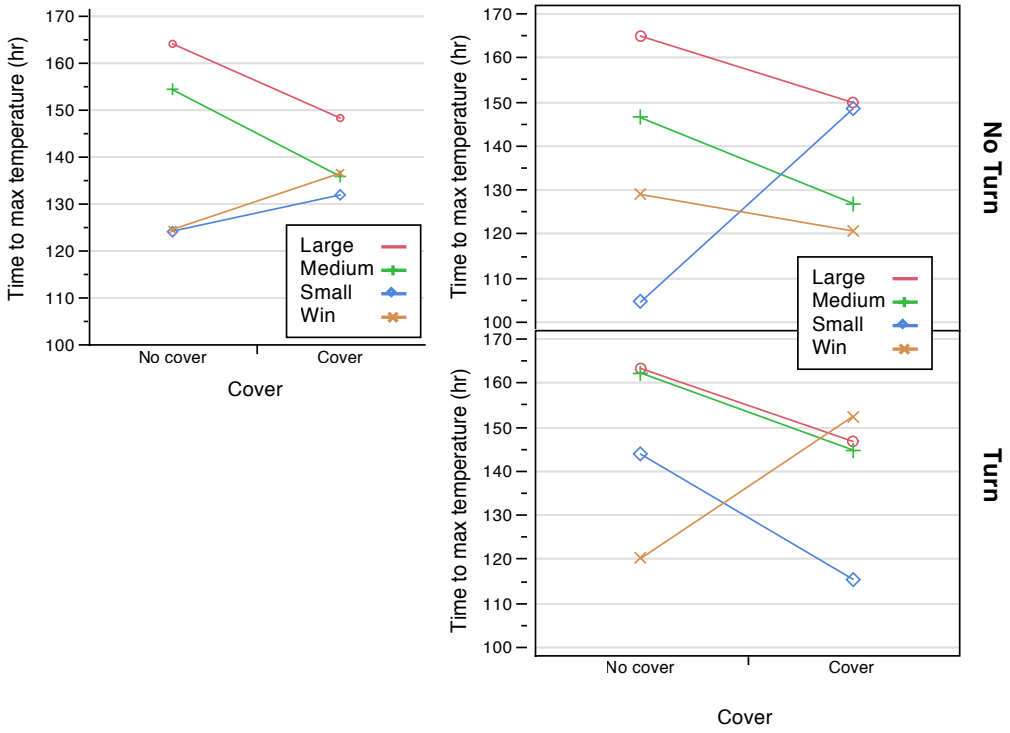

Figure 7.14. Experiment 2.3. Plots of time to reach maximum temperature (LS means ± SE) illustrating interaction between the effects of Heap Size/Shape and Cover (Left) (P=0.04), Turning, Heap Size/Shape and Cover (Right) (P=0.006).

There was significant interaction between the effects of heap size/shape and cover with covers decreasing the time required to achieve maximum temperatures in large and medium heaps, but increasing it in small heaps and windrows (Figure 7.14, Left). However, this effect was qualified by a 3-way interaction between Turning, Heap Size/Shape and Cover (Figure 7.14, Right) showing that the effect of covering had opposite effects on time to reach maximum temperatures in small heaps and windrows.

7.3.6 Overall temperature analysis (repeated measures)

The summary of the analysis of variance table for the repeated measures analysis of litter temperature is shown in Table 7-6. The overall effects of Depth, Heap Size/Shape, Covering and Turning are illustrated in Figure 7.15.

Table 7-6. Expt 2.2.1. Summary of ANOVA table for repeated measures analysis of litter temperature (˚C).

Source	DF	F Ratio	Prob > F
Initial Moisture %	1	3.2765	0.0918
Day	6	661.5816	<.0001*
Turn	1	0.0636	0.8046
Depth	3	376.4103	<.0001*
Heap size	3	2.1897	0.1350
Cover	1	0.1967	0.6642
Day*Turn	6	8.5886	<.0001*
Day*Depth	18	26.5712	<.0001*
Day*Heap size	18	3.2627	<.0001*
Day*Cover	6	2.1223	0.0489*
Turn*Depth	3	0.6470	0.5850
Turn*Heap size	3	1.1913	0.3491
Turn*Cover	1	5.4491	0.0350*
Depth*Heap size	9	14.4261	<.0001*
Depth*Cover	3	4.5089	0.0038*
Heap size*Cover	3	0.9866	0.4276
Day*Turn*Depth	18	1.6479	0.0440*
Day*Turn*Heap size	18	1.0335	0.4189
Day*Depth*Heap size	54	0.4095	0.9999
Turn*Depth*Heap size	9	5.9997	<.0001*
Day*Turn*Depth*Heap size	54	0.5843	0.9925
Day*Turn*Cover	6	0.1312	0.9924
Day*Depth*Cover	18	0.4191	0.9841
Turn*Depth*Cover	3	5.1504	0.0016*
Day*Turn*Depth*Cover	18	0.3927	0.9891
Day*Heap size*Cover	18	1.7066	0.0337*
Turn*Heap size*Cover	3	2.0665	0.1511
Day*Turn*Heap size*Cover	18	2.2930	0.0018*
Depth*Heap size*Cover	9	8.9000	<.0001*
Day*Depth*Heap size*Cover	54	0.3856	1.0000
Turn*Depth*Heap size*Cover	9	7.3021	<.0001*
Day*Turn*Depth*Heap size*Cover	54	0.5817	0.9929

Mean temperatures varied significantly with depth in the heap, ranging from 45.3˚C at 100 cm depth to a 56.1˚C at 25cm depth (Figure 7.15, Left). The overall effects of Heap size/shape, Covering and Turning were not significant (Figure 7.15). However, each of these had significant interactions with Day as shown in Figure 7.16.

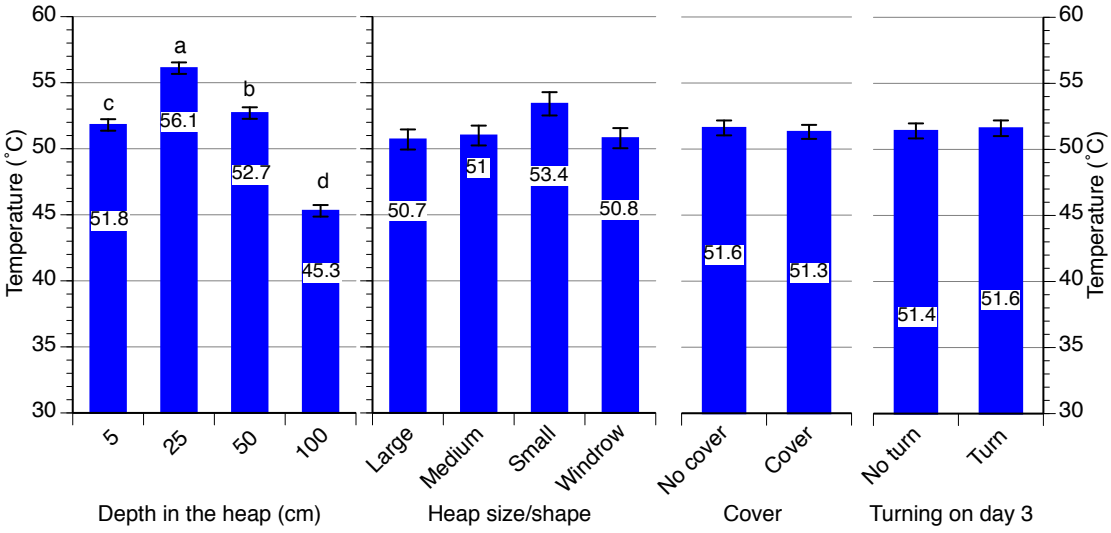

Figure 7.15. Experiment 2.3. Overall effects of Depth in the heap (Left), Heap Size/Shape (Centre Left), Covering (Centre Right) and Turning (Right) on the mean temperature (LS means ± SE). Means not having a letter or sharing a common letter within figures differ significantly (P<0.05).

The effect of depth was significantly modulated by day post heaping with the outer layers heating more quickly than the core (Figure 7.16, Top Left). Temperatures were still increasing at the end of the experiment at all depths other than 5cm. Temperature profiles over time were also influenced significantly by heap size/shape (Figure 7.16, Top Right) with small heaps heating more quickly than the other forms up to day 5. Temperatures largely plateaued from days 5–7 in the small heaps and windrows whereas they continued to increase in the larger heaps (Figure 7.16, Top Right). Covering led to reductions in overall temperatures from days 5-7 but not beforehand (Figure 7.16, Bottom Left). Turning had no effect up to day 3, but caused in a decline in temperatures at day 4 after which temperatures increased at an accelerated rate overtaking those in unturned heaps by day 6 (Figure 7.16, Bottom Right).

Other significant 2-way interactions are shown in Figure 7.17. The effects of covering and turning neutralised each other with covering increasing temperatures in unturned heaps but decreasing them in turned heaps (Figure 7.17 Left). The effects of heap size on temperatures were least at 25cm depth and greatest at the surface and 100 m depths. Windrows were coolest at the surface, but they and small heaps were warmer at 50 and 100 cm (Figure 7.17 Centre). Covering had very little overall effect but there was some

depression of temperature at 100 cm as opposed to a slight elevation at 25cm (Figure 7.17 Right).

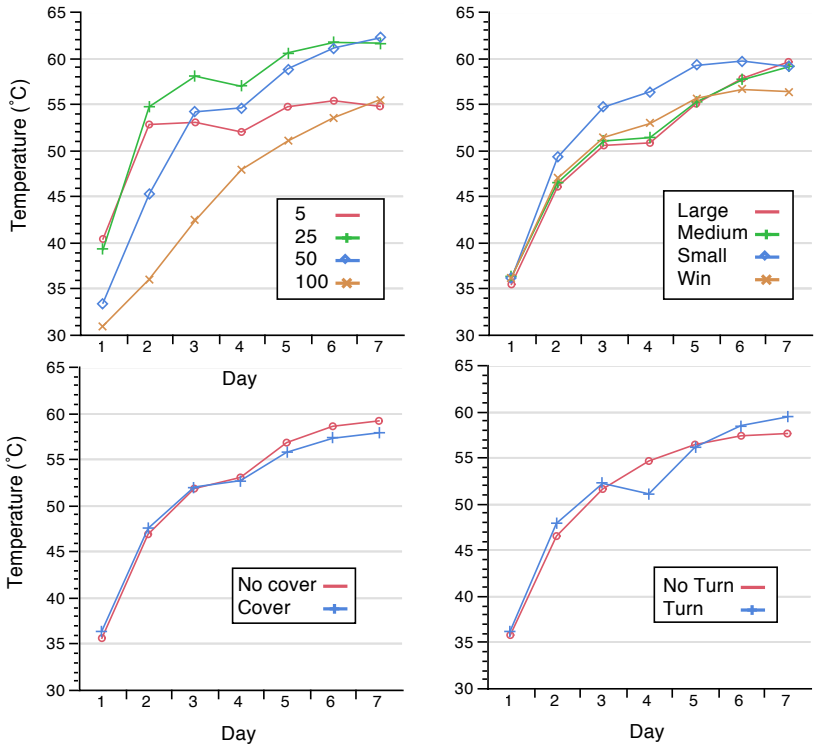

Figure 7.16. Experiment 2.3. Plots of temperature (LS means ± SE) illustrating interaction between the effects of Day and Depth (P<0.0001, Top Left), Day and Heap Size/Shape (P<0.0001, Top Right), Day and Cover (P=0.05, Bottom Left) and Day and Turning (P<0.0001, Bottom Right).

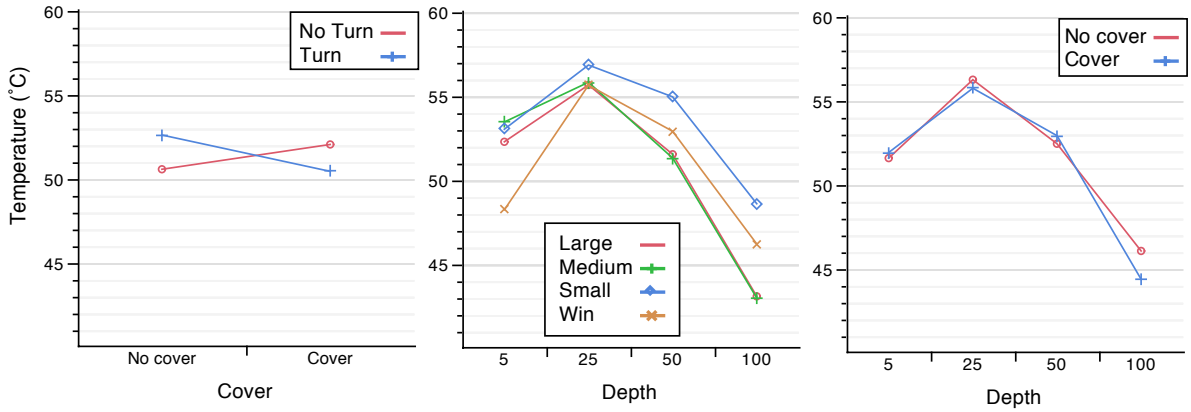

Figure 7.17. Experiment 2.3. Plots of temperature (LS means ± SE) illustrating interaction between the effects of Turning and Cover (P=0.03, Left), Depth and Heap Size/Shape (P<0.0001, Centre) and Depth and Cover (P=0.004, Right).

The significant 3-way interactions extend and combine the 2-way interactions but are not presented graphically for simplicity. The significant interaction between the effects of Day Turn and Depth reflects that turning decreased temperatures on day 4 but increased them on

days 6 and 7 but these effects were restricted to depths other than 100cm for which there was no effect of turning. Most of the turning effect was observed at 25 and 50cm.

The significant interaction between the effects of Turn, Depth and Heap size/shape is a relatively minor effect reflecting a decline in temperatures at 5cm depth with decreasing heap size in unturned, but not turned heaps.

The significant interaction between the effects of Turn, Depth and Cover is a comparatively subtle extension of the 2-way interaction shown at (Figure 7.17 Left) showing that this effect was greatest at 50cm depth and least at 5cm.

The significant interaction between the effects of Day, Heap size and Cover is a subtle extension of the 2-way interactions shown at Figure 7.16, Top Right and Bottom left) and adds little to the interpretation of these.

The significant interaction between the effects of Depth, Heap size and Cover is because the suppressive effect of covering observed at 100cm depth seen in (Figure 7.17 Right) was observed in small heaps only.

The two significant 4-way interactions add little to the understanding provided by the higher order interactions and simply emphasize the complexity of the effects in these heaps.

7.3.7 Association between variables

Pairwise correlations for the major variables analysed are shown in Table 7-7. Temperature variables tended to be significantly associated as might be expected. Heap initial and final moisture content, despite the wide variation in this variable (range 20.8 - 34.5%).

Table 7-7. Table of pairwise correlations coefficients for major variables based on 174 data points from individual dataloggers for temperature associations or 33 data points for individual heaps for associations involving moisture content. Associations are sorted from most to least significant.

Variable	by Variable	Correlation	Count	Signif Prob
Hours above 55˚C	Mean temp. (˚C)	0.8619	174	<.0001*
Max temp. (˚C)	Mean temp. (˚C)	0.8458	174	<.0001*
Hours above 55˚C	Max temp. (˚C)	0.7316	174	<.0001*
Heap initial moisture (%)	Mean temp. (˚C)	-0.5410	31	0.0017*
Heap final moisture (%)	Heap initial moisture (%)	0.4730	31	0.0072*
Heap initial moisture (%)	Time to max temp. (hr)	0.4226	31	0.0179*
Heap initial moisture (%)	Hours above 55˚C	-0.3849	31	0.0325*
Heap final moisture (%)	Max temp. (˚C)	-0.3430	31	0.0589
Heap initial moisture (%)	Max temp. (˚C)	-0.3283	31	0.0714
Time to max temp. (hr)	Mean temp. (˚C)	-0.1307	174	0.0857
Heap final moisture (%)	Mean temp. (˚C)	-0.3081	31	0.0918
Heap final moisture (%)	Time to max temp. (hr)	0.2954	31	0.1067
Max temp. (˚C)	Time to max temp. (hr)	0.1224	174	0.1075
Heap final moisture (%)	Hours above 55˚C	-0.1356	31	0.4670
Hours above 55˚C	Time to max temp. (hr)	-0.0505	174	0.5079

7.4 Brief discussion and conclusions

7.4.1 Effects of treatments on moisture levels

Overall litter moisture content at the start of this experiment (18.3%) was slightly lower than that at the start of Experiment 2.1 (22.3%) and slightly higher than that at the start of experiment 2.2.1 (15.0%). Moisture was not varied systematically in this experiment as in the previous two experiments. However detailed moisture measurements were made and for the first time Moisture at different levels in the heap were measured at the start and end of the experiment. The main findings were

- Uncovered heaps dried out somewhat at the surface and 5cm depths but not deeper levels over the 7 days of the experiment. The overall drying effect of heaping without covers was limited (1.9 percentage points) (Figure 7.5 and Figure 7.6).
- Covering led to a very large increase in moisture content at the surface, prevented drying out at 5cm depth and had little effect at the deeper layers (Figure 7.6 right). The high moisture content at the surface was clearly visible as wet material from “sweating” under the covers (Figure 7.4).

7.4.2 Effects of covering on temperature

Covers had no significant effect on overall temperatures in this experiment. That is not to say that they did not influence events, simply that the influences cancelled themselves out overall. For example covering led to a small increase in temperature (~2˚C) in unturned heaps, but a similar sized decrease in turned heaps (Figure 7.17 Left). Covering had little effect on temperatures during the first 4 days of the experiment, but a slight cooling effect after this (Figure 7.16 Bottom Left). This cooling was mostly seen deep within the heap (Figure 7.17 Right). Effects of covering on time above 55˚C mimicked those above for mean temperature. Covering did cause a significant overall reduction in maximum temperature achieved, but the effect was small (1.8˚C) and seen primarily in turned heaps (Figure 7.12 Centre). Covering led to a decrease in the time taken to achieve maximum temperature in large and medium heaps but an increase in small heaps and windrows (Figure 7.14 Left). In the same figure it can be seen that there was a significant reduction in the spread of maximum temperatures amongst the heaps of different sizes from around 40˚C in uncovered heaps to ~20˚C in covered heaps.

These effects are mostly likely explained by the effects of covering on moisture translocation within the heap, and the availability of oxygen for aerobic thermophile activity. The increased supply of moisture at the surface of covered heaps did not affect temperatures at 5cm

suggesting that cooling and heating effects of this were in balance. The depression in temperature seen in covered heaps after turning, mostly deep in the heap, and late in the experiment is suggestive of lack of air deep in the piles due to reduced gas exchange at the surface. This meant that covering reduced the ability of heaps to benefit from turning.

In experiment 2.1 with large heaps of moisture content of 22.3% covering also had little overall effect other than accelerating the onset of maximum temperatures as in the present experiment. On the other hand with very dry litter in experiment 2.2.1 (15.0%) during mid winter in a cold climate covering led to a marked increase in mean temperature by 9.7˚C despite a pronounced cooling of the surface by ~7˚C. Adding moisture to the heap removed the benefits of covering in that experiment. Taken together, these results suggest that significant beneficial effects of covering are only likely with very dry litter under cold conditions, and possibly only in smaller heaps. These conditions are probably rarely met in practice in industry.

7.4.3 Effects of turning on temperature

In a previous CRC project we found that turning led to a 2–3˚C increase in litter temperature following turning in large heaps on one farm, but no effect on windrows on another farm (Walkden-Brown *et al.* 2010a). In the present experiment there was no overall beneficial effect of turning on overall temperatures, and no strong association with heap size. Overall, turning led to a significant reduction in temperatures on the day following turning (day 4), followed by a rebound in temperatures to exceed those of unturned heaps on days 6 and 7. These two effects cancelled each other out. Turning did lead to an overall increase in maximum temperature by 2˚C with the beneficial effect mostly in the smaller heaps and in uncovered heaps (Figure 7.12). These results clearly indicate that under the conditions of this experiment, turning did not provide sufficient increases in temperature to warrant the effort involved in doing it. Furthermore with pressure for short turnaround times and the work on the present project showing that pasteurising temperatures are achieved at comparatively shallow depths, the rationale for turning simply to mix the litter is weakened.

7.4.4 Effects of heap size/shape on temperature

In a previous CRC project we found major differences in temperature profiles between large heaps and windrows, that suggested that the size and/or shape of heaps may influence the temperatures achieved (Walkden-Brown *et al.* 2010a). On one farm windrows achieved similar peak temperatures as large heaps, but exhibited a decline in temperatures between

days 4 and 9 while heaps maintained or increased temperatures. On a second farm with windrows only, the temperatures attained were lower, but they were maintained for 10 days rather than decreasing. It stands to reason that differences in the size and/or shape of heaps are likely to influence the extent of aeration of heaps, the rate of drying of parts of the heap, and the insulating properties of the whole heap.

In the present experiment heap size did not have an overall effect on mean temperature, although there were many interactions with other factors. Small heaps exhibited a significantly faster increase in temperature and maintained that between days 2 and 6 (Figure 7.16 Top Right). Interestingly this was not the case with windrows which behaved more like the larger heaps. The smaller heaps and windrows tended to be cooler at 5cm and warmer at 50 and 100cm than the larger heaps with little difference at 25˚C (Figure 7.17 Centre). This contrasting effect at different depths explains the lack of a significant overall effect.

With regards mean time above 55˚C however, there was a significant overall effect of heap size with small and medium heaps having longer periods above this temperature than large heaps and windrows (Figure 7.9 Centre Left). A similar pattern was observed with maximum temperatures with small heaps having significantly higher maxima than windrows by 3.7˚C (Figure 7.11 Centre Left). Small heaps and windrows also achieved their maximum temperature in less time than large heaps, with medium heaps intermediate (Figure 7.13 Centre Left).

In Experiment 2.1 which had large litter heaps, litter temperatures achieved slightly lower maximum temperatures than those achieved in the small heaps used in Experiment 2.2.1 but the temperatures were maintained for much longer. This can be clearly seen by comparing Figure 5.5 with Figure 6.1 (Right).

Collectively these results suggest that smaller heaps heat quickly to higher maximum temperatures, but then cool somewhat more quickly than larger heaps. Thus the shorter the time available for pasteurisation, the greater the advantage to smaller heaps. This benefit was not evident in the windrows in the present experiment which remained cooler than small heaps throughout and like them, began to decline in temperature late in the experiment (Figure 7.8, Left). This may be due to a greater surface area for the limited mass, resulting in excessive heat loss and drying of the outer surfaces.

7.4.5 Temperature distribution within the heaps

This experiment differs from the previous two experiments because it excluded surface temperatures and included a 100 cm deep measurement. However the effects of depth in the heap are broadly similar to those reported for the earlier experiments in that increasing depth in the heap greatly slows the increase in temperature, but leads to more sustained temperatures. In the present experiment temperatures at 5cm depth maintained a temperature of around 55˚C for the duration of the experiment whereas in the earlier experiments declines in temperature were evident at this depth. This may be due to the fact that the piles were in modern insulated sealed sheds in late spring with relatively warm shed temperatures (20-30˚C, Figure 7.8) Profiles at 25 and 50cm in the present experiment were very similar to those at 20 and 50cm in Experiment 2.1, neither of which showed the decline in temperature after day 3 evident at these depths in Experiment 2.2.1. Temperatures were very slow to increase at 100 cm depth being up to 20˚C cooler than some other layers on day 2 and only reaching 55˚C on day 7.

One anticipated effect of covers was a reduction in the range of temperatures observed across the different depths, ie an improved temperature distribution within the heap. However this was not achieved, possibly because the cooling effect of covers deep in the heap.

7.4.6 Moisture effects on temperature

Although moisture was not manipulated systematically in the present experiment the initial and final moisture contents at different depths were measured, and mean initial moisture content of heaps was fitted as a covariate in the analyses. Initial moisture content only had a significant effect on Maximum temperature with each unit increase in litter moisture content (%) increasing time to maximum temperature by 2.3 ± 1.1 hr (P=0.03). It is not clear how this was mediated, but possibly by increased sweating under the covers or by restricting aeration deeper in the heap. There was a strong trend (P=0.09) towards an effect of initial moisture content on mean temperature in the repeated measures model with each unit increase in litter moisture content (%) decreasing mean temperature by 0.33 ± 0.18 ˚C. The negative association is somewhat surprising given the very good response to additional moisture obtained in uncovered heaps in Experiment 2.2.1, but consistent with the lack of major moisture effects in the slightly damper litter in Experiment 2.1. In the outright association between variables reported in Table 7-7 heap initial moisture content was significantly negatively associated with mean heap temperature, and hours above 55˚C, and positively associated with time to maximum temperature, all indicative of a negative effect on heat generation within the heap.

7.4.7 Summary of major findings

1. Covering heaps with initial moisture content of (18.3%) led to a very large increase in moisture content at the surface clearly visible as wet material from “sweating” under the covers.
2. Covering reduced drying out of the litter at 5cm depths but not deeper levels in the heap.
3. The overall effect of heaping on moisture loss in uncovered heaps was modest, being a 1.9 percentage point reduction in moisture content over 7 days.
4. Higher initial moisture content of the litter was associated with an increase in time to achieve maximum temperature and a trend towards lower mean temperatures.
5. There were no strong beneficial effects of covering heaps on litter temperature. Effects were subject to other effects in the experiment that tended to cancel out the overall effect. These results plus those of the two previous experiments suggest that significant beneficial effects of covering are only likely with very dry litter under cold conditions, and possibly only in smaller heaps.
6. Turning of litter on day 3 reduced temperatures markedly on day 4 and increased them on days 6 and 7 with these two effects cancelled each other out over a 7-day pasteurisation period. The shorter the period of litter pasteurisation the less the benefit from turning.
7. Sustained elevated temperatures at the 5cm depth in this experiment also provide less incentive to turn heaps, by demonstrating that the “cool rind” of the heap is very thin under these conditions.
8. Smaller heaps heat quickly to higher maximum temperatures, but then cool somewhat more quickly than larger heaps. Thus the shorter the time available for pasteurisation, the greater the advantage to smaller heaps.
9. This benefit was not evident in windrows which remained cooler than small heaps throughout and also began to decline in temperature late in the experiment.

7.5 Summary and comparison of results from the 3 major litter experiments

To tease out the differences in results between the 3 main on farm litter experiments, the conditions under which the experiments were run, temperatures achieved at 20-25cm depth and the most important effects of the treatments applied are summarised below.

7.5.1 Experiment 2.1 – Sydney

This experiment involved 2nd use litter with initial moisture content of 22% in large heaps in late Autumn (late May) in Sydney Treatments included water addition and covering. Over all treatment groups heaped litter reached peak temperatures at 20 cm depth of 58.3˚C in 100hrs (4 days) with temperature at that depth exceeding 55˚C for 100 hours or 60% of the week-long duration. The average temperature at that depth was 52.9˚C.

- Covers had no overall effect on mean heap temperatures. There were subtle depth-dependent increases in temperature, but only in heaps with no additional moisture.
- Increasing moisture content from 22% to 28% or 34% induced no overall increase in temperature, but resulted in slightly warmer outer layers and removed the beneficial effect of covering. Moisture addition led to significant ammonia problems with the batch of chickens subsequently placed on the litter.
- Thus neither covering nor additional moisture had a significant overall effect on temperature. The interaction between them was also non-significant.

7.5.2 Experiment 2.2.1 – UNE/Kirby

This experiment used single use litter that had had a low bird density on it for only 42 days, so it probably had a higher C:N ratio than standard single use litter or 2nd use litter. Initial moisture content was approximately 16%. The experiment used small heaps in winter (early June) in an uninsulated shed in Armidale. Mean daily temperatures within the shed were 12.6˚C. Treatments included water addition and covering. Over all treatment groups heaped litter reached peak temperatures at 20 cm depth of 62.4˚C in 44 hrs (<2 days) with temperature at that depth exceeding 55˚C for 78 hours or 46% of the week long duration. The average temperature at that depth was 52.9˚C, exactly the same as for Experiment 2.1. So it was a quicker rise to a higher peak than in Expt 2.1 but with high temperatures not sustained for as long.

- Covers increased overall temperatures by approximately 10˚C in unwatered heaps but had no effect on overall temperature on heaps in which moisture content was increased to approximately 28%. There was a modest depth-dependent increase in temperature, but only in heaps with no additional moisture.
- Increasing moisture level from approximately 16% to approximately 28% induced an increase in overall temperature of approximately 9˚C but only in uncovered heaps.
- Covering and addition of 12% extra moisture under these conditions induced increases in temperature of similar magnitude but they were not additive.

7.5.3 Experiment 2.3 – Tamworth

This experiment used single use litter with mean initial moisture content of 18.3%. The experiment used heaps of a wide range of sizes in insulated tunnel ventilated sheds in late spring (late November) in Tamworth. Mean daily temperatures within the shed were 25.5 ˚C (Mean min. 19.5˚C, Mean Max. 33.3˚C) with a steady increase in shed temperature during the experiment. Treatments included covering, turning litter on day 3 and different heap shapes and sizes. Over all treatment groups heaped litter reached peak temperatures at 25 cm depth of 63˚C in 139 hrs (5.8 days) with temperature at that depth exceeding 55˚C for 121 hours or 72% of the week long duration. The average temperature at that depth was 56.1˚C, or 3.2˚C higher than in the previous two experiments at a similar depth (20cm). So in this experiment there was a long rise to the highest peak temperature of the 3 experiments with the high temperature then well sustained until the end of the experiment.

- Covers induced no significant overall effect on heap temperatures.
- Moisture content of individual heaps (range 12.7 – 26.3%) was negatively associated with peak temperature and overall mean temperature.
- Turning of litter on day 3 had no significant overall effect on heap temperatures. It led to a significant reduction in temperature on the day after turning, with a rebound increase resulting in higher temperatures on days 6 and 7 with the two effects cancelling themselves out.
- Heap size and shape did not have a significant effect on overall temperatures but broadly speaking smaller heaps heated up more quickly to a higher maximum, but also cooled more quickly. They were also cooler near the surface (5cm) and warmer at depth (50-100cm) than the larger heaps. These effects are likely explained by improved aeration in smaller static heaps, but lower insulating properties.

7.5.4 Discussion and conclusions

From the results of these experiments the following conclusions can be drawn.

1. Covering heaps only produced marked increase in litter temperatures when litter was very dry (16% moisture), had a relatively low faecal (and thus N) content, heaps were small, and ambient conditions were very cold. These are the conditions of Expt 2.2.1. on a small experimental University farm. On commercial farms with a range of larger heap sizes and 1st and 2nd use litter with mean initial moisture contents of 18-22%, there was no clear beneficial effect of covering heaps. On the latter farms, reasons for the lack of a beneficial effects of covers may include:
    - Less important insulating effect of the covers (higher ambient shed temperatures and larger heaps)
    - Higher ammonia production due to higher faecal content in the litter with potentially toxic effects on microbial growth. Covers would presumably increase ammonia concentrations within the heaps
    - Smaller mean particle size and porosity due to higher faecal content and bird time/density on litter. Covers could exacerbate low O_2 availability.
    - Larger heap size and higher moisture content could also affect porosity and free air space so once again covers could exacerbate low O_2 availability.
2. Higher moisture content was only associated with a marked increase in litter temperatures when very dry litter (16% moisture) with a relatively low faecal content was brought up to 28% moisture in small heaps under very cold ambient conditions (Expt 2.2.1). Placing covers on such heaps totally ablated the beneficial effects of the additional moisture. On a commercial farm with large heap size and 2nd use litter with mean initial moisture contents of 18%, there was no clear benefit of increasing moisture content to 28% or 34% (Expt 2.1). On another farm with a range heap sizes and initial moisture contents ranging from 13-26˚C the association between initial moisture content and temperature was negative rather than positive. The findings on the commercial farms were unexpected given recommended optimum moisture contents of around 50% for composting organic materials in general, and some reports of increases in composting temperature with increasing moisture content of broiler litter (Lavergne *et al.* 2006) although the results were also very variable in the latter report. Reasons for the lack of a beneficial effects of higher moisture content in the range 20-34% on commercial farms may include:

- Reduction in porosity/free air space associated with smaller mean particle size and smaller heaps than in Expt 2.2.1. Pressure from large heaps may exacerbate the negative effects of moisture on free air space.
- Enhanced ammonia production due to higher faecal content in the litter and the additional moisture with potentially toxic effects on microbial growth. This could possibly explain the wide disparity between optimum moisture levels recommended for composting most organic materials with relatively low N content, compared with broiler litter. Ammonia has been shown to be effective at reducing on the survival of *E. coli* O157:H7 and *Salmonella Typhimurium* in cattle manure inducing reductions of up to 6 logs in 6 hrs at 378˚C (Park and Diez-Gonzalez 2003).

Small particle size, reduced porosity and elevated ammonia production may also contribute to the observed difference in moisture content recommendations for more bulky wastes, and the observations in poultry litter.

3. There are few beneficial effects on temperature of turning litter on day 3 if total pasteurisation time is 7 days as in Expt 2.3. At shorter pasteurisation times, effects are likely to be negative.
4. Smaller heaps heat up more quickly to higher maximum temperatures than larger heaps but also cool more quickly. Thus there is should be an inverse relationship between turnaround time and heap size for pasteurisation. The shorter the pasteurisation the more effective smaller heaps will be.

8 Strand 2. Standard Operating Procedure for Litter Pasteurisation

Based on the available literature, Australian experience and research covered in the previous section the following SOP is proposed for litter pasteurisation.

8.1 Objective

- To markedly reduce pathogen load in litter by exposure to raised temperatures and ammonia concentrations.
- To achieve this by rapidly and uniformly heating the heaped litter to 55˚C for 3 days or more. This will greatly reduce pathogen load but not eliminate it.

8.2 Guiding Principles

Most of the heat and ammonia in heaped litter are generated by a mixture of aerobic mesophilic and thermophilic bacteria and fungi which require nutrients, water, oxygen and a suitable pH. The closer to optimum these conditions are, the quicker and higher the temperatures achieved. Some of the background and guiding principles for the process are summarised below:

8.2.1 Microbial basis for generating pasteurising temperatures

The pasteurising heats of 50˚C and above obtained in heaped poultry litter above are due largely to aerobic microbial activity requiring oxygen. Aerobic oxidation of carbon substrates releases large amounts of heat energy, water and CO_2 while anaerobic fermentation yields much less energy and CO_2 and large amounts of energy rich methane (CH_4) (Haug 1993a). Aerobic oxidation of glucose produces 677 kcal/mol of energy while anaerobic oxidation produces only 96 kcal/mol of energy. Putrefactive odours are a common by-product bacteria of anoxic and anaerobic composting with sulphur and nitrogen acting as electron acceptors rather oxygen.

The following description of the microbial kinetics during composting is adapted from (Beffa *et al.* 1996). A large variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms (including bacteria, actinomycetes, yeasts, molds and various other fungi) have been reported in composting and other self-heating organic materials at temperatures between 20-60°C. At an early phase of the composting process (temperatures between 20-40°C) mesophilic/thermotolerant fungi, principally yeasts and molds, and acid producing

bacteria are the dominant active degraders of fresh organic waste. Mesophilic bacteria (which prefer temperatures of 20-40˚C) are predominant in the early stages of the process, soon giving way to thermophilic (high temperature) bacteria, which inhabit all parts of the stack where the temperature is satisfactory. Thermophilic fungi usually appear after 5 to 10 days followed by actinomycetes. Mesophilic microorganisms are killed or inactivated during the initial thermogenic stage (temperatures between 40-60°C), where the number and species diversity of thermophilic/thermotolerant bacteria, actinomycetes and fungi increase. The optimal temperature for thermophilic fungi is 40-55°C, with a maximum at 60-62°C. Fungi are killed or are present as spores at temperatures above 60°C. Thermophilic actinomycetes are generally more tolerant than fungi to high temperatures but at temperatures above 60°C their number and the species diversity also decreases, and their importance in the degradation process becomes negligible. Thermophilic bacteria are very active at 50-60°C, and at temperatures above 60°C the degradation process is performed essentially by these microorganisms.

8.2.2 Target temperatures and durations

Heat inactivation of pathogens relies on complex time-temperature relationships (Haug 1993a). Higher temperatures require shorter periods to cause inactivation. Moist heat and higher levels of hydration are more effective than dry heat, or low moisture microbial forms. In general parasites and their eggs are inactivated more readily than bacteria which in turn are inactivated more readily than viruses although there is wide variation and overlap between these. Heat in the temperature range able to be achieved during thermophilic litter composting is a potentially effective means of inactivating many pathogens including viruses, bacteria, fungi, protozoa and metzoan parasites. Exceptions include prions, bacterial spores (genera *Clostridia* and *Bacillus*) and the eggs of some helminth parasites and certain protozoal cysts. In general, vegetative bacteria are destroyed after 5-10 min at 60-70˚C and pasteurization at 70˚C for 30 minutes destroys most pathogens (including viruses) found in sewage sludge (Haug 1993a).

Temperature acts primarily by denaturing proteins and irreversible protein cross-linking and coagulation after denaturation is solvent dependent, requiring higher temperatures as material becomes more desiccated. This likely explains both the greater efficacy of moist heat over dry heat for sterilization and the extended survival of very resistant life forms such as spores, lyophilized virus etc. Viruses which are non cellular, are inactivated by a) the collapse mechanism leading to breakdown of hydrogen bonds and collapse of the secondary structure of DNA or protein capsid (DNA viruses) or b) the chain break mechanism resulting in a break or change the nucleic acid chain at a single point following some chemical reaction (mostly RNA viruses) (Woese 1960).

Regarding bacterial inactivation in litter using composting, the thermophilic temperatures acheived are well above the thermal death points of mesophilic pathogens, such as *E. coli* O157:H7 and *Salmonella* spp. (Chen and Jiang 2014). In several studies enteric bacteria such as *Salmonella* spp., *E. coli*, *Campylobacter* spp., vegetative *Clostridum perfringens* and *Listeria monocytogenes* were undetectable in composted litter or reduced to undetectable levels by poultry litter composting (Brodie *et al.* 1994; Kwak *et al.* 2005; Macklin *et al.* 2008; Silva *et al.* 2009).

The die-off of pathogens during composting may not be uniform and persistence of pathogens in poultry compost has also been reported in many studies with the surface of fresh compost being identified as the critical location for pathogens to extend survival (Chen and Jiang 2014). In open air full composting environments regrowth of bacterial pathogens due to the recontamination is a risk but this is not a major risk for short term litter pasteurization in sheds.

Regarding virus inactivation in poultry litter Newcastle disease virus and avian influenza virus in chicken faeces, feed and litter in porous nylon bags were inactivated by day 3 in composting litter which reached temperatures of 50˚C to 65˚Cby day 7 (Guan *et al.* 2009). Infectious laryngotracheitis virus was reduced to undetectable levels by normal litter composting for 5 days or heating at 38˚C for 48 hours (Giambrone *et al.* 2008). Walkden-Brown *et al.* (2010a) reported that Fowl Adenovirus 8 was largely inactivated in litter after 6–7 days of litter pasteurisation by heaping while chicken anaemia virus and infectious bursal disease virus were largely inactivated after 6-10 days. Marek's disease virus retained significant infectivity at days 9–10. There was little evidence of any litter transmission of infectious bronchitis virus or vaccinal Newcastle disease virus at all. Coccidial oocysts appeared to be inactivated by first sampling after 3 days of pasteurisation.

The selection of heating 55˚C for 3 days in this SOP is based on this information and broad guidelines provided in USA and Australian regulations relating to the inactivation of pathogens in sewage sludges and composts as summarised below.

8.2.2.1 USA – Environmental Protection Agency (EPA)

In its Part 503 Biosolids Rule the EPA differentiates between Class A and Class B treated sludges (EPA 2012). If pathogens (*Salmonella* sp. bacteria, enteric viruses, and viable helminth ova) are below detectable levels, the biosolids meet the Class A designation. Biosolids are designated Class B if pathogens are detectable but have been reduced to levels that do not pose a threat to public health and the environment as long as actions are taken to prevent exposure to the biosolids after their use or disposal. To meet Class A and B conditions using composting the following requirements must be met (EPA 2012):

- **Class A.** Using either the within-vessel composting method or the static aerated pile composting method the temperature of the biosolids is maintained at 55˚C or higher for 3 days. Using the windrow composting method, the temperature of the biosolids is maintained at 55°C or higher for 15 days or longer. During the period when the compost is maintained at 55°C or higher, the windrow is turned a minimum of five times.
- **Class B**. Using either the within-vessel, static aerated pile, or windrow composting methods, the temperature of the biosolids is raised to 40˚C or higher and maintained for 5 days. For 4 hours during the 5-day period, the temperature in the compost pile exceeds 55°C.

8.2.2.2 Australia – Standards Australia

Clause 3.2.la) of the AS4454 Australian Standard on Compost and Produce Standards in Australia (Standards Australia 2012) specifies the following process criteria for pasteurisation on the basis of all material being subjected to sufficiently high temperature for a sufficient duration to cause thermal death:

- Appropriate turning of outer material to the inside of the compost pile/windrow so the whole mass is subjected to a minimum of three turns with the internal temperature reaching a minimum of 55˚C for three consecutive days before each turn
- Where higher risk materials are included in the compost feedstock (including manures, animal waste, food or grease trap wastes) the core temperature of the compost mass shall be maintained at 55˚C or higher for a period of 15 days or longer; and during this period of high temperature the compost pile/windrow shall be turned a minimum of five times (consistent with US EPA 503 Rule).

8.2.3 Chemical composition

A carbon to nitrogen ratio (C:N) between 15 and 30 is recommended. Above 30 microbial growth is impaired. Below 15, high temperatures are achieved but nitrogen is in excess and given off as ammonia. Broiler litter typically has a C:N of 10-15 and this ratio reduces with increased litter reuse. Adding a high C low N source will reduce ammonia emissions.

8.2.4 Moisture Content

Optimum moisture content in most compostable material is generally between 40 and 60%. Excessive moisture limits porosity and oxygen availability and insufficient inhibits microbial growth. Used poultry litter typically has a dry matter content of 20-35%. Poultry CRC research in Australia has shown variable temperature responses to moisture addition to litter, with significant temperature responses only seen in very dry litter (<20% moisture content).

In the USA on farm responses to moisture addition to litter of 25-26% initial moisture content have also been variable with increasing moisture level often not producing the expected increased temperatures and creating subsequent issues related to ammonia production and litter and caking (Lavergne *et al.* 2006). Excessive ammonia production following addition of water to litter to bring moisture content up to 28-34% has also been observed in Australia. It is possible that excessive ammonia production during poultry litter composting inhibits thermophilic bacteria at lower moisture contents than optimal for other materials with a higher C:N ratio. A guide to estimating moisture content of litter provided in Table 1.

Figure 8.1. Descriptions provided to litter of different moisture contents (McGahan *et al.* 2014)

Description	Moisture content (%)
Dusty	<15
Dry to friable	15-20
Friable to moist	20-30
Sticky, beginning to cake	30-40
Wet and sticky, heavy caking	40-50
Very wet and sticky	>50

8.2.5 Oxygen

Pasteurisation in heaped litter is largely an aerobic process as outlined above. Oxygen can become limiting if particle size is small and porosity poor, or if moisture content is too high. Compression in large heaps will reduce porosity and oxygen availability deep in heap or windrow. Turning increases oxygen availability but cools the heap significantly producing a saw-tooth like temperature profile.

8.2.6 pH

Compost microorganisms operate best under neutral to acidic conditions, with pH's in the range of 5.5 to 8 although pH up to 9 supports adequate microbial composting. Composting chicken litter tends to be slightly alkaline (pH > 8) and to acidify slightly during the composting process. Ammonia production increases rapidly as pH increases above 8. The composting process is somewhat self-buffering and deliberate modification of pH is rarely justified.

8.3 Litter pasteurisation practices

There are a number of practices that can be implemented to potentially influence litter pasteurisation.

8.3.1 Cake removal (de-caking)

Litter cake is typically removed to reduce moisture content and condition litter. Removing cake is also likely to increase the C:N ratio. Cake removal may be done before or after heaping and pasteurisation. The effects of inclusion or removal of cake on the thermal properties of heaped litter have not been investigated. However recent Poultry CRC research (Experiment 2.3 in this report) has shown that pasteurisation for 7 days had no effect on reducing the size of cake pieces. It is probably more practical and beneficial to decake prior to pasteurisation.

8.3.2 Turning of litter to improve aeration and mix layers

Long composting cycles typically involve turning, both to aerate the core and to mix in the cooler drier outer layers to produce a more uniform product. The benefits of turning for shorter litter pasteurisation periods is less clear. Poultry CRC research in Australia has shown that over a 9-day period, turning large heaps (height 2.5-2.8 m) at day 3 resulted in a sustained increase in mean temperature following turning whereas there was no benefit in turning smaller windrows (height 0.8-1.2m) (Walkden-Brown *et al.* 2010a). However more recent work over a shorter 7-day pasteurisation period and a range of heap sizes presented in this report has shown that turning of litter on day 3 led to a significant reduction in temperature on the day after turning, with a rebound increase resulting in higher temperatures on days 6 and 7 with the two effects cancelling themselves out. The imperative to mix to ensure more uniform exposure to pasteurising temperatures is also reduced with short pasteurisation times as shown in the same CRC studies. Over a 7-day pasteurisation period in heaps of a wide range of sizes the time spent at 55˚C or higher was significantly greater at a depth of 5cm from the surface than at 100 cm deep in the heap. This indicates that the thin, cool “rind” on pasteurised litter heaps is thin.

8.3.3 Covering heaps with tarpaulins

In the three on-farm studies covered in recent CRC research presented in this report significant overall benefits of covering on temperatures were only seen under conditions when litter was very dry (16% moisture), heaps were very small, and ambient conditions were very cold (mean temperature in the shed of 12.6˚C). Under higher moisture conditions in the same experiment there was no beneficial effect of covering. Under a wider range of conditions on two other commercial farms, no major benefit of covering was observed. This contrasts with a single report from the USA in which covering increased temperatures in heaps of higher moisture content (37-40%) but only following moisture addition. Ambient temperatures were as cold as those seen in the Australian study in which a response to covering was observed.

8.3.4 Passive and forced aeration

Passively aerated systems are designed to eliminate the need for physical turning during normal composting. Aeration is achieved through perforated plastic pipes embedded at 12- to 18- inch intervals in the base of each windrow. Air is drawn into the pipes from outside the pile and is forced through the pile from the chimney effect created by the hot gases escaping from the windrow. The effects of such systems on temperature profiles in pasteurizing litter have not been evaluated.

Forced aeration involves air being forced through the pile from the base through a system of pipes. This can speed up the whole compost process but is capital-intensive. One small CRC study (Expt 2.2.2 in this report) investigated the effects of forced ventilation on temperatures in heaped litter and found that while aeration led to increases in mean temperatures over 7 days of 3.1 to 9˚C at 0, 5 and 50 cm depths, but not 10cm.

8.3.5 Addition of water

As noted in section 8.2.3 addition of moisture to heaped litter has had very variable results in both Australia and the USA with negative effects on final litter moisture and ammonia production from the pasteurised litter. The one unequivocal improvement observed in our studies involved very dry litter (16% moisture) in which increasing moisture content to 28% led to a large increase in average temperatures of 8.9˚C. In covered heaps in the same experiment no response to additional moisture was observed demonstrating that the two effects are not additive. On a commercial farm with large heap sizes and 2nd use litter with mean initial moisture content of 18%, there was no clear beneficial increasing moisture content to 28% or 34%. Significant adverse effects due to excessive ammonia were observed in chicks reared on pasteurised litter including that from the high moisture treatments. On another farm with a range heap sizes and initial moisture contents ranging from 13-26˚C the association between initial moisture content and temperatures during pasteurisation was negative rather than positive.

8.4 Method

1. Record the disease status of the last placement on the litter to be pasteurised and note any unusual disease problems encountered. Extend pasteurisation period or opt for full cleanout and new litter following a major infectious disease problem.
2. Record the period of time available for pasteurisation. Longer times will result in a greater reduction of pathogens.

   a. If the time available is 9 days or longer using larger heaps (height ~2m) and turning the litter treatment at day 3 or 4 will result in higher temperatures.

   b. If the time available is less than 9 days, smaller heaps or windrows will provide higher mean temperatures and turning is unlikely to provide additional thermal inactivation of pathogens. A heap or windrow height of ~1m is appropriate for pasteurisation periods of 7 days. For shorter periods even smaller heaps may be contemplated.

3. Estimate the moisture content of the litter to be pasteurised. Use Fig 8.1 as a guide.

   a. If moisture content is very low (dusty litter), litter is first use, and external conditions are cold (mean temperature <15˚C) consider addition of 5% moisture OR covering the heaped litter with tarpaulins to improve pasteurisation. Do not do both.

4. If possible de-cake the litter and heap or windrow the decaked litter using available equipment.

5. Limit ventilation to maintain temperatures in the shed but be aware of the increase in ammonia concentrations that this will incur.

6. Use a datalogger or temperature probe to record temperatures at 25 cm depth in the heap daily. Temperatures should exceed 55˚C on at least 3 of the daily measurements.

7. Spread the litter and ventilate for a minimum of two days prior to chick placement. Consider incorporation of an effective litter amendment at this point to reduce ammonia production during brooding, particularly if the litter is moist (>25% moisture).

8. Pre-heat for 24 hr before brooding to drive off further ammonia and provide a warm dry insulating surface for the chicks.

9 Strand 3. Kinetics of shedding of FAdV-8, IBDV, ILTV and CAV in faeces

9.1 Introduction

To determine the survival of viral pathogens in litter, the shedding patterns of the virus in litter must be determined so that litter can be obtained at or around the time of peak shedding. This pattern may differ due to the presence or absence of maternal antibody. This section of the report presents results of shedding profiles in faeces of FAdV-8, IBDV, ILTV and CAV in faeces in both maternal antibody free SPF chicks, and in broiler chickens infected at days 3 and 16 of age, with maternal antibody levels expected to be in decline by the latter infection date. Detailed information on the shedding profiles of MDV in dander of commercial broiler (Islam and Walkden-Brown 2007) commercial layer (Islam *et al.* 2014) and SPF chickens (Islam *et al.* 2013c) are available from within our research group so the focus in this work was on the other 4 viruses under study.

Specific objectives for this activity were:

- To determine the shedding profiles in faeces of FAdV-8, IBDV, ILTV and CAV vaccine and/or field strains in faeces of maternal antibody free SPF chickens
- To determine the shedding profiles in faeces of FAdV-8, IBDV, ILTV and CAV vaccine and/or field strains in faeces of commercial broiler chickens infected at two ages to assess the effect of maternal antibody on shedding profiles.
- Provide infective litter for subsequent experiments on virus survival in litter

The SPF experiment (Experiment 9.1) was carried out in the isolator facility at UNE while the broiler experiment (Experiment 9.2) was carried out in the isolation pens at UNE. This experiment produced the infective litter used in Chapter 10 to determine effects of temperature and time on infectivity of these viruses in litter.

The experiments were supervised and implemented by Dr Fakhrul Islam with assistance from students working on each of the virues, namely Robin Achari for FAdV, Kanchana Jayasundara for IBDV and Mamdouh Alsharari for CAV. Visiting Scientist Prof. Parimal Roy carried out serology for ILTV under supervision of Sue Burgess. Sue Burgess performed qPCR assays for MDV, ILTV and CAV and assisted students with assays for the other viruses. This report contains data on faecal shedding and serological responses to infection. For most viruses other measurements were also made and these will be reported as the work is completed and formally published.

9.2 Materials and methods

9.2.1 Experiment 9.1 Determination of shedding profiles of Fowl adenovirus 8 (FAdV), infectious bursal disease virus (IBDV), infectious laryngotracheitis virus (ILTV) and chicken anaemia virus (CAV), in SPF chickens (Expt. LT13-C-Shed1)

AEC No: AEC13-045 **Start date:** 12/7/2013 **End date:** 9/8/2013

The experiment was conducted in the UNE isolator facility. A single isolator was used to for each virus so there was no replication at isolator level and the individual chicken was the experimental unit. Isolators were fitted with solid floors with pine shavings litter (Figure 9.1). The experimental chickens comprised 135 SPF white leghorn type (Lohmann LSL classic, Valo) chickens hatched at University of New England (UNE) from SPF eggs provided by Australian SPF Services Pty Ltd, Woodend Vic, Australia.

Figure 9.1. Experiment 9.1. SPF chicks in isolators with solid floors with shavings.

Nine isolators (8 viruses + 1 negative control) were used in the experiment, 15 chickens in each. Two strains of each of viruses were used. Chickens were infected individually at day old (day 0) as described below. Six chickens from each isolator were identified using wing tags for a longitudinal component of the experiment study. The remaining chickens were serially sacrificed to determine tissue tropism. Various samples were collected at different intervals up to four weeks to quantify the viruses using qPCR. Faecal samples were collected twice a week from individual chickens. Blood sample were collected weekly for serological analysis.

Viruses used and their administration are summarised below.

- *Fowl adenovirus 8:* One field isolate from NSW DPI (EMAI FADV96, kindly provided by Dr Edla Arzey) was used. The virus supplied was from CEL cell culture in M199

with hepes supplemented with 2% foetal calf serum. The titre was 2 x 10^8 $TCID_{50}$/ mL. On Dr Arzey's recommendation 20µl of the virus was diluted (with sterile PBS) to make 4ml of the inoculum. Each chicken received 200µl of inoculum orally (2 x 10^5 $TCID_{50}$/chick).

The second strain was FAdV-8 Strain *E. surient* (Intervet FAV Vaccine). Each vial contains a freeze-dried pellet equivalent to 1000 doses of living, *E. Surient* strain of fowl adenovirus vaccine.

- *Infectious bursal disease virus*: Australian field isolates were kindly provided by Dr Sandra Sapats of CSIRO Geelong. IBDV Australian classical field strain 06/95 is a filter sterilized 20% bursal homogenate in PBS chick passage 2, 10/8/98 with a titre of ~10^7 CID_{50}/ml. IBDV Australian variant field strain 02/95 (Victorian origin) is a filter sterilized 20% bursal homogenate in PBS chick passage 3, 15/9/00 titre ~10^7 CID_{50}/ml. Each virus was diluted to 1:10 to make 5ml. Each chicken received 200µl orally, equating to a dose of ~$10^{5.3}$ CID_{50}/chick.
- *Infectious laryngotracheitis virus*: Strain SA2 was available in a vial of 1000 doses, which contains a viral dose of $10^{4.1}$ pfu per chick. The contents were diluted with sterile PBS to make 10ml. 200µl of the diluted content was given to each chicken orally, so that each chicken received 2.5 x 105^4 pfu of vaccinal virus. This dose is approximately 20 times the minimum recommended dose. The vaccine was Poulvac Laryngo SA2® batch 1201859 expiry 15 Nov 2014.

  Strain A20 was available in a vial of 2000 doses, which contains a vaccinal dose of $10^{3.5}$ pfu per chick. A20 vaccine was diluted to make 10ml of inoculum. Each chicken was given 200µl orally, so that each chicken received 1.23 x 105 pfu of vaccinal virus. This dose is approximately 40 times the minimum recommended dose. The vaccine was a Poulvac Laryngo A20® batch 1201870 expiry 08 Mar 2014.
- *Chicken anaemia virus*: CSIRO strain CAV 269/7 passage 4 was in a 1.5ml vial with potency of 10^7 $TCID_{50}$/ml. The whole content of a vial was added to 2.5ml of sterile PBS to make 4ml. Each chicken received 200µl (equivalent to $10^{5.9}$ TCID50) orally.

  CAV strain 3311(Steggles) is an old vaccine and a vial with expiry date 2000 was used. The total volume of the vaccine was 10mL, containing 1000 doses at ≥38 CID_{50}/dose. The contents were thawed and 4mL (undiluted) was used to infect the chickens, each of which received 100µL orally, thus 10 x the vaccine dose.

From the 6 individually marked birds faecal samples were collected a various regular intervals by placing individual chicks in a container inside the isolator and collecting the

resultant faeces for qPCR evaluation of faecal load in faeces. Blood was sampled on days 7, 14, 21 and 28 post infection for determination of specific antibody to each virus using ELISA kits as described in Section 3.4.7. Litter and dust samples were collected at various intervals for virus enumeration by qPCR. Three chickens from each isolator (treatment) were sacrificed to collect tissues at approximately weekly intervals for virus enumeration by qPCR.

9.2.2 Experiment 9.2 Determination of shedding profiles of FAdV-8, IBDV, ILTV and CAV in commercial broiler chickens infected at two ages (Expt. LT13-C-Shed2)

AEC No:AEC 13-118 **Start date:** 24/9/2013 **End date:** 29/10/2013

This experiment aimed to determine the shedding profiles in faeces of FAdV-8, IBDV, ILTV and CAV and in faeces of commercial broiler chickens and the effect of maternal antibody on this. A second important aim was to provide infective litter for subsequent experiments on virus survival in litter. For this purpose only MDV was included as a 5th virus in the experiment.

The experiment was conducted in the UNE isolation sheds on campus. Each is a self-contained shed with dimensions of 2.5m x 1.5 m with a wooden floor, pine shavings bedding, automatic nipple waterers and self feeders. Pens are separated by 20-30 m from each other. Six sheds were used, one for each virus plus an unchallenged control group. Five sheds (negative control, FAdV-8, IBDV, ILTV and CAV) were divided to contain birds of two ages (Figure 9.2). The remaining shed (MDV) contained birds of only one age (group 1).

Figure 9.2. Experiment 9.2 Photo of the experimental setup in one isolation shed showing the division of the pen for the birds of two ages.

Commercial Ross broiler chicks hatched in Tamworth arrived at UNE on 24/9/13 (Group 1, 90 chicks) and 10/10/2013 (Group 2, 75 chicks). From group 1, 15 birds were placed in one of 6 isolation sheds, one for each virus under test plus the negative control. In all but the MDV shed birds had access to half of the floor space with the pen divided by a wire partition. From Group 2, 15 chicks were placed in each of the 5 isolation sheds for FAdV-8, IBDV, ILTV, CAV and negative control.

MDV challenge occurred to group 1 birds 3 days after arrival (day -13) while challenge for the other viruses occurred on 10/10/13 (day 0), the date of arrival of batch 2 chicks. Chicks were thus challenged at 0 and 16 days of age on a single challenge date. Unlike experiment 9.1 only one viral strain was used for each virus with virus and challenge summarised below.

- *Fowl adenovirus 8:* The field isolate EMAI FADV96 was used. As in Experiment 9.1. Each chicken received 200µl of inoculum orally (2 x 10^5 $TCID_{50}$/chick).
- *Infectious bursal disease virus*: IBDV Australian variant field strain 02/95 was used. As in Experiment 9.1 each chick received 200µl orally, equating to a dose of ~$10^{5.3}$ CID_{50}/chick.
- *Infectious laryngotracheitis virus*: Strain SA2 was used (Poulvac Laryngo SA2®). As in Experiment 9.1 each chicken received $10^{5.4}$ pfu of vaccinal virus in 200µl.
- *Chicken anaemia virus*: CSIRO strain CAV 269/7 passage 4 was used. As in experiment 9.1 a dose of $10^{5.9}$ in 200µl was administered orally to each chick.
- *Marek's disease virus.* Approximately 5 g of isolator dust containing virulent MDV strain MPF57 collected from a previous challenge experiment and stored subsequently at -80˚C was used. On day -13, the when chicks in the MDV challenge treatment 3 days old they were placed in a ventilated box, dusted with the infective dust and left for an hour. This is an effective method for infecting chickens with MDV (Walkden-Brown *et al.* 2007).

As in experiment 9.1, six chickens from age group (except MDV) were colour marked for a longitudinal study of serology and faecal excretion of virus. Depending on the virus birds were bled at frequent intervals for determination of specific antibody to each virus using ELISA kits as described in Section 3.4.7. Faeces were collected at regular intervals by exerting gentle abdominal pressure and stimulating the area around the cloaca. Litter and dust samples were collected at various intervals for virus enumeration by qPCR. Three chickens from each isolator (treatment) were sacrificed to collect tissues for virus enumeration by qPCR at approximately weekly intervals.

At 19 days post infection (dpi) (29/10/13) litter from all 5 sheds was collected and thoroughly mixed using a cement mixer with repeated mixing to ensure all viruses were in all samples. It was bagged in 52 woven synthetic bags (Woolworths reusable grocery bags) of approximately 3 kg each.

9.3 Results

All infections were successful in both experiments as determined by detection of the relevant agent in tissues (data not shown).

9.3.1 FAdV-8

9.3.1.1 FAdV-8 antibody titres

Antibody titres are shown in Figure 9.3. In SPF chicks in Expt 9.1 challenge with FAdV-8 resulted in significant increases in titre at days 21 and 28 (P<0.0001) (Figure 9.3 Left). The effect of challenge virus was almost significant (P=0.06) with higher titres for the vaccine strain at 21 dpi. The interaction between dpi and challenge virus was not significant (P=0.3).

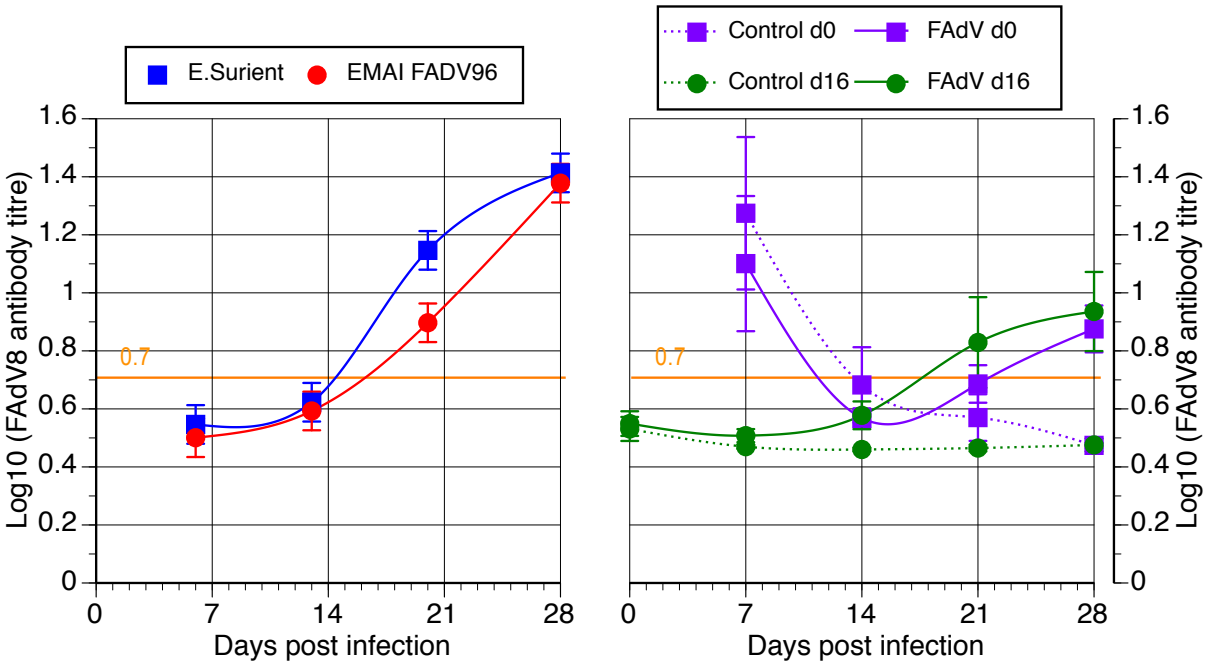

Figure 9.3. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) Log10 titres against FAdV-8 showing the effect of different challenge strains in SPF chicks (Left) and effects of challenge with EMAI FADV96 or no challenge (Control) on hatch day (d0) or 16 days of age in broiler chicks (Right). Data are not available for day 0 in chicks challenged at hatch. The orange line represents the threshold for positive samples in the serology kit used.

In broiler chicks in Experiment 9.2 unchallenged d0 control chicks showed a rapid decline in antibody to below the positive threshold at 14 dpi, continuing to decline to 28 dpi (Figure 9.3 Right). Unchallenged control chicks from the older group exhibited low titres throughout the

experiment confirming adequate biosecurity for control chickens. Birds challenged with FAdV-8 on day 0 showed a decline in antibody similar to the controls to 14 dpi, but then titres increased thereafter while continuing to decline in the controls. Birds challenged with FAdV-8 on day 16 showed a gradual increase in antibody levels, but at a slower rate than observed in the SPF chicks. Age at challenge (P=0.012), and the interactions between age at challenge x FADV challenge (P=0.025) and FADV challenge x dpi (0.021) were all significant.

9.3.1.2 FAdV-8 in faeces

FAdV viral genome copy number per gram of faeces are shown in Figure 9.4. In SPF chicks in Expt 9.1 challenge with FAdV-8 resulted in rapid increases in viral load for both viruses with peak detection in faeces on days 5 and 7 for the *E. surient* and EMAI strains respectively. Levels then declined to low, but detectable levels by days 21 and 28 (Figure 9.4 Left). The effect of dpi was highly significant (P<0.0001) but the effect of challenge virus (P=0.93) and interaction between the two effects (P=0.53) were not.

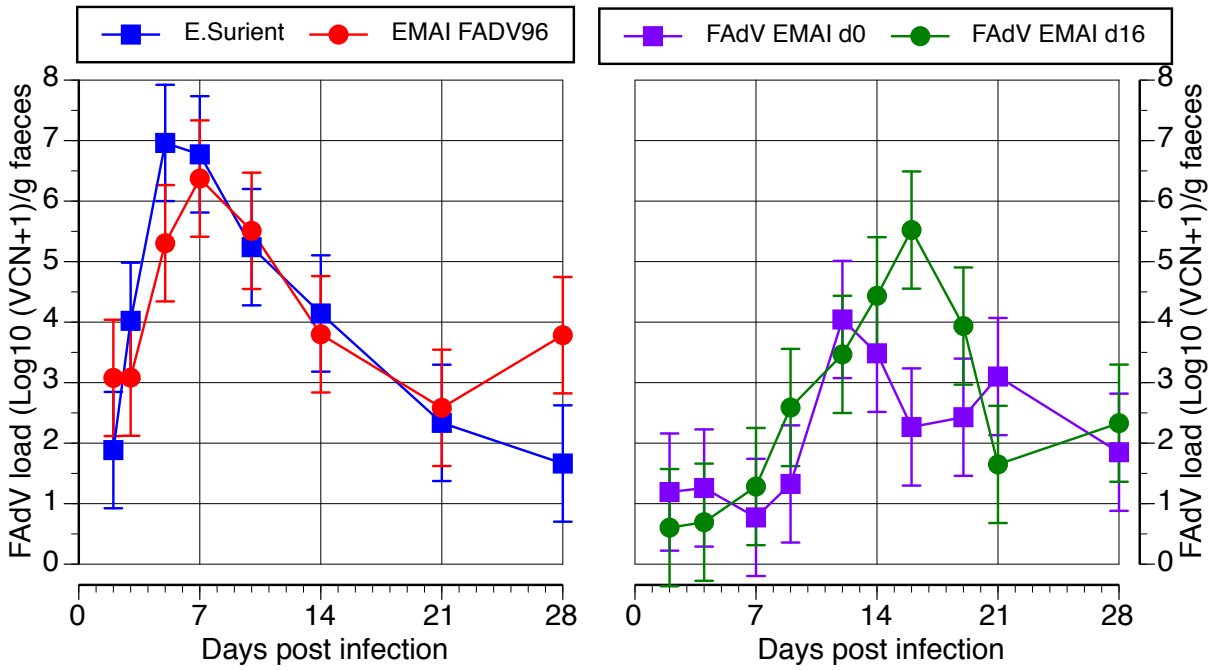

Figure 9.4. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) Log10 FAdV viral copy number per gram of faeces showing the effect of different challenge strains of FAdV-8 in SPF chicks (Left) and effects of age at challenge with EMAI FADV96 in broiler chicks (Right).

In broiler chicks in Expt 9.2 challenge with FAdV-8 at hatch or day 16 of age resulted in a much slower increase in viral load in faeces with peak detection in faeces on days 12 and 16 respectively compared with day 7 in SPF chicks (Figure 9.4 Right). Peak viral load was also lower, particularly in the chicks challenged at hatch in which it was over 2 logs lower than

that observed in SPF chicks. The effect of dpi was highly significant (P<0.001) but the effect of age at challenge (P=0.26) and interaction between the two effects (P=0.42) were not.

9.3.2 IBDV

9.3.2.1 IBDV antibody titres

Infections were successful and induced significant bursal atrophy (data not shown). Antibody titres are shown in Figure 9.5. Titres were normally distributed and did not require transformation. In SPF chicks in Expt 9.1 challenge with two isolates of IBDV resulted in significant increases in titre to day 21 in both cases (P=0.007) with significantly higher titres observed following challenge with strain 02/95 than 06/95 (P=0.002) (Figure 9.5 Left). There was no significant interaction between these two effects (P=0.92).

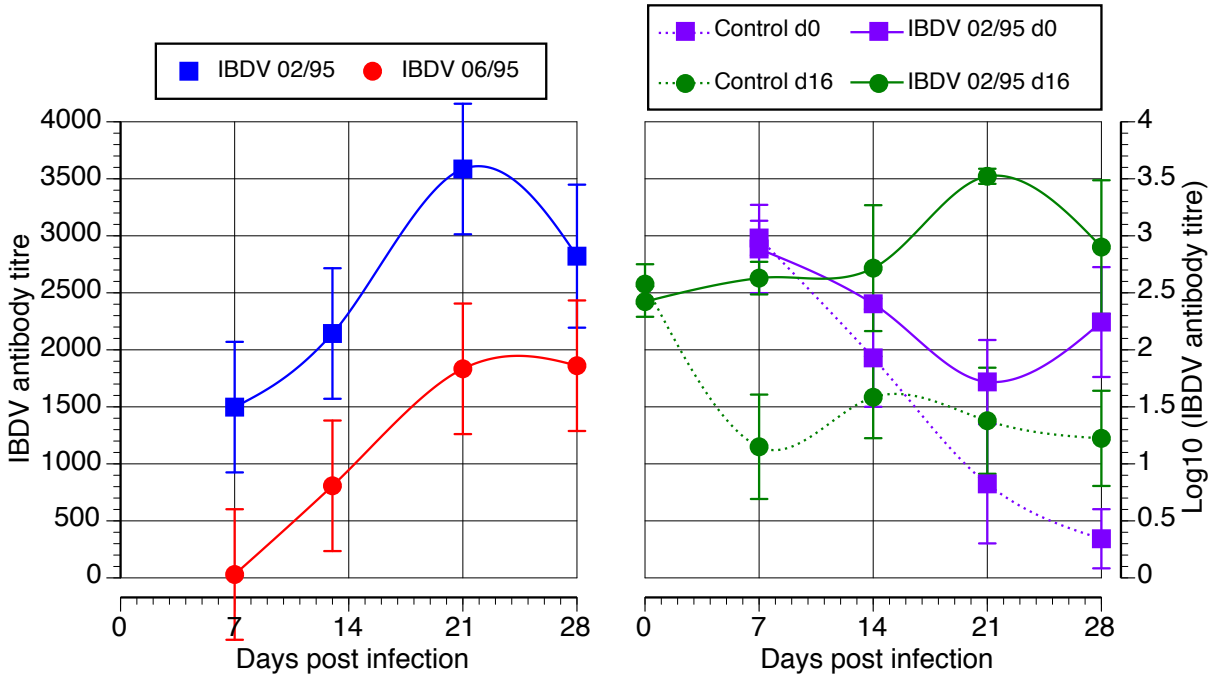

Figure 9.5. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) titres against IBDV showing the effect of different challenge strains in SPF chicks (Left) and effects of challenge with IBDV strain 02/95 or no challenge (Control) on hatch day (d0) or 16 days of age in broiler chicks (Right). Data are not available for day 0 in chicks challenged at hatch. Only data in the right panel required transformation.

In broiler chicks in Experiment 9.2 the younger unchallenged control chicks showed (d0 group) showed a steady decline in antibody to 28 days of age (Figure 9.5 Right). Unchallenged control chicks from the older group exhibited an initial decline in titre between days 0 and 7 with values varying at low levels beyond this date. Birds challenged with IBDV on day 0 showed a decline in antibody to 21 dpi at a slower rate than the controls, but titres then increased to 28 dpi while continuing to decline in the controls.

Birds challenged with IBDV on day 16 showed a gradual increase in antibody levels, at a slower rate than observed in the SPF chicks (Figure 9.5 Right). However the peak titre at 21 dpi was similar in the two groups. The effects of IBDV challenge (P=0.0008) and the interaction between IBDV challenge and dpi (P=0.014) were statistically significant but those of challenge age (0.33), dpi (0.06) and the interaction between IBDV challenge and challenge age (P=0.08) were not.

9.3.2.2 IBDV in faeces

IBDV viral genome copy number per gram of faeces are shown in Figure 9.6. In SPF chicks in Expt 9.1 challenge with IBDV of both strains resulted in peak viral load at the time of first measurement (2 dpi). Virus in faeces then reduced sharply to trace levels at 14 and 18 dpi before becoming undetectable (Figure 9.6 Left). Viral load in faeces was significantly higher for Strain 02/95 than 06/95 (P=0.015). The effect of dpi was highly significant (P<0.0001) but the interaction between challenge strain and dpi (P=0.076) were not.

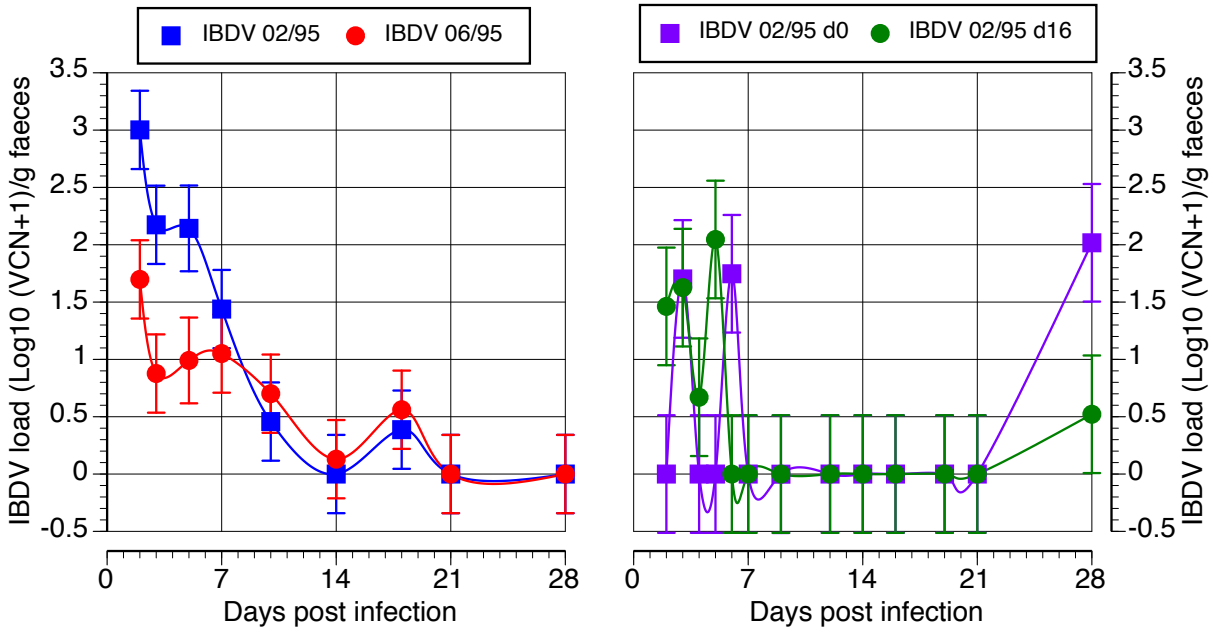

Figure 9.6. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) Log10 IBDV viral copy number per gram of faeces showing the effect of different challenge strains of IBDV in SPF chicks (Left) and effects of age at challenge with IBDV 02/95 in broiler chicks (Right).

In broiler chicks in Expt 9.2 challenge with IBDV strain 02/95 at hatch (d0) or day 16 of age resulted in a slower increase in viral load in faeces with peak detection in faeces on days 5 and 6 respectively compared with day 2 in SPF chicks (Figure 9.6 Right). Peak viral load was also lower by approximately 1 log than observed in SPF chicks. Virus declined to undetectable levels by 7 dpi and remained that way until 28 dpi when virus once again commenced being shed. The effect of dpi was highly significant (P=0.003) but the effect of

age at challenge (P=0.75) was not. However there was significant interaction between the two effects (P=0.040) reflecting higher IBDV levels in chicks challenged at 16 dpi during the first week, but the reverse being true at 28 dpi.

9.3.3 ILTV

9.3.3.1 ILTV antibody titres

Infections were successful and induced significant clinical signs of ILT in both SPF chicks and broilers, particularly those challenged at hatch. Antibody titres are shown in Figure 9.7. In SPF chicks in Expt 9.1 challenge with two vaccine strains of ILTV resulted in rapid increases in titre to day 21 in both cases (P<0.0001) with no difference in titres between strains (P=0.31) and no significant interaction between these two effects (P=0.23) (Figure 9.7 Left).

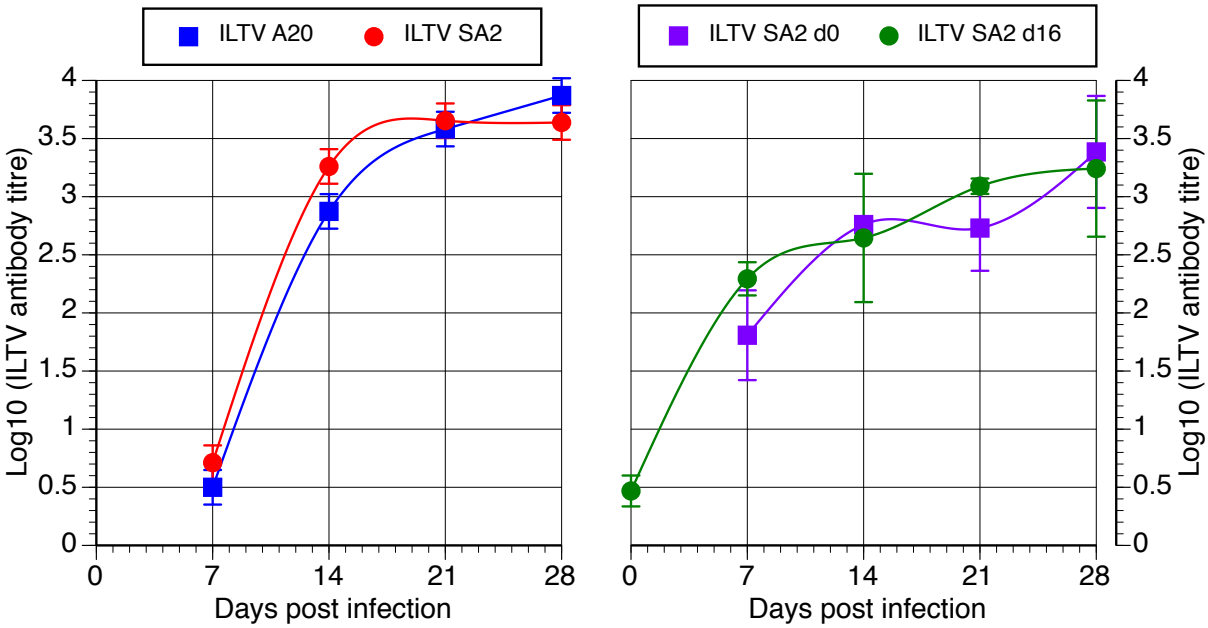

Figure 9.7. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) titres against ILTV showing the effect of different challenge strains in SPF chicks (Left) and effects of challenge with ILTV strain SA2 on hatch day (d0) or 16 days of age in broiler chicks (Right).

In broiler chicks in Experiment 9.2 there was little difference in the increase in antibody titre between chicks challenged at hatch (d0) or at 16 days of age (Figure 9.7 Right). This is suggestive of a lack of maternal antibody directed against ILTV although the increase in titre was slower in both groups than in SPF chicks and somewhat lower overall. Unfortunately sera from the unchallenged control chicks were not assayed for antibody against ILTV. The effect of dpi was highly significant (P<0.001) but the effect of age at challenge (P=0.27) was not. Neither was there significant interaction between the two effects.

9.3.3.2 ILTV in faeces

ILTV viral genome copy number per gram of faeces are shown in Figure 9.8. In SPF chicks in Expt 9.1 challenge with ILTV of both strains resulted in high viral load in faeces from the first measurement (2 dpi) with a peak in shedding at 5 dpi for both challenge strains. Virus load in faeces then reduced gradually to nadir values at 21 and 28 dpi for A20 and SA2 respectively (Figure 9.8 Left). Substantial amounts of ILTV in faeces continued to be detected at 28 dpi. The effect of dpi was highly significant (P<0.0001) but the effect of challenge strain (P=0.53) and the interaction between challenge strain and dpi (P=0.077) were not.

In broiler chicks in Expt 9.2 challenge with ILTV strain SA2 at hatch (d0) or day 16 of age resulted in a much lower levels of virus detected in faeces than in SPF chicks but peak shedding had similar timing being at days 4 and 7 for chicks challenged at days 16 and 0 respectively (Figure 9.8 Right). Peak viral load was lower by approximately 4 logs than observed in SPF chicks. Virus declined to undetectable levels by 21 dpi in chicks challenged at d0, but trace levels remained at 21 and 28 dpi in chicks challenged at 16 days of age. The effect of dpi was highly significant as was the effect of age at challenge (P<0.0001).

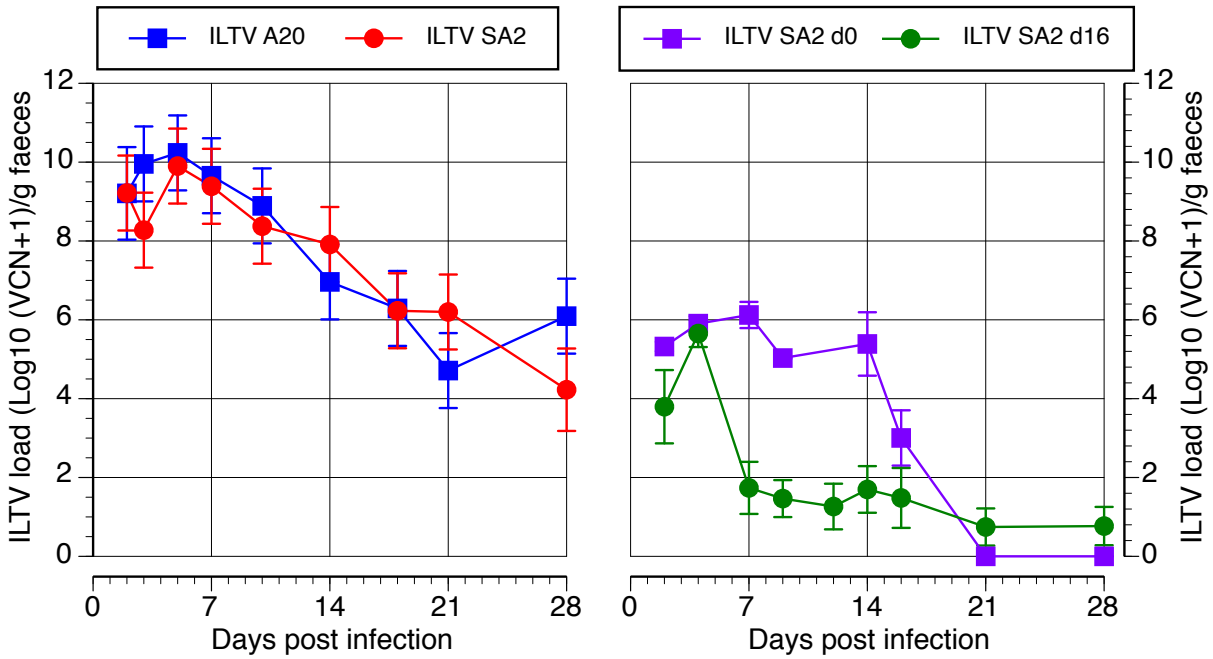

Figure 9.8. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) Log10 ILTV viral copy number per gram of faeces showing the effect of different challenge strains of ILTV in SPF chicks (Left) and effects of age at challenge with ILTV SA2 in broiler chicks (Right).

9.3.4 CAV

9.3.4.1 CAV antibody titres

Infections were successful as determined by detection of virus in target tissues. Challenge also induced a reduction in haematocrit in SPF chicks. Antibody titres are shown in Figure 9.9. In SPF chicks in Expt 9.1 challenge with two strains of CAV resulted in rapid increases in titre to between days 14 and 28 in both cases (2-2.5 logs, P<0.0001) with no difference in titres between strains (P=0.53) and no significant interaction between these two effects (P=0.14) (Figure 9.9 Left).

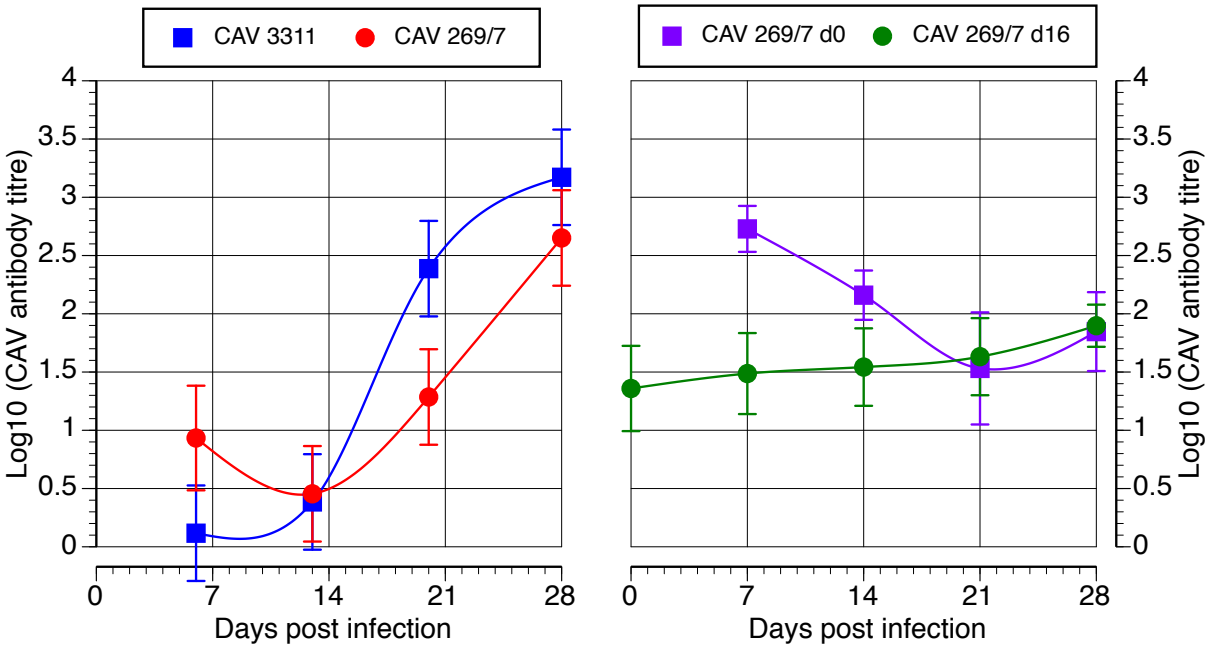

Figure 9.9. Experiments 9.1 (Left) and 9.2 (Right). Mean (± SEM) titres against CAV showing the effect of different challenge strains in SPF chicks (Left) and effects of challenge with CAV strain CSIRO 269/7 on hatch day (d0) or 16 days of age in broiler chicks (Right).

In broiler chicks in Experiment 9.2 chicks challenged at hatch (d0) with CSIRO CAV strain 269/7 clearly had maternal antibody present and antibody levels declined to 21 dpi before increasing slightly by 28 dpi. Challenge in older birds (16 days of age) resulted in a slow increase in titre, much more gradual than that seen in SPF chicks (Figure 9.9 Right). The effect of age at challenge bordered on statistical significance (P=0.07) as and there was a trend towards interaction between the effects of dpi and age at challenge (P=0.15). The overall effect of dpi was not significant (P=0.46).

9.3.4.2 CAV in faeces

Unfortunately due presumably to methodological issues to date, no CAV has been detected in faeces of chicks from either experiment despite CAV detection in multiple tissues and a clear serological response to infection.

9.3.5 Discussion and conclusions

Successful infections were induced with all 4 challenge viruses for which shedding kinetics were investigated. Challenge strain differences were noted in antibody response or faecal shedding for IBDV, but not for the FAdV, ILTV and CAV strains tested. Effects of maternal antibody could be inferred from measurement of antibody titres in Experiment 9.2 and inferred from differences from the results of this experiment with that of Experiment 9.1 using SPF chicks free of maternal antibody. Clear effects of maternal antibody were evident for FAdV (delayed and lower peak in virus shedding in faeces), IBDV (delayed and lower peak in shedding) and CAV (greatly reduced CAV antibody response to infection) but not ILTV despite much lower shedding in broiler chicks than SPF chicks.

Shedding of the FAdV-8 in faeces was detected from 2 dpi consistent with the reported incubation period (Adair and Fitzgerald 2008). In both experiments the EMAI showed a non-significant increase in shedding after 21 dpi. This is consistent with a second rise in faecal virus concentration reported elsewhere observed in earlier published work (Grgic *et al.* 2006; Adair and Fitzgerald 2008). The presence of maternal antibody did not prevent infection but delayed peak faecal shedding by 7-9 days and reduced the amplitude of the peak by 1.5-3 logs depending on the amount of maternal antibody present. This has significant implications for planning of faecal collection for experiments investigating viral survival in faeces.

In the case of IBDV, the shedding of IBDV in faeces observed in our SPF experiment is broadly similar to an earlier report in infected SPF chickens using virus isolation (Takase *et al.* 1982) but the peak is earlier and the duration of shedding is longer than the 5-6 days reported by these authors, perhaps reflecting a more sensitive detection method. However it is also longer that the detection period in faeces of 5-8 days in faeces reported by (Zhao *et al.* 2013) using PCR. Strain differences influenced both the antibody level and the shedding rate in faeces with strain 02/95 having higher responses for both. As with FAdV-8 presnece of maternal antibody influenced faecal shedding delaying the peak by 3 days, reducing it by a log and shortening the period of detectable shedding from 18 days to 6 days. However as with FAdV there was evidence in broilers, but not SPF chickens of reactivation of shedding after 21 dpi.

In the case of ILTV, the shedding of IBDV in faeces observed in both experiments is novel. Levels in faeces were the highest of all the viruses tested. The two vaccine strains tested produced very similar shedding profiles and there was no evidence of maternal antibody presence or effects in the broiler chickens, despite much lower levels of virus in the faeces. The significantly lower faecal shedding observed in broiler chicks challenged at day 16 rather than at hatch, is more likely due to age resistance as has been demonstrated previously (Fahey *et al.* 1983). ILTV primarily targets the respiratory tract although in our experiments and those of (Wang *et al.* 2013) if has been found in a wide range of tissues. ILTV is thought to enter the host is via the nasal, oral or conjunctiva with the source of infection suspected to be aerosolized exudates from the respiratory tract (Seddon and Hart 1936; Bagust *et al.* 2000). Given that a wide range of tissues may be infected with the virus and transmission of ILT has been demonstrated by intra-tracheal inoculation of suspensions of liver and spleen tissue from affected birds (Beach 1931) alternate modes of shedding and transmission including transmission from faeces is possible. The origin of faecal ILTV is most likely swallowed material from the respiratory tract, although detection of the virus in kidneys by Wang *et al.* (2013) and in Experiment 9.2 means that excretion via this route is also possible. The infectivity of faecally shed ILTV and its role in the epidemiology of ILT remain to be determined.

In the case of CAV we have been unable to detect the virus in faeces despite successful infection. We are continuing to work on resolving this. It is not clear whether it is because it is not shed in faeces or we are failing to detect it. We have detected the virus widely in litter and poultry dust. CAV spreads both horizontally and vertically. Horizontal transmission is thought to be based on the presence of virus in the feces of chickens for 5-7 weeks after infection (Yuasa *et al.* 1982; Hoop 1992; Schat and van Santen 2008). However virus is present in feathers leading to the suggestion that shedding also occurs from the feather follicle epithelium (Davidson and Skoda 2005) and respiratory infection cannot be ruled out.

9.3.5.1 Conclusions

1. Shedding profiles of FAdV, IBDV, and ILTV, but not CAV in faeces were successfully defined.
2. Shedding in SPF chickens was always earlier and to a higher level than in broiler chickens.
3. Measurement of antibody levels and challenge of broilers at 2 ages provided strong evidence of a maternal antibody effect, except for ILTV where the reduced and delayed shedding in broilers is likely due to age and breed effects.

10. Strand 3. Temperature-time relationships for viral inactivation in litter and alternative methods of assessment.

10.1 Introduction

Under Australian conditions the most practical means of reducing viral pathogen load in litter between batches is through partial composting of litter in static heaps or windrows. Composting has always had an important role in the inactivation of human, animal and plant pathogens and weed seeds, and it is clear that the inactivation is due primarily to the effects of temperature during the thermophilic stage of composting (Haug 1993b; Bohm 2007) although ammonia generated during litter composting could also contribute to inactivation of some viruses (Burge 1983; Cramer *et al.* 1983). Temperature effects are moisture and time dependant, and for many of the most important bacteria, the temperature-time relationships for inactivation are well understood. For viruses temperature-time data are less complete (Islam and Walkden-Brown 2010). Detailed temperature-time information for inactivation is required for accurate prediction of inactivation of any pathogen using variable heat processes including inactivation of poultry viruses during partial composting. It is clear that there is not a universal set of time-temperature relationships for the different poultry viral pathogens, which have widely divergent thermolability (Islam and Walkden-Brown 2010).

Some progress has been made in defining temperature-time inactivation of poultry viruses during composting. Guan *et al.* (2009) described the change in nucleic acid detection and loss of infectivity over time for Newcastle disease virus (NDV) and Avian influenza virus (AIV) in litter at ambient temperatures and during composting. Avian influenza virus appears to be readily inactivated in composting litter (Lu *et al.* 2003a; Elving *et al.* 2012) as does ILTV Giambrone *et al.* (2008). Under Poultry CRC Project 06-15 a chick bioassay for detecting virus infection in litter was developed (Islam *et al.* 2013a) and used to measure the efficacy of different types of between-batch litter composting on inactivation of Marek's disease virus (MDV), Chicken anaemia virus (CAV), Fowl Adenovirus (FAdv), Infectious Bronchitis virus (IBV) and Infectious Bursal Disease virus (IBDV) at different stages of the composting process (Walkden-Brown *et al.* 2010a; Walkden-Brown *et al.* 2010b). However these studies failed to produce a comprehensive set of temperature x time interactions for inactivation of key chicken viruses.

This project aims to improve our understanding in this area by applying a range of temperatures to virus contaminated litter under controlled laboratory conditions to evaluate

the feasibility of using cheaper and more rapid PCR based measures of infectivity to replace the use of the chick bioassay.

Earlier in this project we have determined that viral nucleic acids for the key viruses under study could be detected and quantified in litter samples on which infected chickens had been maintained, using qPCR. A remaining problem is to determine whether the amplified nucleic acid material reflects the presence of live infective organisms, or inactivated organisms for which nucleic acids are still detectable. However it seems likely that persistence of inactivated viral nucleic acids in microbiologically active materials such as composting litter is short. Using intact NDV and AIV, Guan *et al.* (2009) showed that viral nucleic acids were undetectable after 3 days in composting litter but were detectable at 21 days in litter and manure maintained at room temperature. Evidence suggests that under warm, microbiologically active conditions, virus infectivity will correlate closely with qPCR quantification of viral nucleic acids.

In the experiments described below the objectives were therefore

- To determine temperature-time inactivation relationships for FAdV, IBDV, ILTV, CAV and MDV in poultry litter using the chick bioassay method; and
- To evaluate the extent to which qPCR enumeration of viral nucleic acids in litter to correlates with infectivity determined by bioassay and thus may provide a cheaper and quicker method for evaluating composting effects on pathogen survival.

These objectives were tested in three experiments:

- Experiment 10.1. Preliminary study into the effects of bag type on moisture loss and viral nucleic acid recovery from litter subjected to temperature treatments in ovens.
- Experiment 10.2 (non-SPF bioassay). Litter contaminated naturally with FAdV, IBDV, ILTV, CAV and MDV in Experiment 9.2 was subject to a range of temperatures for different times and durations and virus survival assessed in a chick bioassay using non-SPF chickens, and by viral nucleic acid enumeration by qPCR. The use of non-SPF chicks was due to failure of a hatch of SPF chick eggs at UNE.
- Experiment 10.3 (SPF bioassay). The same litter as used in experiment 10.1 was subject to a range of temperatures for different times and durations and virus survival assessed in a chick bioassay using SPF chickens, and by viral nucleic acid enumeration by qPCR.

Experiment 10.1 was carried out by Prof Parimal Roy a visiting scientist at UNE. qPCR tests for ILTV and FAdV were carried out by Sue Burgess and Robin Achari respectively.

Experiment 10.2 was supervised and implemented by Dr Fakhrul Islam with assistance from students working on each of the viruses, namely Robin Achari for FAdV, Kanchana Jayasundara for IBDV and Mamdouh Alsharari for CAV. Visiting Scientist Prof. Parimal Roy assisted with analysis of samples for ILTV. Sue Burgess performed qPCR assays for ILTV, CAV and MDV and serology for MDV and assisted students or Prof Roy with assays for the other viruses.

Experiment 10.3 was supervised and implemented by Prof Steve Walkden-Brown with assistance from students working the viruses, namely Kanchana Jayasundara for IBDV, Mamdouh Alsharari for CAV and Hai Tran Minh for MDV. Sue Burgess performed qPCR assays for ILTV, CAV and MDV and assisted students with assays for the other viruses. ELISA tests were carried out by the students or Sue Burgess.

10.2 Materials and methods

10.2.1 Experiment 10.1 Preliminary study into the effects of bag type on moisture loss and viral nucleic acid recovery from litter subjected to temperature treatments in ovens.

The experiment was conducted in December 2013. It attempted to address the issue of what sort of bag should litter be kept in when undergoing treatment in ovens, to best simulate the conditions in heaped litter. It was a 3 x 2 x 5 factorial experiment testing the following effects on bags containing 1 kg of litter placed in ovens:

- Bag type. Sealed plastic bag, plastic bag with 10 paper punch holes in it, plastic bag with 20 paper punch holes in it or woven synthetic reusable shopping bag (Woolworths bag).
- Temperature. 25˚C and 60˚C
- Storage time at temperature. 0, 3, 5, 10, 15, 20 days

To estimate moisture loss under the different conditions Approx 1 kg of uninfected control litter from Expt 9.2 was placed in 8 bags (2 of each bag type) weighed and placed in ovens at 25 and 60˚C. At the storage times noted, bags were weighed and loss of moisture calculated.

To determine temperature-time relationships on qPCR detection of ILTV and FAdV in litter a further 6 bags were prepared containing known virus contaminated litter from experiment 9.2 (mixed litter plus ILTV treatment litter combined). Two sealed plastic bags, 10-hole punched bags and Woolworths bags and one of each incubated at 25˚C or 60˚C. At the times noted above 10g subsamples of litter were removed from each bag for qPCR enumeration of viral

genome copy number for ILTV and FAdV. Unfortunately samples were not collected at time 0, so decline is measured from day 3.

10.2.2 Experiment 10.2 Effects of litter storage at a range of temperatures for 5,10 and 20 days on reduction in viral load as determined by bioassay in non-SPF chicks and qPCR of the litter material (LT13-C-Bioassay).

AEC No: AEC13-119 **Start date:** 4/11/2013 **End date:** 28/1/2014

The objective of this experiment was:

- To determine temperature-time inactivation relationships for FAdV, IBDV, ILTV, CAV and MDV in poultry litter using a chick bioassay method; and
- To evaluate the extent to which qPCR enumeration of viral nucleic acids in litter to correlates with infectivity determined by bioassay.

This experiment was supposed to start on 29/10/2013 with hatching of SPF chicks at UNE for the bioassay component of the experiment coinciding with litter collection and mixing in that experiment. However this hatch and hatchings for 1/11, 8/11 and 18/11 to accommodate the different litter treatment times also failed despite successfully hatching SPF eggs recently for experiment 9.1.

In light of this development a decision was made to use IsaBrown cockerels, with exposure to the litter delayed until maternal antibody protection could reasonably be expected to have declined to negligible levels (approx. 28 days). To accommodate this change of plans the 50 bags of litter from Experiment 9.2 were frozen at -20˚C immediately after mixing and maintained until use.

The experimental design involved the following treatments

- Negative control isolators for each batch of chickens and exposure time (5 isolators). Fresh pine shavings.
- Positive control isolators (2 isolators) exposed to freshly thawed virus contaminated mixed litter from experiment 9.2.
- A 5 x 3 factorial component using 15 isolators and comprising
    - Litter exposed to 5 temperatures (25, 35, 45, 55 and 65 ˚C) in ovens
    - For 3 exposure times to each temperature (5, 10 and 20 days)

Litter samples remained in the original ~3 kg Woolies woven reusable shopping bags that they were packed into after mixing in experiment 9.2 and subsequently frozen. Samples were thawed before being placed in the ovens set at the different temperatures all on the same day (day 0, 2/12/2013) that same the chicks were exposed to the positive control litters. Litters were subsequently removed on days 5, 10 and 20 and chickens exposed to them in the relevant treatments.

The experiment was conducted in the UNE isolator facility. The experimental chickens comprised commercial IsaBrown cockerels transported from Tamworth to UNE on the day of hatch. To accommodate the experimental needs 3 batches of chickens were used

- Batch 1 (210 birds) hatched on 4/11/2013 (Litter heating treatments 0 and 5 days)
- Batch 2 (120 birds) hatched on 11/11/2013 (Litter heating treatments 10 days)
- Batch 2 (120 birds) hatched on 18/11/2013 (Litter heating treatments 20 days)

Chicks were initially placed in isolators on solid floors with fresh pine shavings on them. At 28-34 days of age they were exposed to the treated litters by removal of the existing litter and replacement with the contaminated treated litter. Details of precise dates and ages of chicks at different treatments are provided in Table 10-1.

Table 10-1. Expt. 10.2. Summary of chick arrival, treatment and termination times

Chick batch	Arrival date	Litter exposure treatment (d)	Exposure date	Exposure age (d)	Sero-conversion test date	Sero-conversion test age (d)	Exposure period
1	4/11/2013	0	2/12/2013	28	6/01/2014	63	35
1	4/11/2013	5	7/12/2013	33	11/01/2014	68	35
2	11/11/2013	10	12/12/2013	31	16/01/2014	66	35
3	18/11/2013	20	22/12/2013	34	26/01/2014	69	35

Measurements. A susbset of chicks from each batch of chicks was blood sampled prior to exposure to litter to determine the level of antibody directed against the viruses of interest. On day 35 after exposure to the treated litters all birds were blood sampled to detect sero-conversion to the viruses of interest using the ELISA kits described in Section 3.4.7. Chicks were then sacrificed, weighed, immune organs weighed or scored for atrophy and birds examined post mortem for gross pathology.

A sub-sample of litter from each temperature treatment was collected prior to placement in the isolators for qPCR detection and enumeration of viral nucleic acids of the viruses of interest.

10.2.3 Experiment 10.3 Effects of litter storage at a range of temperatures for 5,10 and 20 days on reduction in viral load as determined by bioassay in SPF chicks and qPCR of the litter material (Expt LT14-C-BIO2SPF).

AEC No: AEC14-074 **Start date:** 7/10/2014 **End date:** 11/11/2014

The objectives of this experiment were as per the previous experiment.

- To determine temperature-time inactivation relationships for FAdV, IBDV, ILTV, CAV and MDV in poultry litter using a chick bioassay method; and
- To evaluate the extent to which qPCR enumeration of viral nucleic acids in litter to correlates with infectivity determined by bioassay.

In Experiment 10.2 the failure of our SPF eggs to hatch necessitated the running of the proposed bioassay experiment using non-SPF chicks. Most of these chicks had background levels of maternal antibody to the viruses of interest and could have been potentially infected with some of them as well. With the return of availability of hatched SPF chicks at certain times of the year, this experiment was planned to overcome a number of weaknesses in the previous study, namely:

- Use of non-SPF chicks with attendant risks of background infection and maternal antibody interfering with sensitivity to infection and interpretation of results. The present study will use hatched SPF chicks flown from Melbourne.
- Using different challenge times and ages for the different litter heat treatments, necessitating 3 different batches of chickens to be used with attendant problems with variability and complexity in the experiment. In the present study all litter treatments will be timed to finish on the day the chicks arrive and be administered on that day.

Due to resource constraints and costs of SPF chickens, a simpler design with fewer birds was used with 12 birds per isolator and only 4 litter heating temperatures (25˚C, 35˚C, 45˚C and 55˚C).

This experiment used 180 SPF chicks in 15 isolators (12/isolator). Chicks were exposed on the day of hatch (day 0, 7/10/2014) to the same infective litters as used in experiment 10.2. Treatments were;

- Negative control – fresh pine shavings (1 isolator)
- Positive control – Fresh (frozen thawed) litter from Expt 10.2 (2 isolators)
- Litter stored at 25˚C for 5, 10 or 20 days (1 isolator each)
- Litter stored at 35˚C for 5, 10 or 20 days (1 isolator each)

- Litter stored at 45˚C for 5, 10 or 20 days (1 isolator each)
- Litter stored at 55˚C for 5, 10 or 20 days (1 isolator each)

Litter was from the same batch of 52 bags of mixed virus contaminated litter collected in Experiment 9.2 on 29/10/13 and stored at -22˚C since. Bags of litter for the respective treatments were thawed then placed in aerated ovens set at 25, 35,45 and 55˚C on days -20, -10 and -5. The positive control litter was was removed from the freezer and thawed on day 0.

In contrast to the previous experiment which used solid floors and two bags of virus contaminated litter per isolator (~6 kg) in the present experiment the original bioassay method developed earlier (Islam *et al.* 2013a) was used which involved placing 1 bag of litter (2-3 kg) of litter into two scratch trays with the remainder of the floor being punched stainless steel.

At day 35 after exposure post litter exposure, birds were bled, sera retained and assayed for antibodies against FAdV, IBDV, ILTV, CAV and MDV as in Expt 10.2. Litter was subsampled at the completion of heat treatments, just prior to being placed in the isolators. Viral nucleic acids were quantified in these samples following nucleic acid extraction using the abbreviated method.

10.3 Results

10.3.1 Experiment 10.1 – Preliminary experiment on bag types

10.3.1.1 Moisture loss

The initial moisture content of the component litters used to create the mixed samples was 30% (70% dry matter). The rate of moisture loss was significantly affected by bag type ($P=0.001$), storage temperature ($P<0.001$), day of storage ($P<0.001$) and the interaction between bag type and temperature ($P=0.015$) and between storage temperature and day of storage ($P=0.003$). These effects are shown in Figure 10.1.

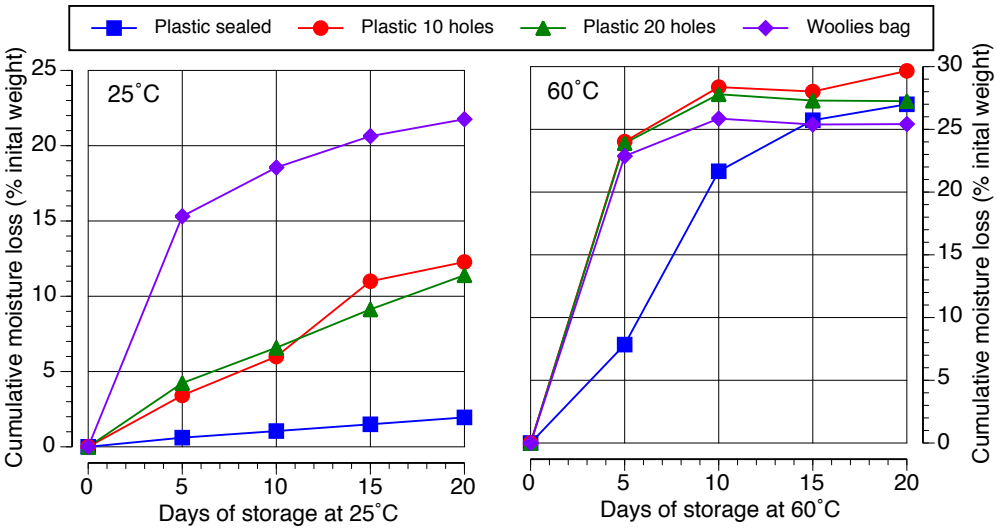

Figure 10.1. Experiment 10.1 Effect of storage of litter at 25˚C (Left) and 60˚C (Right) on cumulative moisture loss when stored in different bag types.

Unsurprisingly sealed (taped closed) plastic bags had the slowest rate of drying, but at 60˚C the final level of drying at 20 days was similar to the other treatments. Punching holes in the bag increase the rate of drying with little difference between 10 and 20 holes. The Woolies woven shopping bags showed the greatest rate of drying at 25˚C, but a similar rate to plastic bags with holes at 60˚C (Figure 10.1). These data suggest that the woven shopping bags are the most porous and have the least confounding between the effects of temperature and drying.

10.3.1.2 ILTV genome copy number

ILTV genome copy number was significantly affected by bag type (P=0.006), storage temperature (P=0.003), but not days of storage (P=0.18). There was significant interaction between the effects of bag type and temperature (P=0.006). These effects are shown in Figure 10.2. At 25˚C there was little decline in ILTV genome copy number over 20 days irrespective of bag type although there was greater trend towards decline with sealed plastic bags. At 60˚C there was a decline in between days 5-20 in all bag types but the decline was precipitous in the sealed bag with no viral genome recovered after day 5. In the other bag types the decline was approximately one log.

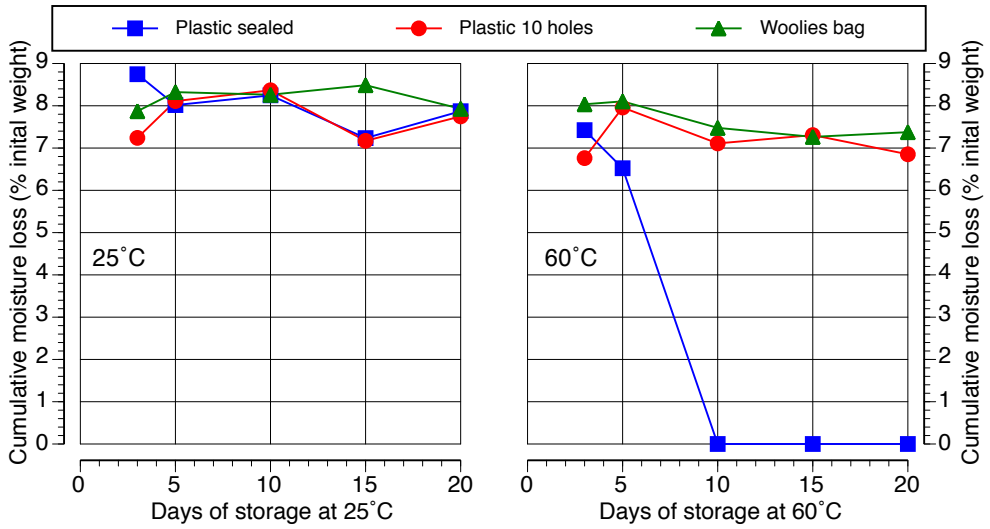

Figure 10.2. Experiment 10.1. Effect of storage of litter at 25˚C (Left) and 60˚C (Right) on detection of ILTV genome using qPCR after various durations of storage

10.3.1.3 FAdV genome copy number

Unlike the case with ILTV the genome copy number for FAdV was not significantly affected by any of the treatments or time. There was a trend towards reduced copy number in the sealed plastic bag type (P=0.21, contrast with the other two bags P=0.09). Data are shown in Figure 10.3.

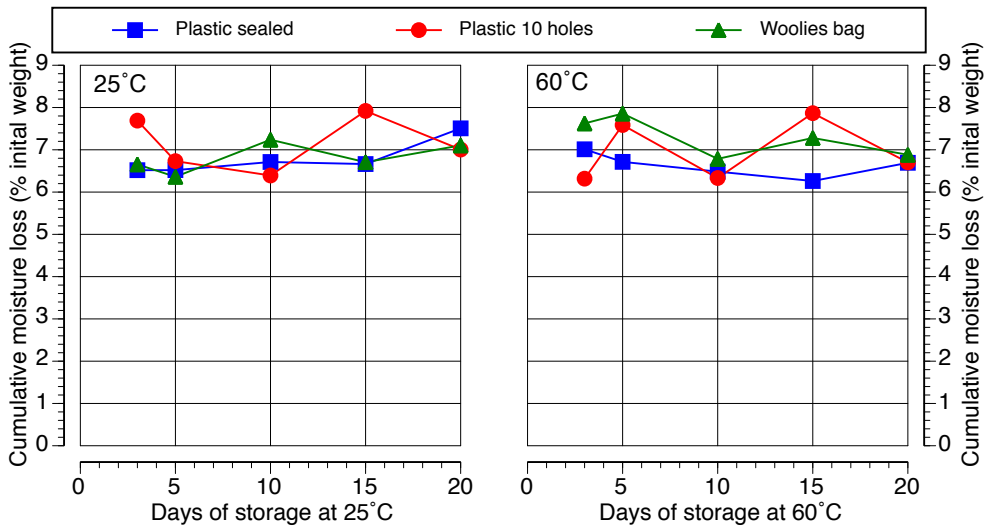

Figure 10.3. Experiment 10.1. Effect of storage of litter at 25˚C (Left) and 60˚C (Right) on detection of ILTV genome using qPCR after various durations of storage

10.3.2 Experiment 10.2 – Virus inactivation study. Non-SPF bioassay and qPCR of litter material

10.3.2.1 Inactivation of FAdV

A small proportion of negative control chicks (IsaBrown cockerels, <10%) were seropositive for FAdV-8 at the end of the experiment, but this did not obscure clear evidence of seroconversion in this experiment (Figure 10.4). More than 80% of chicks seroconverted to FAdV-8 when exposed to stored frozen litter (the "positive control") and high levels of seroconversion were evident in chicks exposed to litter stored at 25˚C for 5, 10 and 20 days. At a litter storage temperature of 35˚C seroconversion occurred in birds placed on litter stored at 5 and 10 days but not 20 days. At 45˚C and above there was no evidence of seroconversion at all, suggestive of viral inactivation at these temperatures.

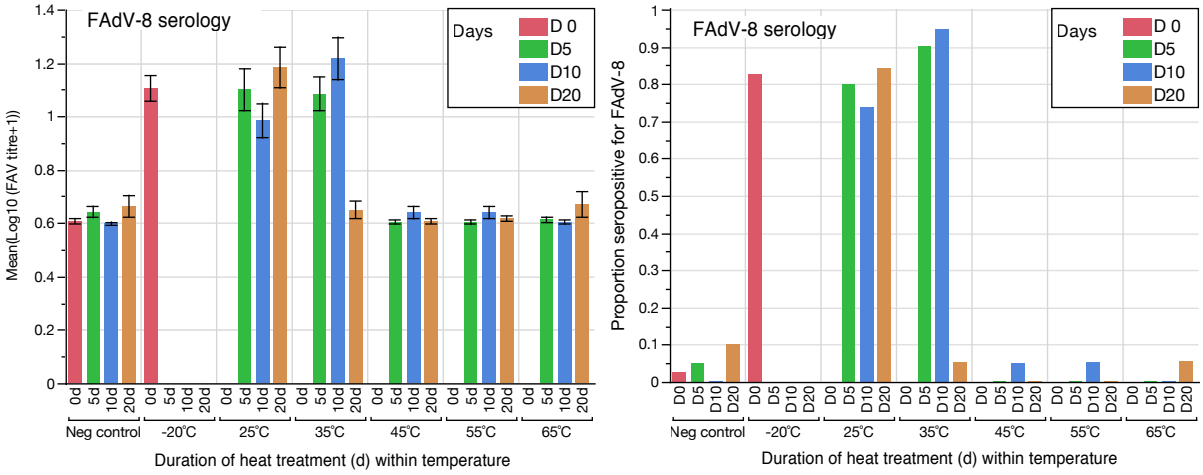

Figure 10.4. Experiment 10.2 FAdV Serology. Mean titre (Left) and proportion of chicks seroconverting (reft) to FAdV 35 days after exposure to litter kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days (n= ~20/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

FAdV viral nucleic acids were assayed in litter following litter preparation and DNA extraction using two methods, the CSIRO method described at 4.6.2 and the abbreviated method described at 4.6.4. Results are shown in Figure 10.5. Using both methods the positive control litter stored at -20˚C contained detectable virus and virus could be detected following storage at temperatures up to 45 ˚C, but not 55˚C or 65˚C. The CSIRO extraction method was clearly more efficient providing higher viral DNA recoveries and allowing detection of viral DNA in samples stored for 20 days at 25˚C or 35˚C which were negative using the abbreviated method. The litter qPCR results show reasonable concordance with the seroconversion data but either over-estimate the amount of infective virus present (eg. at 45˚C) or are a more sensitive detection method for viral presence than the bioassay.

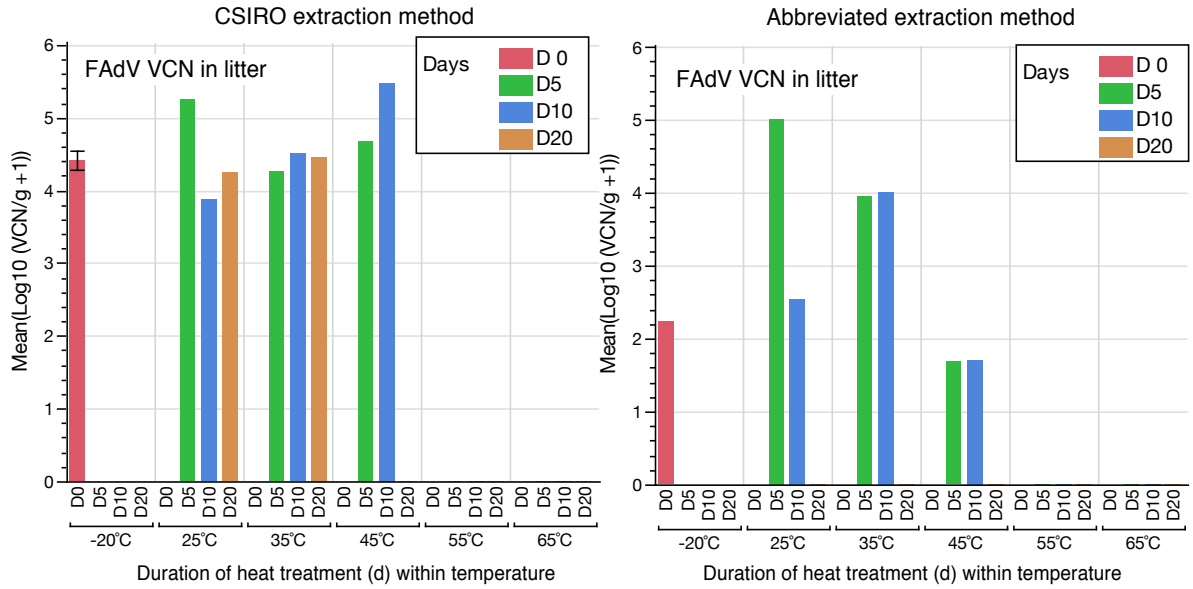

Figure 10.5. Experiment 10.2 Mean FAdV genome copy number per gram of litter (Log_{10}) kept at 35, 25, 45, 55 and 65˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the full CSIRO (Left) or abbreviated (Right) method.

10.3.2.2 Inactivation of IBDV

On arrival at UNE chicks on the day of hatch chicks had high levels of antibody directed against IBDV which declined significantly until the end of the project (Figure 10.6). Low levels of antibody were still detectable when birds were 63-69 days of age.

Only in two treatments did birds show a serological response to litter exposure above background levels. These were for litter stored at 25˚C for 5 and 20 days. Surprisingly the positive control litter (no heat treatment) and the 25˚C for 10 days treatments did not seroconvert. Seroconversion was closely associated with bursal atrophy at post mortem examination, so the serological results are not in doubt.

IBDV viral nucleic acids were assayed using the CSIRO method yielded no detection of IBDV RNA at all whereas use of the abbreviated method described at 4.6.4 identified very low levels of viral RNA as shown in Figure 10.7. As with the serology the positive control was negative for presence of the virus while it was detected at all 3 storage times when stored at 25˚C. At 35˚C, 45˚C and 55˚C virus was detected after 5 day at these temperatures but not thereafter and at 65˚C it was not detected at any point.

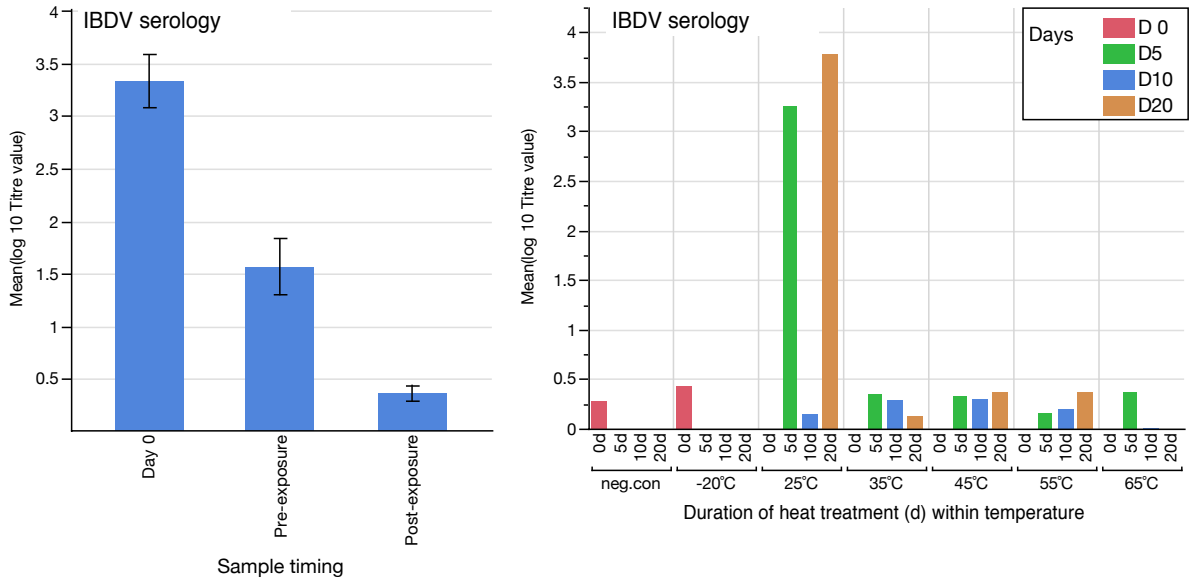

Figure 10.6. Experiment 10.2 IBDV antibody titres showing decline in maternal antibody in control birds (Left) and main experimental data (Right). Control sera are at hatch (n=17), pre-exposure to litter at 28-34 days of age (n=16) and at the end of the experiment when birds were 63-69 days of age (137 samples). Main experiment data show the proportion of chicks seroconverting to IBDV 35 days after exposure to litter kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days (n= ~20/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

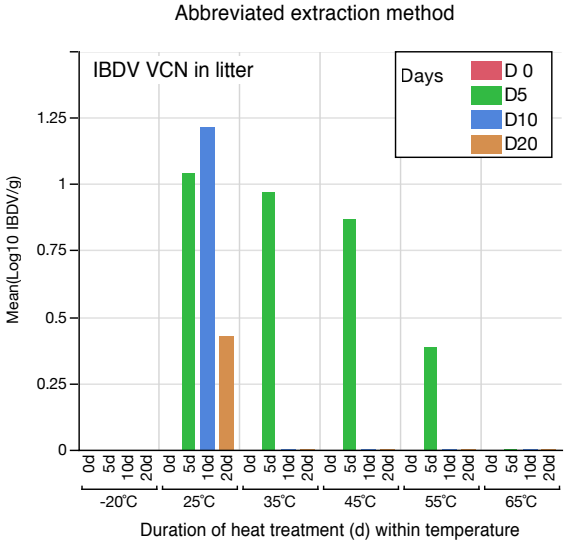

Figure 10.7. Experiment 10.2 Mean IBDV genome copy number per gram of litter (Log_{10}) kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the abbreviated method. When processed by the CSIRO method no samples were positive.

Once again the litter qPCR results show reasonable concordance with the seroconversion data but either over-estimate the amount of infective virus present or are a more sensitive detection method for viral presence than the bioassay.

10.3.2.3 Inactivation of ILTV

Antibody titres directed against ILTV were low and were barely raised above background levels (Figure 10.8). Birds on positive control litter with no heat treatment showed no antibody response to exposure, but surprisingly, there was a statistically significant elevation in ILTV titre in litter stored at high temperatures (45-65˚C) for 20 days (P=0.001). Samples were systematically distributed over the 6 ELISA plates used for the assays so there was no confounding between treatments effects and assay methodology to explain this. These birds were all from batch 3, but the negative control birds and those on litter stored at 25˚C and 35˚C were unaffected. There were no clinical signs of ILTV in the experiment.

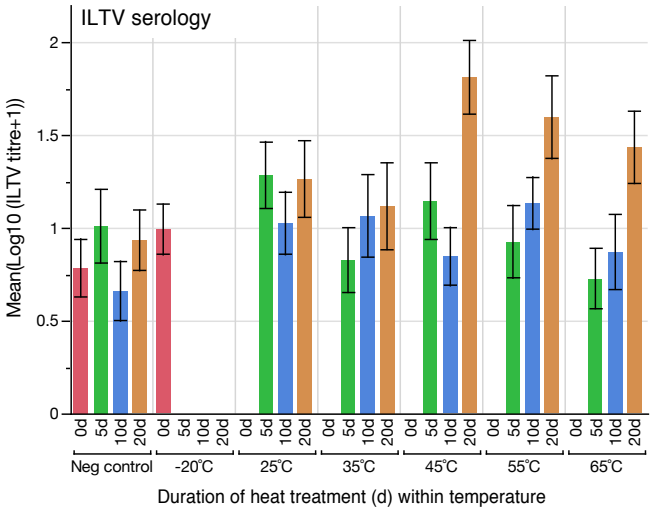

Figure 10.8. Experiment 10.2 ILTV Serology. Mean titre against ILTV 35 days after exposure to litter kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days (n= ~20/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

When the litter samples were subjected to qPCR tests for ILTV following DNA extraction by the CSIRO method viral DNA was clearly present in the positive control samples and samples stored at up to 45˚C for 10 days but not for samples stored longer at that temperature, or stored at a higher temperatures (Figure 10.9). The results were a little less clear with the abbreviated extraction method, with a trace of viral DNA detected at temperatures up to 65˚C.

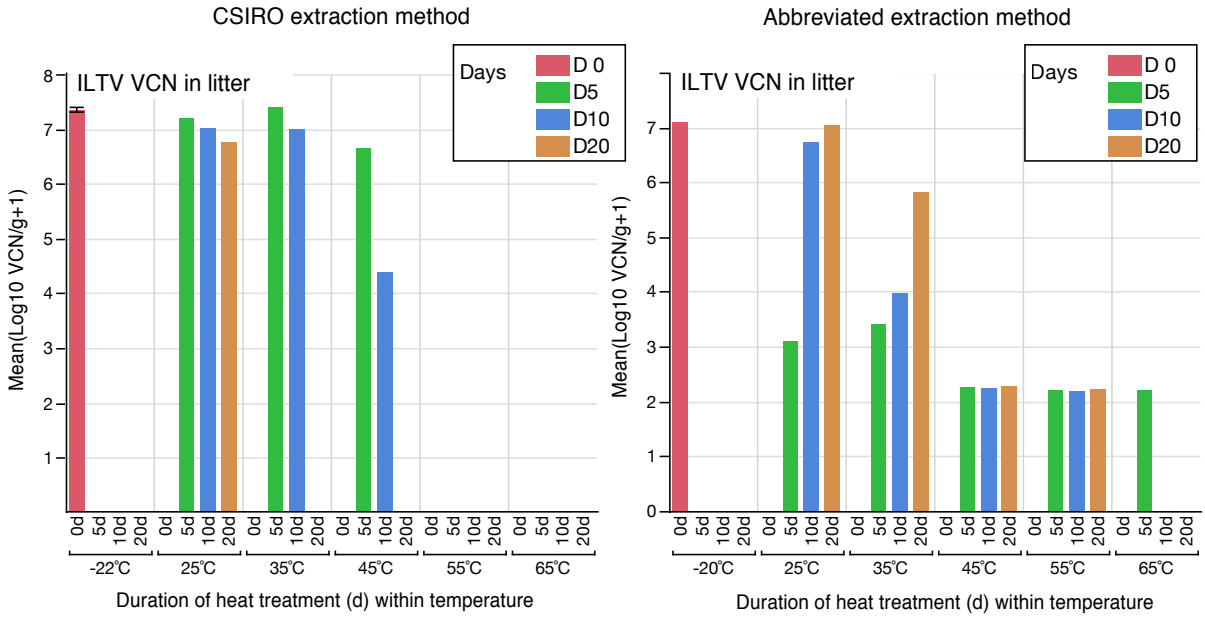

Figure 10.9. Experiment 10.2 Mean ILTV genome copy number per gram of litter (Log_{10}) kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the full CSIRO (Left) or abbreviated (Right) method.

For the ILTV results there is a greater disparity between the ELISA results and the qPCR results than for the previous two viruses. Despite the unexplained titre rises in chicks placed on litter with the hottest treatments for the longest period, the lack of clinical signs, and very low titres (compare these with Figure 9.7) suggest that none of the chickens seroconverted to ILT. On the other hand, viral nucleic acids were plentiful and evidence of reductions in amount was only seen after storage at 45˚C for 20 days and thereafter.

10.3.2.4 Inactivation of CAV

Antibody titres directed against CAV were low with little evidence of a response to litter exposure (Figure 10.10). However negative control birds appeared to show an increase in titre over time and the control birds for the positive control had titres a log lower. With these results it is difficult to determine whether birds are all negative or are all low positives with low grade CAV infection. In experiment 9.2 broiler infected with CAV, but with maternal antibody present had titres similar to these (Figure 9.9 Right).

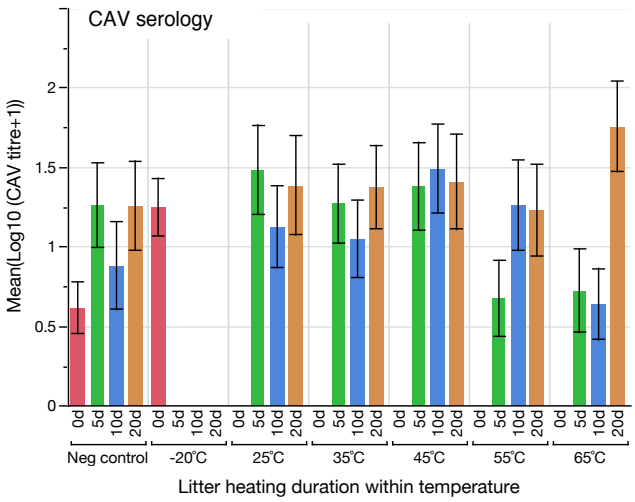

Figure 10.10. Experiment 10.2 Mean titre against CAV 35 days after exposure to litter kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days (n= ~20/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

When the litter samples were subjected to qPCR tests for CAV following DNA extraction by the CSIRO and the abbreviated method, all samples were negative.

10.3.2.5 Inactivation of MDV

Antibody titres directed against MDV are shown in Figure 10.11.

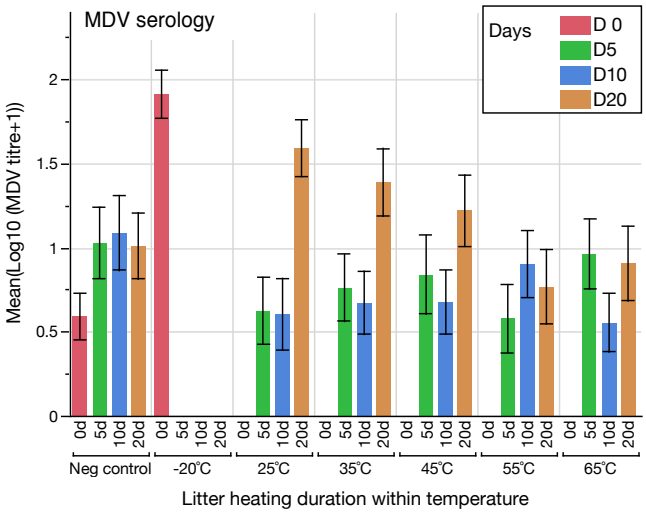

Figure 10.11. Experiment 10.2 MDV Serology. Mean titre against ILTV 35 days after exposure to litter kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days (n= ~20/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

Titres were low and only 4/426 sample exceeded the laboratory benchmark of a titre above 500 for a definite positive. All of those samples were from the positive controls indicating the presence of infective virus in the initial litter. The clear difference between the negative and

positive control samples in Figure 10.11 clearly demonstrates this. On the other hand in there was no increase in titre over the negative controls in chicks exposed to litters of all temperatures for 5 or 10 days indicating lack of infective virus or response to it in these litters. However in the group 3 chickens exposed to litters kept at various temperatures for 20 days there was a clear antibody response to litters kept at 25 and 35 ˚C and possibly 45˚C also.

When the litter samples were subjected to qPCR tests for MDV following DNA extraction by both the CSIRO and abbreviated methods the results were very similar and showed viral DNA detection decreasing by roughly one log per 10˚C increase in temperature, with the effect being greater at the higher temperatures (Figure 10.12). Within temperature categories, longer exposure to high temperatures also reduced the amount of MDV genome detected, with this effect becoming evident at 45˚C and above.

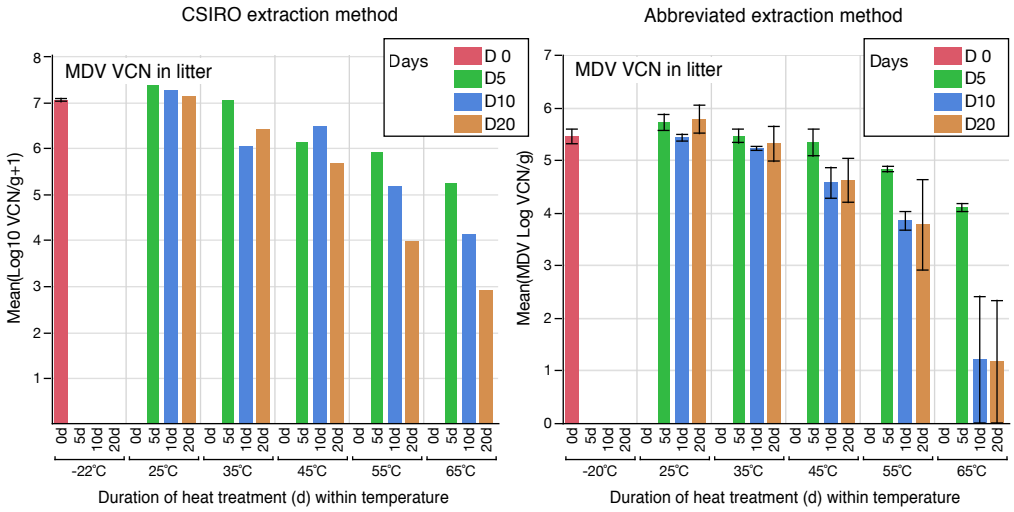

Figure 10.12. Experiment 10.2 Mean MDV genome copy number per gram of litter (Log_{10}) kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the full CSIRO (Left) or abbreviated (Right) method.

As with ILTV, the other herpesvirus in the experiment, viral DNA persisted in heated litter to a far greater extent than infectivity did, as determined by this bioassay.

10.3.3 Experiment 10.3 – Virus inactivation study. SPF bioassay and qPCR of litter material

10.3.3.1 Inactivation of FAdV

SPF chicks exposed to the various litter treatments were all serologically negative for antibodies against FAdV, including those raised on the positive control litter. Enumeration of viral genome copy number in the litter following the abbreviated extraction procedure revealed high levels of viral nucleic acids, with little effect of heating or heating duration (Figure 10.13). In fact the positive control, not exposed to any heat treatment had the lowest level of virus detected. These results stand in marked contrast to what was observed in Experiment 10.2 and are indicative of a loss of infectivity in the litter with storage, but retention or even improved retention of viral genome.

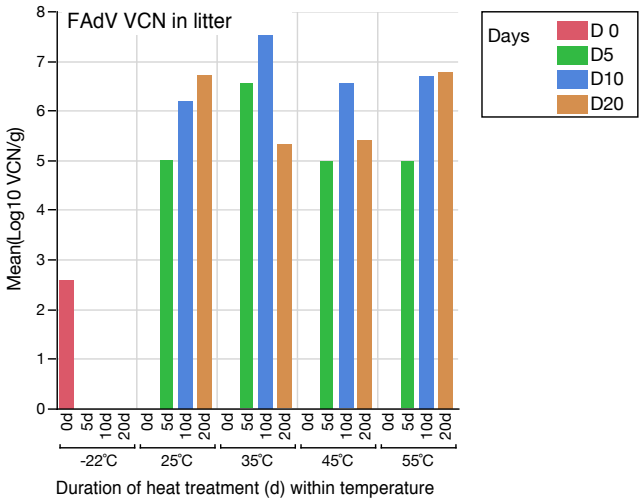

Figure 10.13. Experiment 10.3 Mean FAdV genome copy number per gram of litter (Log_{10}) kept at 25, 35, 45 and 55 ˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the full CSIRO (Left) or abbreviated (Right) method.

10.3.3.2 Inactivation of IBDV

Only one treatment group seroconverted to IBDV n the experiment, that exposed to litter maintained at 25˚C for 10 days (Figure 10.14 Left). The seroconversion was associated with marked bursal atrophy in this group. When the litter was analysed for viral RNA there was amplification of these in some treatments, but at below the level of the lowest standard in the standard curve. Data are therefore presented in terms of ct (critical threshold values) using

the 40-ct variable (Figure 10.14 Left). On this scale each unit increase represents approximately doubling of the amount of virus present. Based on these data the positive control and litters stored at 25˚C, 35˚C and 45˚C contained detectable virus, with the amount declining in line with both increasing temperature and the period of heating.

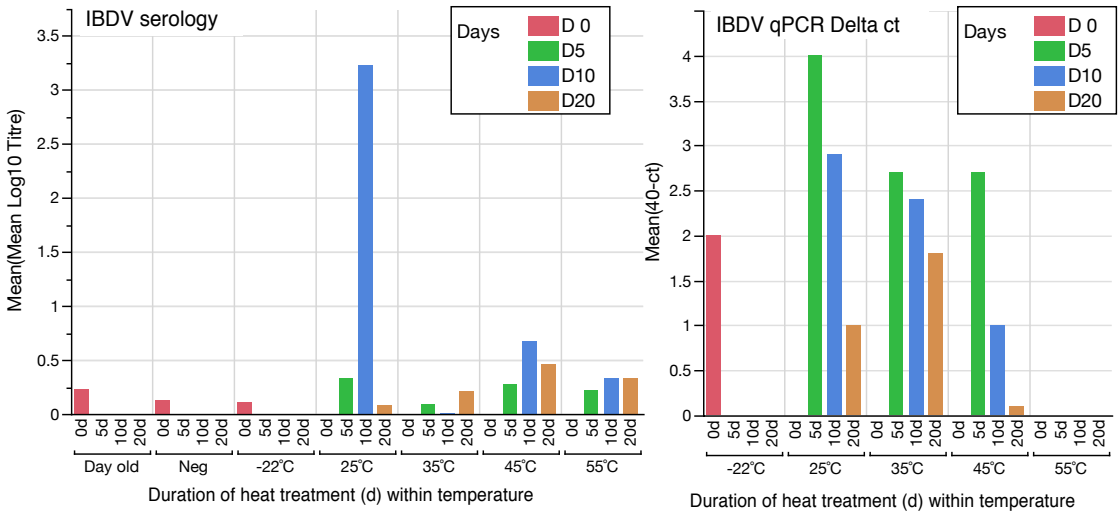

Figure 10.14. Experiment 10.3 IBDV serology (Left) and viral genome copy number detection (Right). Mean titre against IBDV 35 days after exposure of SPF chicks to litter kept at 25, 35, 45 and 55 ˚C for 5, 10 or 20 days (n= ~12/group) including negative control sera from birds not exposed to the litters and day old SPF chicks. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the 0d treatment for all the other groups. The qPCR results are presented as 40-ct with each unit increase represents approximately doubling of the amount of virus present.

These data are broadly consistent with those of Experiment 10.2 with seroconversion only in birds on litter stored at 25˚C and temperature and time dependant recovery of viral genome using qPCR. It is unclear why in both experiments the positive control litter failed to induce seroconversion. In the two experiments this represented 6 different bags of original mixed litter.

10.3.3.3 Inactivation of ILTV

Antibody titres directed against ILTV in the experiment were low and possibly no more than background levels (Figure 10.15 Left) with no systematic effect of litter heat treatment evident. Intriguingly none of the birds on negative control litter had a titre recorded at all. There were no clinical signs of ILT in the experiment.

Analysis of viral genome copy number showed high levels of virus present in all treatments (Figure 10.15 Right), but with no systematic effect of temperature or duration. This contrasts with the findings of experiment 10.2 in which there was loss of detection at 55˚C and above.

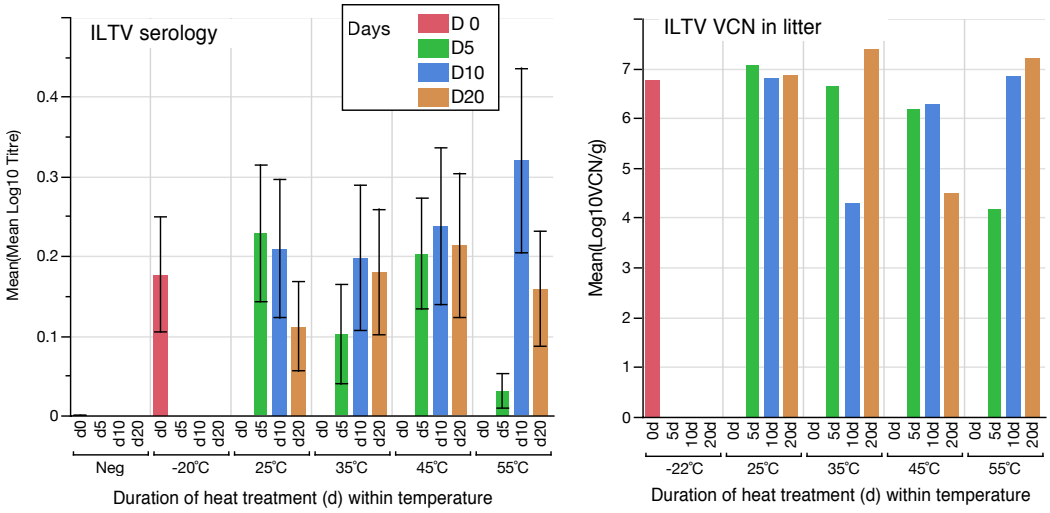

Figure 10.15. Experiment 10.3 ILTV serology (Left) and viral genome copy number detection (Right). Mean titre against ILTV 35 days after exposure of SPF chicks to litter kept at 25, 35, 45 and 55 ˚C for 5, 10 or 20 days (n= ~12/group) including negative control sera from birds not exposed to the litters and day old SPF chicks. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the 0d treatment for all the other groups. The viral copy numbers were determined in samples processed by the abbreviated method.

10.3.3.4 Inactivation of CAV

Antibody titres directed against ILTV in the experiment are shown in Figure 10.17.

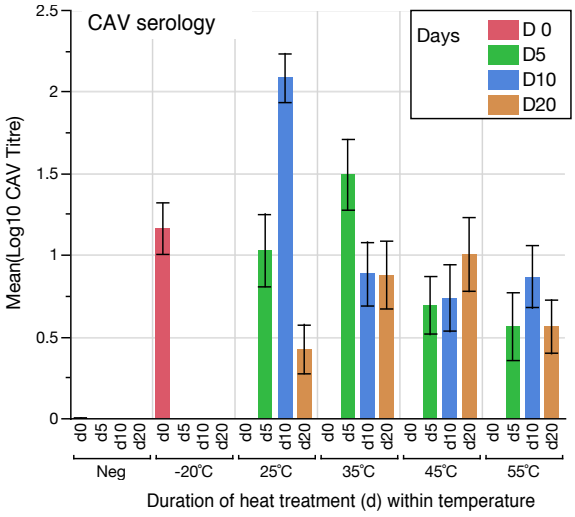

Figure 10.16. Experiment 10.3 CAV serology. Mean titre against CAV 35 days after exposure of SPF chicks to litter kept at 25, 35, 45 and 55 ˚C for 5, 10 or 20 days (n= ~12/group). Negative control sera are from birds of the same age, not exposed to the litters. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups.

Negative controls did not seroconvert while positive controls did. All litter treatments showed a evidence of seroconversion with antibody titres showing a broad trend to decline with increasing litter temperature and duration at a given temperature. Interestingly, the birds in the 25˚C 10d litter treatment had concurrent IBDV infection and showed the highest antibody

response to CAV. Samples from this experiment have not been tested for CAV genome copy to date.

10.3.3.5 Inactivation of MDV

SPF chicks exposed to the various litter treatments were all serologically negative for antibodies against MDV, including those raised on the positive control litter.

Analysis of viral genome copy number showed high levels of virus present in all treatments (Figure 10.17), but with no systematic effect of temperature or duration. This contrasts with the findings of experiment 10.2 in which there was a progressive loss of detection with increasing storage temperature and duration of storage.

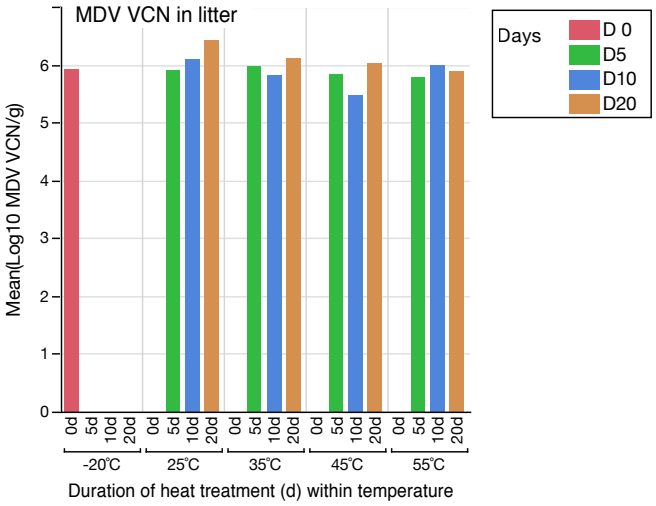

Figure 10.17. Experiment 10.2 Mean ILTV genome copy number per gram of litter (Log_{10}) kept at 25, 35, 45, 55 and 65˚C for 5, 10 or 20 days. The -20˚C treatment is stored litter not subject to any heat treatment, so is equivalent to a positive control and represents the D0 treatment for all the other groups. Samples were processed either by the full CSIRO (Left) or abbreviated (Right) method.

10.4 Summary and Discussion

Overall this part of the project was disappointing and failed to produce unequivocal time-temperature relationships for the viruses under test. Nevertheless there were some very clear findings and useful information obtained. It should be noted that these experiments produced a large number of samples at a time when some of the methodology was still being developed, so it was not the case that we had full understanding of the outcomes of each experiment prior to the next one.

Experiment 10.1 showed that storage of litter in ovens was associated with significant temperature-dependant moisture loss and that this could be influenced by the selection of storage container used. We selected a bag type that provided aeration of sample as occurs in composting litter, but this bag type (Woolies shopping bag) was associated with considerable drying out during storage. This could be expected to preserve viral nucleic acids from microbial and enzymatic degradation. When litter samples for this experiment were assayed for ILTV genome copy number this was confirmed, but only at high temperatures (65˚C). At 25˚C there was no difference in viral genome recovery between the different bag types, but at 65˚C recovery was greatly reduced in after 5 days of storage in sealed bags retaining more of the moisture. This was not true for FAdV for which viral genome recovery was significantly reduced at this temperature, although there was a trend towards this. The key finding, consistent with existing knowledge was

- At high temperatures higher moisture levels are associated with a higher rate of degradation of viral nucleic acids.

The main findings of Experiment 10.2 in non-SPF chickens and experiment 10.3 in SPF chickens but using the same frozen litter from Expt 10.2 are summarised in Table 10-2 and Table 10-3 respectively. A summary of the overall findings for each virus is discussed below.

10.4.1 FAdV

The results of experiments 10.2 and 10.3 were markedly different for reasons that are not clear. In Experiment 10.2 using litter that had been frozen for 5 weeks before treatments were applied to it there was a serological response to contaminated litter that ceased when litter was heated at 35˚C for more than 10 days or at 45˚C and above. Viral genome copy number was detected up to 10 days at 45˚C but not thereafter. These data provide fairly clear evidence of time-dependant inactivation of the virus in the 35-45˚C range with time-dependant loss of detection of virus genome in the 35-45˚C range. We have previously reported that FAdV-8 was largely inactivated in litter after 6–7 days of litter pasteurisation by heaping (Walkden-Brown et al. 2010a).

In experiment 10.3, using litter that had been stored frozen for 11 months all ability to infect chickens had been lost, and viral genome recovery was high and unaffected by litter treatment. While the reasons for this discrepancy cannot be known, it is possible that drying out of the samples in the freezer, or temperature cycling, damaged the viability of the virus but enhanced the persistence of the viral nucleic acids. Unfortunately dry matter content of the starting materials was not assessed. Freezing is not included in a long list of effective methods virus inactivation (World Health Organisation 2004). Lower temperatures generally enhance virus survival and most viruses can be stored frozen to maintain their infectivity for

long periods of time (Sobsey and Meschke 2003). However, freezing temperatures may cause structural damage and thus decrease survival for some viruses.

10.4.2 FAdV

The results of experiments 10.2 and 10.3 were markedly different for reasons that are not clear. In Experiment 10.2 using litter that had been frozen for 5 weeks before treatments were applied to it there was a serological response to contaminated litter that ceased when litter was heated at 35˚C for more than 10 days or at 45˚C and above. Viral genome copy number was detected up to 10 days at 45˚C but not thereafter. These data provide fairly clear evidence of time-dependant inactivation of the virus in the 35-45˚C range with time-dependant loss of detection of virus genome in the 35-45˚C range. We have previously reported that FAdV-8 was largely inactivated in litter after 6–7 days of litter pasteurisation by heaping (Walkden-Brown *et al.* 2010a).

In experiment 10.3, using litter that had been stored frozen for 11 months all ability to infect chickens had been lost, and viral genome recovery was high and unaffected by litter treatment. While the reasons for this discrepancy cannot be known, it is possible that drying out of the samples in the freezer, or temperature cycling, damaged the viability of the virus but enhanced the persistence of the viral nucleic acids. Unfortunately dry matter content of the starting materials was not assessed. Freezing is not included in a long list of effective methods virus inactivation (World Health Organisation 2004). Lower temperatures generally enhance virus survival and most viruses can be stored frozen to maintain their infectivity for long periods of time (Sobsey and Meschke 2003). However, freezing temperatures may cause structural damage and thus decrease survival for some viruses.

Table 10-2. Summary of the key outcomes of Experiment 10.2 (non-SPF chickens, Dec 2013)

Issue/Question	FAdV	IBDV	ILTV	CAV	MDV
Background Ab in chicks?	Y	Y	Y	Y	Y?
Background infection	N	N	N	Y?	N
Serological response to contaminated litter?	Y	Y	Y?	Y	Y
Response varies with litter treatment?	Y	Y	Y?	Y?	Y
Conditions inhibiting response	>35˚ for 10d	≥35˚C	<20d or <45˚C?	Reduced ≥55˚C?	All except +ive control
Viral genome recovered from contaminated litter?	Y	Y	Y	N	Y
VCN varies with litter treatment?	Y	Y	Y	N/A	Y
Conditions inhibiting detection	>45˚ for 10d	>55˚C for 5d	>45˚ for 10d	N/A	No absolute inhibition. Steady reduction with temperature and time

Table 10-3. Summary of the key outcomes of Experiment 10.3 (SPF chickens, Oct 2014)

Issue/Question	FAdV	IBDV	ILTV	CAV	MDV
Serological response to contaminated litter?	N	Y	N	Y	N
Response varies with litter treatment?	N/A	Y	N/A	Y	N/A
Conditions inhibiting response	N/A	≥35˚C	N/A	No absolute inhibition. Trend to reduction with temperature and time	N/A
Viral genome recovered from contaminated litter?	Y	Y	Y	Not done yet	Y
VCN varies with litter treatment?	N	Y	N	N/A	N
Conditions inhibiting detection	None	>45˚C for 20d	N/A	N/a	None

10.4.3 IBDV

In contrast to FAdV, the results for IBDV are very similar between the two experiments. One problem clearly is that the amount of IBDV in the litter was low and patchy in distribution, something that probably arose from the discarding of some very wet litter from the IBDV treatment in experiment 9.2 prior to mixing of the litters. Probably for this reason birds on the positive control litter did not seroconvert in either experiment, but on the other hand, in both experiments birds seroconverted with bursal atrophy in the 25˚C treatments but not in any other suggestive of an inactivating effect of heat treatment in those.

In both experiments low amounts of IBDV were detected in the treated litter samples but not reliable at temperatures above 45˚C. Once again direct detection of viral nucleic acids persisted beyond when infectivity could be demonstrated. IBDV is a very stable and persistent virus (Eterradossi and Saif 2008) with reports of it withstanding cooking temperatures for moderate periods of time, but in our previous work in heaped litter the evidence was that IBDV was largely inactivated after 6-10 days (Walkden-Brown *et al.* 2010a).

10.4.4 ILTV

The results for this virus were the most difficult to interpret. In Experiment 10.2 there was evidence of seroconversion to ILT, but in chickens exposed to litter given the most extreme heat treatments making the results improbably. In experiment 10.3 it was clear that there was no seroconversion so it may be that the results seen in 10.2 are an artefact.

Clearly there was a lot of ILT virus in the litter in both experiments but whereas in experiment 10.2 recovery of viral genome was temperature and time dependant, declining to absence or

very low levels at 55˚C or above, this was not seen in Experiment 10.3. This was also seen for FAdV and may reflect the same underlying reasons, possibly drying of the litter during prolonged freezing had a protective effect on the nucleic acids.

IITV is not thought to be particularly resistant to high temperatures or survival in the environment (Jordan *et al.* 1967) and a previous report indicated that the virus was reduced to undetectable levels by normal litter composting for 5 days or heating at 38˚C for 48 hours (Giambrone *et al.* 2008). Our results are consistent with this and are suggestive of rapid loss of infectivity with prolonged persistence of viral nucleic acids. In Experiment 9.2 which produced the experimentally infected litter, peak shedding of ILTV in faeces was from days 0-7 but there was substantial shedding days 7-14 also, so litter collected on day 19 should have contained infective virus. However initial brooding temperatures in the isolators are 35˚C (with considerable variation around this temperature) which are close to the 38˚C which has been reported to inactivate ILTV within 48hr (Giambrone *et al.* 2008). This is a likely explanation for the findings.

10.4.5 CAV

In experiment 10.2 there was possible background infection with CAV which made serological results difficult to interpret. To further complicate matters, no CAV was detected in litter. It is unclear whether the latter is due to methodological issues or an absence of virus in the litter.

In Experiment 10.3, with SPF birds free of CAV the serological results were clearer. Birds exposed to infective litters in all treatments seroconverted with little association between litter treatment and antibody titre, with the exception that in the one isolator that also seroconverted to IBDV, there was a much higher CAV titre. These data are consistent with CAV being one of the most resistant poultry viruses to inactivation (Schat and van Santen 2008) and a virus that plagues SPF facilities. Nevertheless, in previous work we have shown that litter pasteurisation by heaping largely inactivated CAV and after 6-10 days, as determined by chick bioassay in SPF chicks.

10.4.6 MDV

For MDV, there was definite seroconversion in the positive control group in experiment 10.2 but not for the other litter treatments, suggestive of loss of infectivity with heat treatment of any kind. The litter contained high levels of MDV genome with steady declines in genome recovery with increasing temperatures and times of storage at these temperatures.

In experiment 10.3 in SPF chick there was no evidence of any seroconversion at all but once again high levels of MDV genome were detected in the litter. However in this experiment there was no evidence of a decline in MDV genome copy recovery with higher temperatures.

MDV is generally considered to be stable in the environment surviving, for many months at room temperature (Calnek and Hitchner 1973; Carrozza *et al.* 1973) but not many studies have examined survival in litter at higher temperatures. It has been reported to survive for 21 days at 37˚C (Calnek and Hitchner 1973) but not for 28 days at the same temperature. In another long term study with storage of infective dust at 37˚C infectivity was reduced by 28 days, but persisted out to 90 days (Blake *et al.* 2005). In our previous CRC project we found that MDV retained infectivity after litter pasteurisation, with declines in infectivity being time, but not temperature-dependant (Walkden-Brown *et al.* 2010a).

In the present experiments the level of infectivity was very low suggestive of a failure to induce strong infections and high levels of shedding in the chickens in Experiment 9.2. Litter was collected 32 days after initial challenge, by which time adequate shedding should have occurred (Islam and Walkden-Brown 2007; Islam *et al.* 2008; Islam *et al.* 2013b). It is possible that some of the viral genome recovered in the litters is from the infecting dust, rather than that being shed, but the high levels detected are more suggestive of active shedding of the donor chickens.

10.5 Conclusions and implications

1. Detection of viral genome by qPCR does not correlate well overall with loss of viral infectivity in litter. Viral nucleic generally acids persist longer than the period of infectivity defined by a chick bioassay.
2. Quantification of viral genome in litter is therefore useful of as a marker of past infection status of birds on the litter, but not necessarily of the risks associated with the litter itself. It should not be used as a marker of viral inactivation in litter treatments.
3. The moisture content of litter material will influence both virus inactivation and persistence of viral nucleic acids, so it is critical that litter treatments simulating heaping, try to replicate the temperature, oxygen and moisture conditions of actual heaped litter. In retrospect it would have been preferable to place bags of infective litter and vials of infective clean virus, in various parts of real litter heaps to determine time-temperature relationships.
4. Developing methods to prepare litter in a way that would allow isolation of virus into cell culture or chick embryos without concurrent bacterial and fungal contamination could

provide a cheaper and more practical alternative to the chick bioassay for assessing the virus infective status of litter given various treatments.

5. There was only one virus (CAV) for which there was evidence of survival at all of the temperature and time combinations tested. For the other viruses there was either no litter transmission (ILTV), limited transmission (MDV) or the treatments reduced the infectivity of the litter (FAdV, IBDV) suggesting that for most of the viruses tested the pasteurising temperatures obtained in heaped litter will provide significant reductions in infectivity.
6. Prolonged freezing of virus in breathable bags led to a decline in virus infectivity for the dsDNA viruses FAdV, ILTV and MDV, but not the circovirus CAV or the RNA virus IBDV.
7. It is planned to use these results, together with other published findings, to build a predictive model of pathogen destruction within litter heaps, following on from the model predicting temperatures.

11. Strand 4. Field validation of project findings

11.1 Introduction

The aim of Strand 4 of the project was to evaluate practical application of the project findings on farm and to develop a decision support spreadsheet to assist with decision-making regarding litter pasteurisation.

The on farm validation component of this work is reported in this Section. It involved working on two farms in Sydney with the following broad objectives

- Determination of decay rate viral nucleic acids in poultry litter following heaping under commercial conditions
- Determine whether temperature-time relationships for viral nucleic acids developed in the lab apply in the field.
- Further investigate temperatures close to the surface of heaps which are potential areas for pathogen carryover.

The on farm studies were carried out in Sydney by Dr Fakhrul Islam with litter extraction and qPCR assays done by Sue Burgess and Kanchana Jayasundara (IBDV).

We expected potential poultry viruses such as MDV, FAdV and IBDV would be present in the end of batch poultry litter and possibly some other viruses such as ILTV and CAV. We hypothesized that the viral nucleic acids would be detectable at day 0, immediately after emptying the shed but the number of genome copies would decline during litter pasteurization by composting.

11.2 Materials and methods

11.2.1 Experimental design and procedures

The experiment was carried out on two Sydney farms from 16th to 25th March 2014. One heap in each of two sheds from each farm was included in the study thus making 4 heaps/sheds in total. Litter samples were collected at the time of making the heaps from each shed (two samples from each shed) and these samples were considered as day 0 samples. Once the heaps were made, iButtons were inserted at depths of 5, 10, 25 and 50 cm inside eac litter heap. iButtons were also placed in the shed to record the shed environmental temperatures. Photos of the heaps inside the sheds are provided in Figure 3.1 and the size of the heaps summarized in Table

Figure 11.1 Heaps inside the sheds

Table 11-1. Expt. 11.1. Summary of heap sizes and initial moisture content on Farms 1 & 2

Farm	Shed	Height (m)	Length (m)	Width (m)	Estimated volume (m^3)	Initial moisture (%)
1	1	1.62	6.2	5.1	22.6	23.54
1	2	1.55	5.02	4.04	14.0	28.39
2	1	1.53	5.71	4.8	18.3	21.13
2	4	1.44	5.54	4.45	15.9	20.97

Litter samples were also collected at days 3, 6 and 9 following heaping. Equal amounts of litter were collected from four spots of each heap, at depths of 0 (surface), 5, 10, 25 and 50cm in a 10 litre bucket. Litter samples were thoroughly mixed for about 5 minutes, bagged in zip lock bags and stored at -20˚C until use. On day 9 the temperature data loggers (iButtons) were removed and the experiment ended.

Nucleic acids were extracted from the litter samples using the complete CSIRO litter preparation and extraction method (Section 4.6.2) and the abbreviated method (4.6.4). Viral nucleic acids were quantified using real-time PCR for the respective viruses.

11.2.2 Details of farms and litter management

11.2.2.1 Farm 1, Londonderry NSW

Londonderry is a suburb in the Greater Western Sydney. It is a growing residential area with acres of farming lands. There were other poultry farms within one kilometer of Farm 1. The farm has four sheds of which Sheds 1 and 2 were used in the experiment (Figure 3).

Shed 1 had dimensions of 62.5 x 12 m with a capacity of 11,500 broiler chickens (RSPCA standard) while Shed 2 had dimensions of 76.5m x 12m with a capacity of 14,000 to 15,500 broiler chickens.

This farm reuses litter throughout the year and changes litter completely once a year or if they face any particular problem (disease outbreak, very wet litter etc.). Litter is usually removed from half of the shed (brooding area) and new pine shavings are used in this area. The current litter being composted has had 3 batches of broilers reared on it.

The size of the

11.2.2.2 Farm 2, Schofields NSW

Schofields also a suburb within the Greater Western Sydney region, is about 45 km from the Sydney CBD. It is a growing residential area but there are also many farms nearby. Again the farm has four grower sheds of which Sheds 1 and 4 were used in the experiment.

Shed 1, the smallest of all, had dimensions of 55 x 12 m. It can hold around 10,000 broiler chickens while the larger Shed 4 can hold about 15,000 chickens. This farm has been reusing litter for many years and has not fully cleaned the sheds for many batches (full clean out occurred about 18 months ago). As for farm 1 there is partial reuse of litter with new litter provided in the brooding area only.

11.3 Results

11.3.1 Litter moisture content

The initial litter moisture content on Farm 1 (25.9%) was significantly higher at day 0 (at the time of heaping) than on Farm 2 (21.2%) (P=0.003). Values were very similar by day 9 (19.0% and 19.2% respectively) meaning a decline in dry matter by 6.9 and 2 percentage units respectively (Figure 11.2).

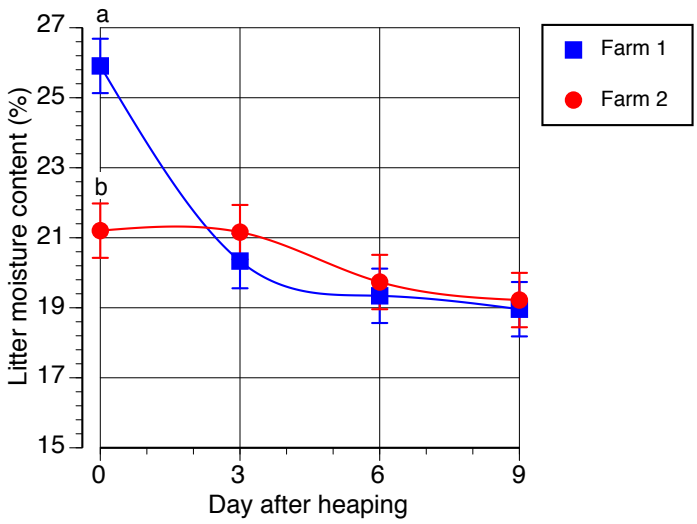

Figure 11.2 Moisture content (LSM ± SE) of litter on the two farms during the litter pasteurising event.

11.3.2 Litter temperature

Temperature profiles at various depths are presented in Figure 11.3 and summary temperature data in Table 11-2.

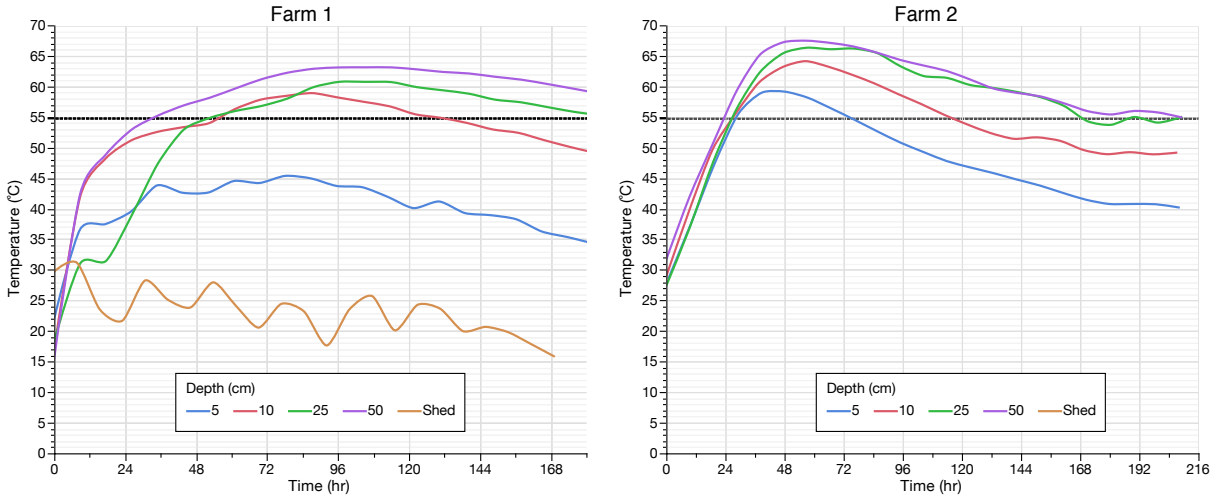

Figure 11.3 Temperature profile inside the heaps by depth. Curves represent spline smoothed curves through the data points. As the two experiments ran concurrently, the shed temperature profiles on Farm 1 may be taken as an approximation of the temperatures experienced on Farm 2. For these data, spline smoothing reduces the actual measured diurnal variation by up to 5˚C.

The heating profiles on the two farms differed considerably although both reached temperatures in excess of 60˚C at depths of 25 cm or greater. On Farm 1 temperatures were slow to increase to peak temperatures that were lower than on Farm 2, but high temperatures were maintained for longer longer so that the average amount of time spent

above 55˚C was slightly higher on Farm 1 (Figure 11.3 and Table 11-2). This effect may be due to the higher initial moisture content on Farm 1. On both farms temperatures were assessed at 5 and 10 cm depths, close to the surface. On Farm 2 temperatures at both depths achieved pasteurizing temperatures above 55˚C, but on farm 1 this was only the case at the 10cm depth with the 5cm depth being intermediate between ambient room temperature and temperatures deeper in the pile (Figure 11.3).

Table 11-2 Key temperature variables on the two farms

Farm	Depth (cm)	Max Temp (˚C)	Time to max temp (hr)	Mean temp (˚C)	Time above 55˚C
Farm 1	Shed			23.3	
	5	45.5	85.0	40.2	0.0
	10	59.0	90.5	52.7	37.4
	25	61.0	114.5	53.0	67.4
	50	63.4	114.0	58.1	74.6
	Mean	**57.2**	**101.0**	**51.0**	**44.9**
Farm 2	5	59.0	55.0	47.6	44.0
	10	64.8	46.5	53.9	27.5
	25	66.3	71.7	57.7	74.7
	50	67.5	65.3	59.2	30.0
	Mean	**64.4**	**59.6**	**54.6**	**44.1**

11.3.3 Viral genomes in the litter

Litter samples from both farms collected at days 0, 3, 6 and 9 days were prepared for DNA extraction using the abbreviated method (Section 4.6.4) and the resultant extract subjected to qPCR for MDV, ILTV and CAV. All samples proved negative so the samples were re-extracted using the complete CSIRO litter preparation and extraction method (Section 4.6.2) and subjected to qPCR for MDV, ILTV, CAV and IBDV. Again all samples were negative. Samples will be assayed for FAdV shortly to complete the work.

11.4 Discussion and conclusions

Both farms used heaps of comparatively large size, and litter that had been used several time previously to rear chickens. The biggest differences between the two farms were in the initial moisture contents and the speed of the temperature response, with Farm 1 staring out with wetter litter and having a slower but more sustained rise in temperature. This is consistent the effects of higher moisture content observed in our earlier experiments 2.2.1 and 2.3.

The failure to detect viral nucleic acids of the viruses of interest in this field site highlights the risks of work such as this. Farms were selected on the basis of being in poultry-dense production areas where infection with a number of the viruses under test could be expected, as found by Islam *et al.* (2013a). This meant that the major objective of the experiment could not be achieved. However it is encouraging from a biosecurity point of view that none of the viruses were detected.

A lesser objective of this work was to again investigate temperatures close to the surface of the heap, something not achieved in earlier CRC project 06-15 (Walkden-Brown *et al.* 2010a). The findings on Farm 2 were consistent with those in earlier experiments 2.1 and 2.2.1 where temperatures at these depths were much closer to those at 20 or 25 cm, than to surface temperatures. However on Farm 1, temperatures at 5 cm only reached a peak temperature of 45˚C, and were clearly sub-optimal for pathogen inactivation. The two dataloggers at this depth in two separate heaps on this farm provided very similar recordings so the finding appears not to be aberrant.

In conclusion, once again, heaping of litter in large heaps produced temperatures well into the pasteurizing range confirming the reliability of this method. In no experiment or situation have we encountered failure to generate high temperatures when litter is heaped or windrowed, even in as small a vessel as a broccoli box.

12. Strand 4. Decision support tool

12.1 Introduction

The aim of Strand 4 of the project was to evaluate practical application of the project findings on farm and to develop a decision support spreadsheet to assist with decision-making regarding litter pasteurisation. This would be in addition to the SOP developed and reported in Section 8 of this report.

Our initial goal was to develop a fairly simple spreadsheet based on project findings, working in collaboration with Mark Dunlop. However we have ended up aiming higher, for a more complete and accurate model for the following reasons:

- The project results made it clear that modelling of the management factors that may influence temperatures in heaps would be a more complex task than originally envisaged
- Mark Dunlop undertook a PhD and necessarily would have limited time to devote to the model
- There is considerable litter pasteurisation data from outside of this project that should be included in developing the model. This includes data from Poultry CRC project 06-15 “Optimising methods for multiple batch litter use by broilers” and from Michael Cressman’s PhD project.
- A skilled modeller, Yan Laurenson joined our research group as a post-doctoral fellow and agreed to take on the lead role in developing a model that would predict the thermal status of heaped litter given initial starting conditions, together with specific useful summary outputs.

The work on model development has been led by Yan Laurenson in consultation with Steve Walkden-Brown, Mark Dunlop and Fakhrul Islam. The data on which the model is built was from 8 on-farm data sets mostly collected by Fakhrul Islam under this project and project, 06-15, but also data collected by Michael Cressman. Data collation into one dataset and validation was done by Fakhrul Islam and Steve Walkden-Brown.

Re-used litter may harbour bacterial, viral fungal and parasitic pathogens. Thus, it may serve to transmit pathogens to the next batch of chickens. As such, the appropriate treatment of litter between batches of chickens is essential to minimise the risks involved with re-using litter. One such treatment method is the composting/pasteurisation of litter in heaps or windrows but differing pathogens vary in their susceptibility to changes in temperature. Thus, in order to determine the appropriate duration of litter composting for optimal reduction in

pathogen burden, it is would be ideal to be able to predict the temperature profile of a heap/windrow and factors influencing this. These would include the impact of ambient temperature (and daily variation therein), initial moisture (% water content), heap/windrow size, and composting practices such as covering and turning. The development of a predictive mathematical model of composting temperatures may thereby aid in determining the appropriate design of composting practices (heap/windrow size, moisture content, duration, covering and turning). There is no such model worldwide at present although (Schmidt *et al.* 2013) have recently measured and attempted to model temperature change in windrowed broiler litter under experimental conditions.

The aim of this study is to develop an appropriate mathematical model to describe composting temperature profiles as part of an effective advice tool. The final advice tool should able to account for the following input information

- Ambient temperature, heap/windrow size, moisture content, covering and turning

To be of use to the end user the model should provide as outputs

- Average, minimum or maximum temperature at any one specified time or period during the process, and the proportion of the heap/windrow reaching specified temperatures and durations.
- Underlying these predictions will be a complete heat map of the heap/windrow as pasteurising proceeds.

This report outlines the progress towards this aim, and the remaining steps towards producing an advice tool.

12.2 Methods

The complete data set comprises 542 temperature profiles over a litter pasteurisation cycle at various depths in approximately 100 heaps subject to various treatments on 8 farms in NSW and QLD. This data set includes over 90,000 individual temperature recordings (usually hourly). The modelling approach is an empirical one approach is to model empirically the available data, component by component, using 75% of the data to develop the model and 25% of the data to validate the model against. The model is being constructed in Excel and progress on each component is reported below.

12.2.1 Ambient (Air/Shed) temperature profile

The goal is to convert min/max temperature data and timing into continuous profiles. Ambient (Air/Shed) temperature is modelled (for time intervals not less than a 10^{th} of a second) such that the user can specify input values for:

- maximum temperature (°C)
- minimum temperature (°C)
- time of maximum temperature
- time of minimum temperature
- time at start

Air/Shed temperature is given by equation 10 according to equations 1 to 9 as follows:

$$\alpha(t) = \arctan\left(\cot\left(\frac{\pi}{24}\cdot(t+24+t_{start}-t_{min})\right)\right) \quad \text{[Equation 1]}$$

$$\beta(t) = \arctan\left(\cot\left(\frac{\pi}{24}\cdot(t+24+t_{start}-t_{max})\right)\right) \quad \text{[Equation 2]}$$

$$\gamma(t) = \left(\frac{1}{\pi}\cdot\left(\alpha(t)\text{-}\beta(t)\right)\right) + \left(\frac{t_{max}-t_{min}}{24}\right) \quad \text{[Equation 3]}$$

$$\omega(t) = \gamma(t)\cdot\left(\frac{\pi}{t_{max}-t_{min}} - \frac{\pi}{24-(t_{max}-t_{min})}\right) + \frac{\pi}{24-(t_{max}-t_{min})} \quad \text{[Equation 4]}$$

$$\rho(t) = \gamma(t)\cdot\left(\frac{t_{max}-t_{min}}{2} - \frac{24-(t_{max}-t_{min})}{2}\right) + \frac{24-(t_{max}-t_{min})}{2} \quad \text{[Equation 5]}$$

$$\tau(t) = 24\cdot\mathbb{Z}\left(\frac{t+(24-(t_{max}-t_{min}))-(t_{min}-t_{start}+0.001)}{24}\right) \quad \text{[Equation 6]}$$

$$\varphi(t) = \omega(t)\cdot\left(\rho(t)+\tau(t)+(t_{min}-t_{start})\right) \quad \text{[Equation 7]}$$

$$a = \frac{C_{max}-C_{min}}{2} \quad \text{[Equation 8]}$$

$$b(t) = \sin\left(\omega(t)\cdot t-\varphi(t)\right) + \frac{C_{max}+C_{min}}{2} \quad \text{[Equation 9]}$$

$$C(t) = a * b(t) \quad \text{[Equation 10]}$$

where t = duration in hours since the start of simulation (hours given in decimals e.g. 1 hour 30 minutes = 1.5 hours); t_{start} = time at start of simulation (24 hour clock given in decimals e.g. 14:30 = 14.5); t_{min} = time at which minimum air temperature occurs (24 hour clock given in decimals); t_{max} = time at which maximum air temperature occurs (24 hour clock given in decimals); C = air temperature (°C); C_{min}= minimum air temperature (°C); C_{max}= maximum air temperature (°C); ω = angular frequency; φ = phase.

Figure 12.1 provides an example of the pattern of modelled air temperature given input values of:

- maximum temperature (°C) = 25

- minimum temperature (°C) = 15
- time of maximum temperature = 15 (i.e. 3pm)
- time of minimum temperature = 6 (i.e. 6am)
- time at start = 0 (i.e. 12am midnight)

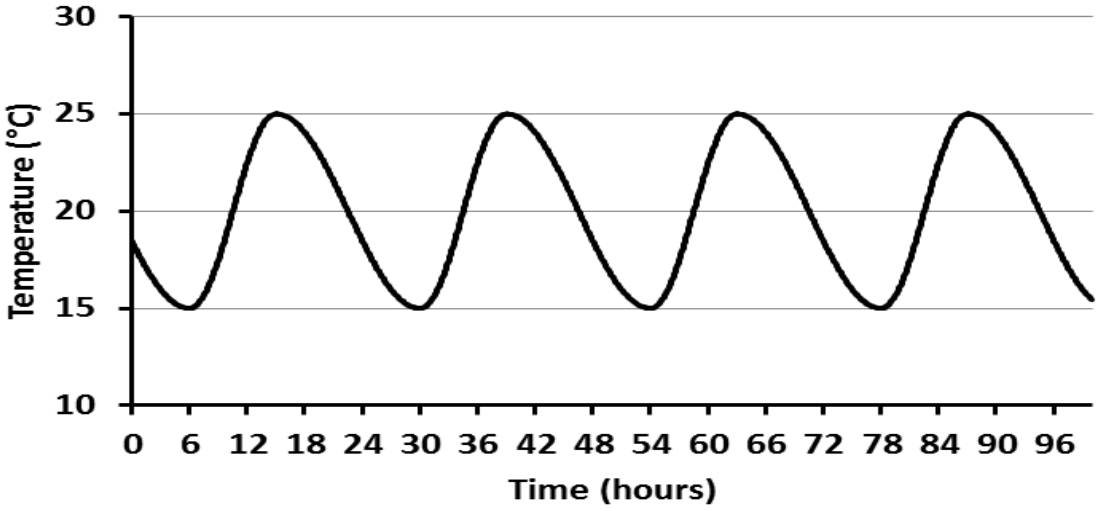

Figure 12.1 Air temperature (°C). See text for model assumptions

The model for air/shed temperature was validated by comparison to shed temperatures recorded at the Hebblewhite, Kirby and Cauchi experimental sites. This data set is given as hourly shed temperature readings for 3 sheds at the Hebblewhite site (sheds 1,2 & 3), 2 data sets for the Kirby site (shed 1), and 1 data set for the Cauchi site (shed 2). The air/shed temperature model given above adequately described the shed temperatures given by the validation data set (R^2 = 0.93) as shown in Figure 12.2.

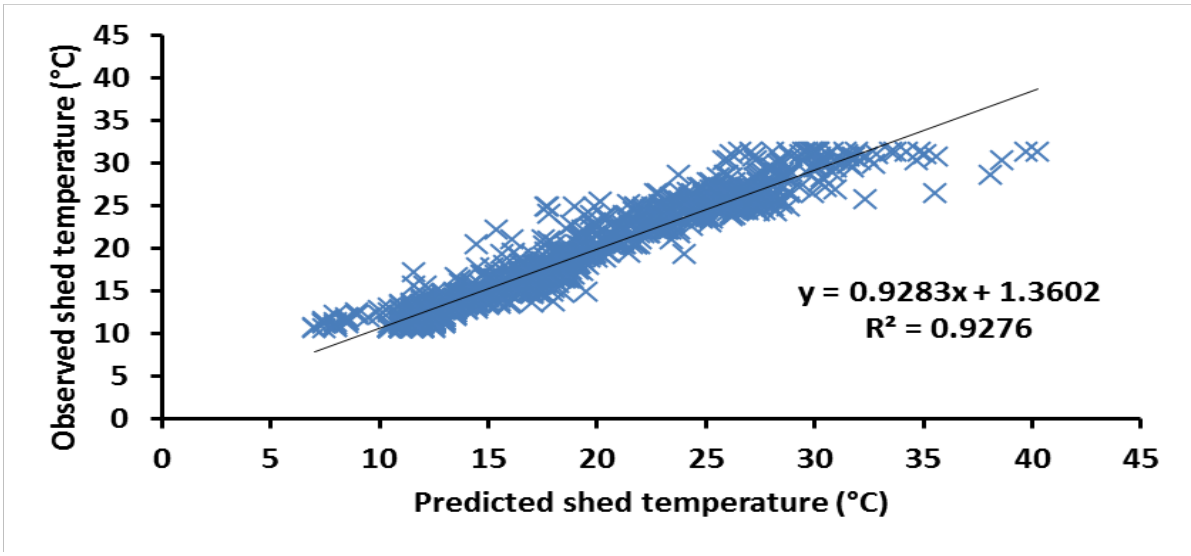

Figure 12.2 Validation of air/shed temperature model

12.2.2 Heap/Windrow Dimension, Volume & Surface Area

12.2.2.1 Heap/Windrow Dimension

A windrow or heap is modelled as a truncated paraboloid, such that the user can specify input values for:

- Peak Height (cm)
- Width (cm)
- Length (cm)

The dimensions (x,y,z co-ordinates) of a windrow or heap are given by equations 11 to 13 as follows:

$$y = h - \left(\frac{h}{25} \cdot \frac{\left(x - \frac{w}{2}\right)^2 + \left(z - \frac{w}{2}\right)^2}{\left(\frac{w}{10}\right)^2} \right) \quad \text{for } x > \left(\frac{w}{2}\right) \,\&\, y \geq 0 \quad \textbf{[Equation 11]}$$

$$y = h - \left(\frac{h}{25} \cdot \frac{\left(z - \frac{w}{2}\right)^2}{\left(\frac{w}{10}\right)^2} \right) \quad \text{for } \left(L - \frac{w}{2}\right) \geq x \geq \left(\frac{w}{2}\right) \,\&\, y \geq 0 \quad \textbf{[Equation 12]}$$

$$y = h - \left(\frac{h}{25} \cdot \frac{\left(x - \left(L - \frac{w}{2}\right)\right)^2 + \left(z - \frac{w}{2}\right)^2}{\left(\frac{w}{10}\right)^2} \right) \quad \text{for } x > \left(L - \frac{w}{2}\right) \,\&\, y \geq 0 \quad \textbf{[Equation 13]}$$

where *h* = peak height (cm); *w* = width (cm); *L* = length (cm).

Figure 3 provides an example of the dimensions of a windrow given input values of:

- Peak Height (cm) = 100
- Width (cm) = 150
- Length (cm) = 250

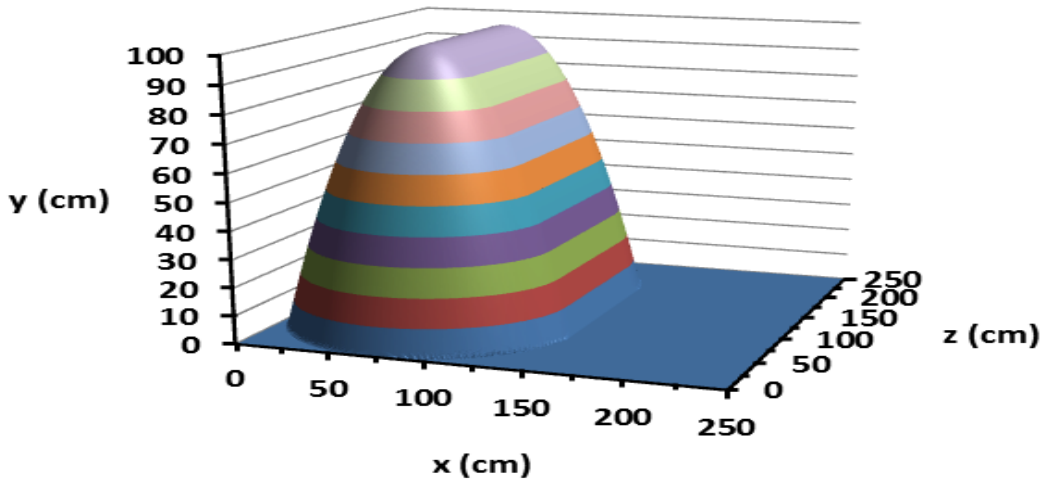

Figure 12.3 Dimensions of a windrow.

12.2.2.2 Heap/Windrow Volume

The volume (cm^3) of a heap or windrow is given by equation 14 as follows:

$$v = \frac{\pi}{2} \cdot h \cdot \left(\frac{w}{2}\right)^2 + \left(\frac{2}{3} \cdot h \cdot w \cdot (L - w)\right) \quad \textbf{[Equation 14]}$$

where v = volume (cm^3); h = peak height (cm); w = width (cm); L = length (cm).

12.2.2.3 Heap/Windrow Surface Area

The surface area (cm^2) of a heap or windrow (not including the base) is given by equation 17 according to equations 15 & 16 as follows:

$$p = \frac{\pi}{6} \cdot \left(\frac{w/2}{h^2}\right) \cdot \left(\left(\left(\frac{w}{2}\right)^2 + 4h^2\right)^{\frac{3}{2}} - \left(\frac{w}{2}\right)^3\right) \quad \textbf{[Equation 15]}$$

$$q = \left(\left(\frac{\sqrt{16h^2+w^2}}{2}\right) + \left(\left(\frac{w^2}{8h}\right) \cdot \left(\ln\left(4h + \sqrt{16h^2 + w^2}\right) - \ln(w)\right)\right)\right) \cdot (L - w) \quad \textbf{[Equation 16]}$$

$$sa = p + q \quad \textbf{[Equation 17]}$$

where *sa* = surface area (cm^2); h = peak height (cm); w = width (cm); L = length (cm); p = surface area of a paraboloid (cm^2); q = surface area of a truncated parabola (cm^2) (i.e. arc length of a parbola multiplied by the length of the truncated section).

12.2.3 Heap/Windrow Temperature profile

Data gathered from 8 experimental sites (Camilleri, Cauchi, Douglas, Hebblewhite, Kirby, Refalo, Sultana & Mercuri) were used to construct a mathematical description of heap/windrow temperature over time (hours) at specific depths. The data analysed included initial moisture (% water content) and shed/air temperature (°C) as quantitative values, and cover/no cover & turn/no turn as binary traits. Further, this data provides temperature (°C) measurements at hourly intervals at depths of 0, 5, 10, 20, 25, 50, 75 & 100cm. 75% of the available data was utilised for model construction and parameterisation, with the remaining 25% being retained for model validation.

A mathematical model resulting from analysis of the available data was constructed to predict temperature (°C) according to depth from surface of heap/windrow, time (hours), initial moisture (% water content), air/shed temperature (°C), heap/windrow volume (cm^3) and surface area (cm^2), cover/no cover.

The temperature (°C) at a specified depth (cm) and time (hours) is given by equation 22 according to equation 18 to 21 as follows:

$$\alpha(t) = a + b(t) \cdot (0.35 \cdot e^{-0.28d}) \quad \textbf{[Equation 18]}$$

$$\beta(t) = \frac{94 \cdot \left(e^{-\gamma d} - e^{-0.34d}\right)}{\left(1 + e^{-0.3t \cdot e^{-0.05\alpha(t)}}\right)} \quad \textbf{[Equation 19]}$$

$$\delta(t) = \frac{\varphi - \left(\varphi \cdot e^{-0.07d}\right)}{\left(1 + e^{-0.1 \cdot \left(t - \left(\omega \cdot e^{-0.02 m_0}\right)\right) \cdot e^{-0.1\alpha(t)}}\right)} \quad \textbf{[Equation 20]}$$

$$\tau(t) = \frac{\left(94 \cdot \left(e^{-\gamma d} - e^{-0.34d}\right)\right) + \left(\varphi - \left(\varphi \cdot e^{-0.07d}\right)\right)}{\left(1 + e^{-0.007t \cdot e^{-0.04d}}\right)} \quad \textbf{[Equation 21]}$$

$$H(t) = \alpha(t) + \beta(t) + \delta(t) - \tau(t) \qquad \text{for } H(t) > C_{min} \quad \textbf{[Equation 22]}$$

where t = time since the start of simulation (hours), $\alpha(t)$ = impact of fluctuating air/shed temperature; a = average air/shed temperature as given by equation 8; $b(t)$ = fluctuation in air/shed temperature; d = depth from the surface of heap/windrow (cm); m_0 = initial moisture (% water content); $H(t)$ = heat/temperature (°C); C_{min} = minimum air temperature (°C); γ, φ, ω depend upon whether the heap/windrow is covered or not (Table 12-1).

Table 12-1 Parameter values affected by covering.

Cover	γ	φ	ω
Yes	0.015	65	0
No	0.01	40	67

The mathematical model given above adequately described the temperature across time at the depths provided within the data set used for parameterisation (R^2 = 0.89, Figure 4) and the data set retained for validation (R^2 = 0.88, Figure 5).

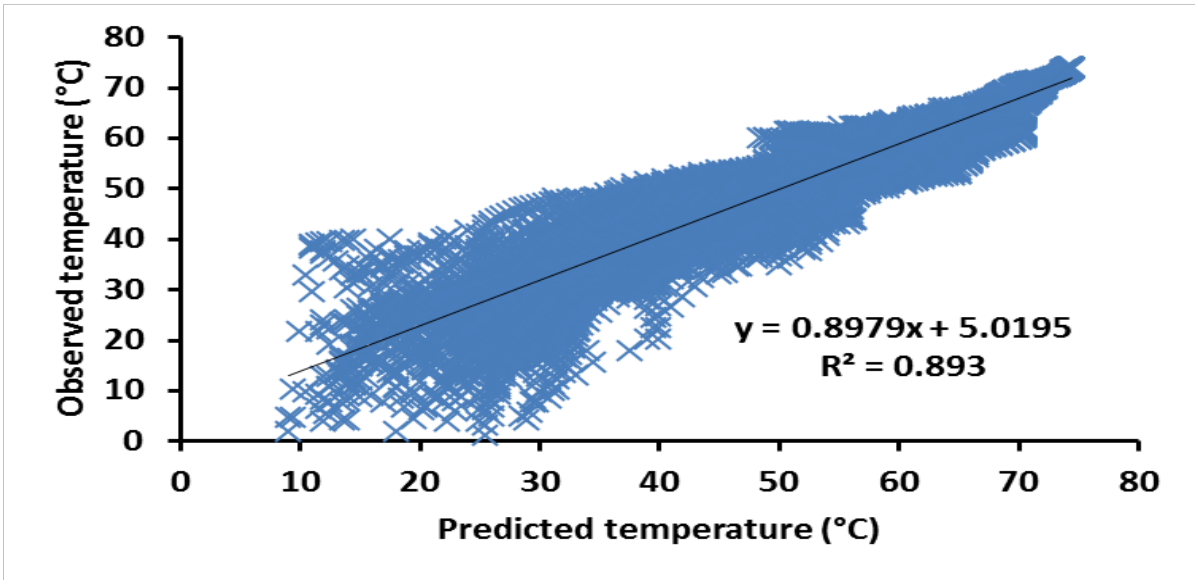

Figure 12.4 Model fit to data used for model construction and parameterisation.

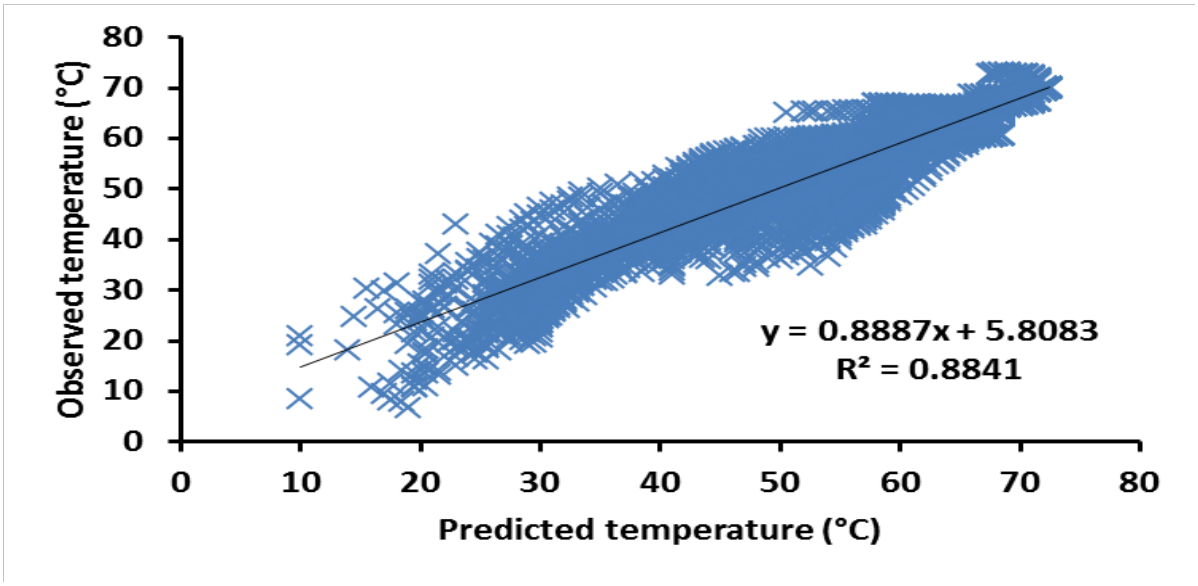

Figure 12.5 Model fit to data used for validation.

Model outputs are provided for 3 ranges of maximum (C_{max}) and minimum (C_{min}) air/shed temperature, 3 level of initial % moisture content (15, 25, 30%), and cover/no cover. Time of maximum air temperature = 15 (i.e. 3pm), Time of minimum temperature = 6 (i.e. 6am), Time at start = 0 (i.e. 12am midnight).

The following series of full page figures shows model outputs as follows

- Figure 12.6 provides model outputs for an initial moisture content of 15%.

- Figure 12.7 provides model outputs for an initial moisture content of 25%.
- Figure 12.8 provides model outputs for an initial moisture content of 35%.

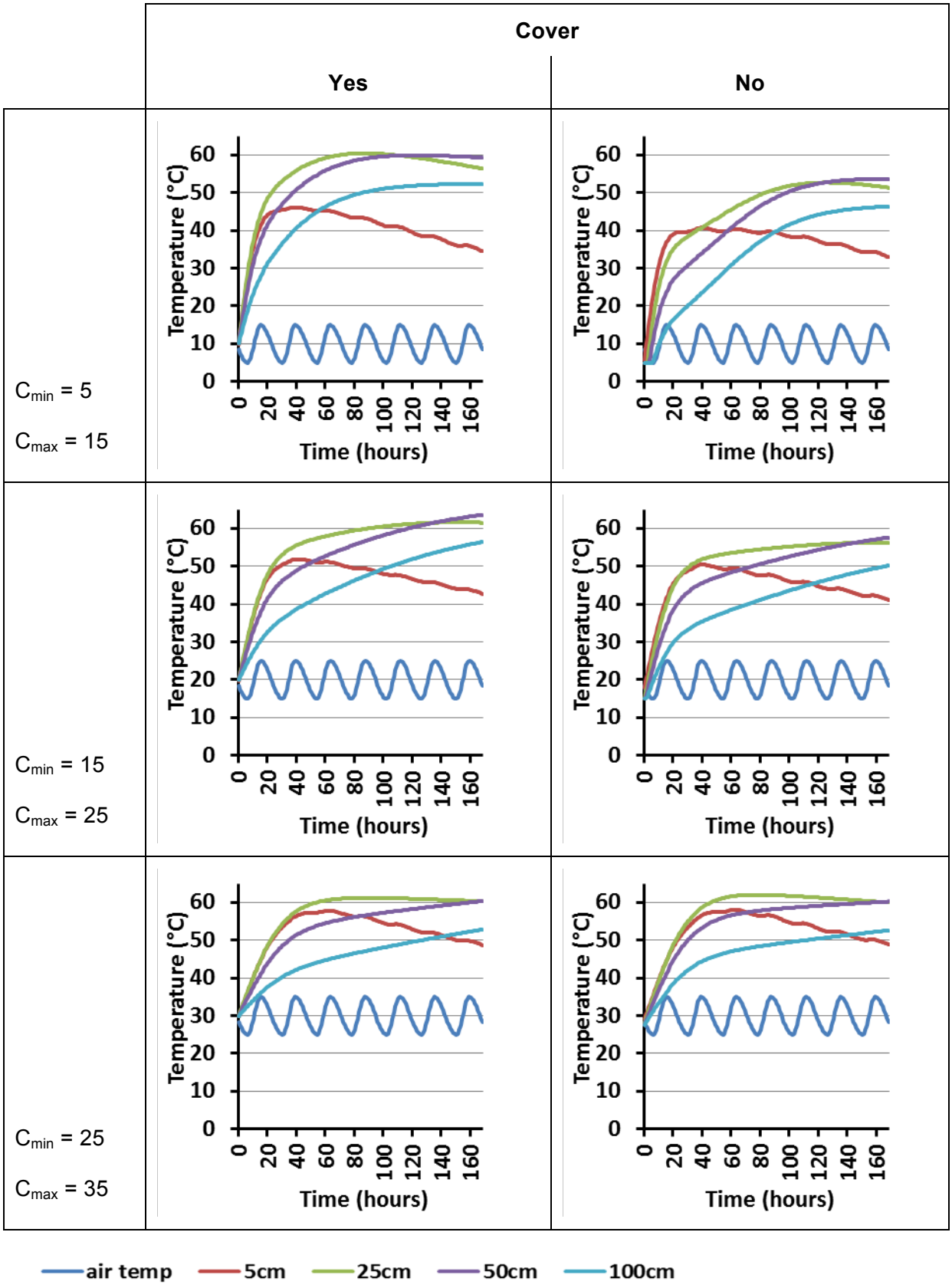

Figure 12.6 Model fit to data used for validation. Model outputs for an initial moisture content of 15%.

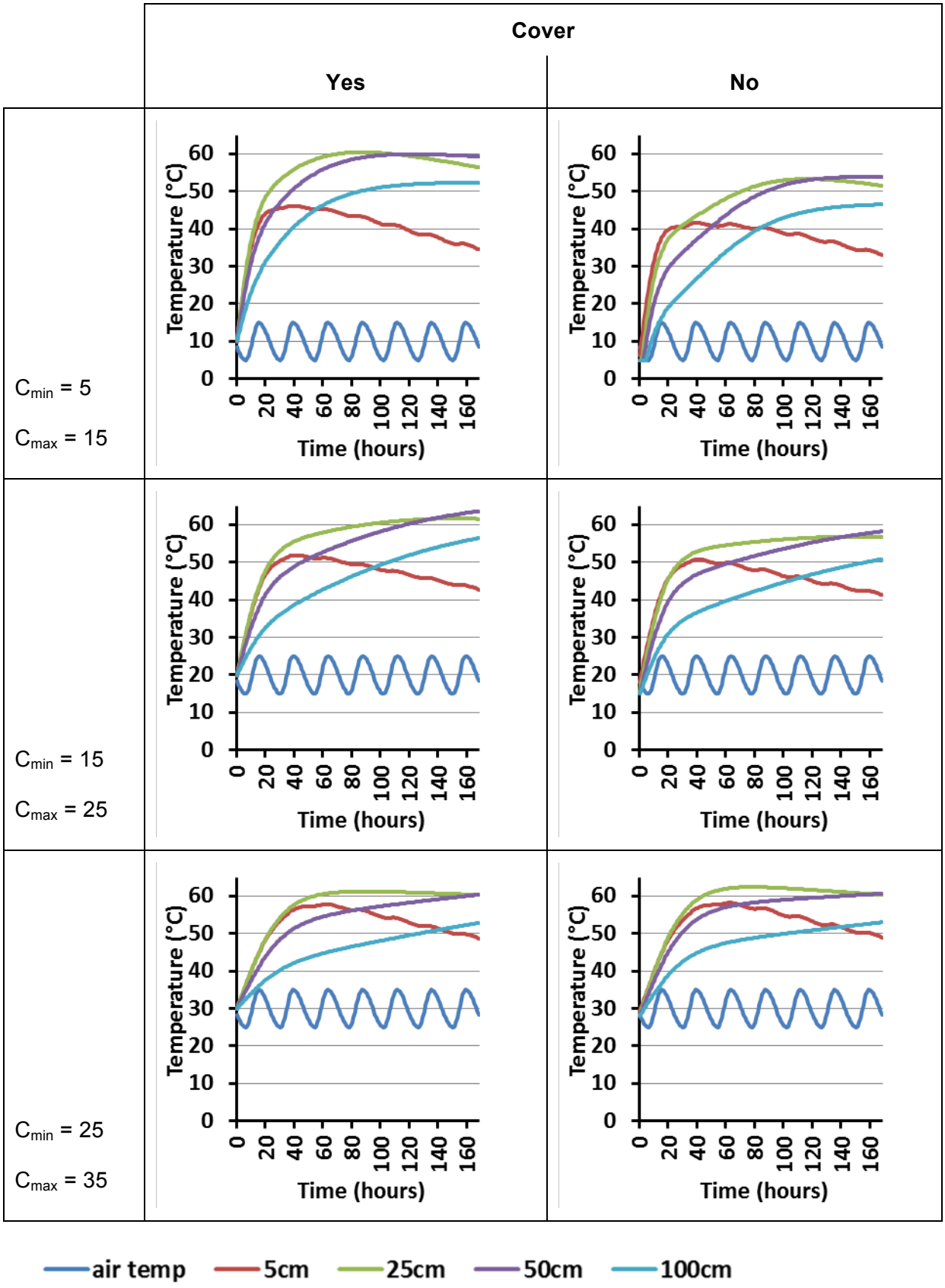

Figure 12.7 Model fit to data used for validation. Model outputs for an initial moisture content of 25%.

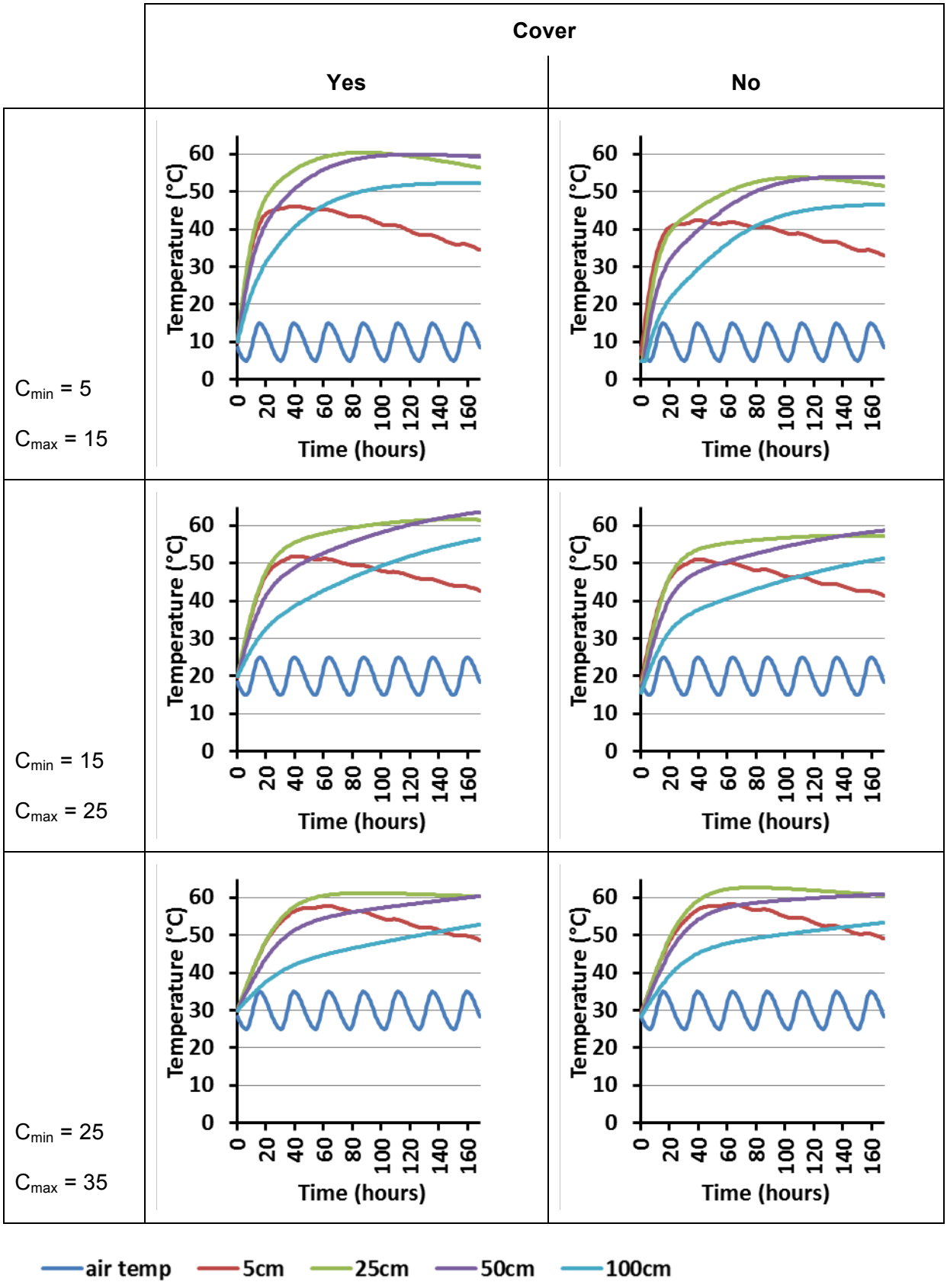

Figure 12.8 Model fit to data used for validation. Model outputs for an initial moisture content of 35%.

12.3 Discussion and remaining steps towards decision support tool

Sufficient validation has been done to determine that the model is adequately robust to produce useful information and is likely to achieve its original objectives. Work is currently in progress to incorporate the impact of turning upon composting temperature profiles. Following completion of a mathematical model to describe the impact of turning, the completed mathematical model will be incorporated into an excel spreadsheet. This will involve mapping predicted temperature profiles onto the heap/windrow dimensions outlined in Section 12.2.2.1, utilising the calculation of heap/windrow volume outlined in Section 12.2.2.2.

The final advice tool will be available as an Excel spreadsheet which will allow **user inputs** for:

- Ambient (Air/Shed) temperature
    - maximum daily temperature (°C)
    - minimum daily temperature (°C)
    - time of maximum temperature
    - time of minimum temperature
- Time at start of composting
- Heap/Windrow dimensions
    - Peak Height (cm)
    - Width (cm)
    - Length (cm)
- Initial moisture (% water content)
- Duration of composting
- Covering (Yes/No)
- Turning
    - Yes
        - Time of turns
    - No

Outputs of the advice tool will include:

- Average heap/windrow temperature over time
- Proportion of heap/windrow at differing temperatures over time
- Proportion of heap/windrow at a specified temperature and duration

Following completion of the advice tool it will be published in an appropriate scientific journal and the advice tool (as an excel spreadsheet) will be available for use. It is estimated that after 4 months work on the model it is about 80% complete and the CRC should have the complete model by the end of June 2015.

13 Supporting information

13.1 Acknowledgements

We are grateful to the following for their contributions to the project:

Collaborators

UNE	Prof Steve Walkden-Brown, Dr Fakhrul Islam, Dr Katrin Renz, Dr Yan Laurenson, Prof Parimal Roy (visiting scientist) Ms Sue Burgess (Technician), Mr Robin Achari (PhD student), Ms Kanchana Jayasundara (PhD student), Mr Mamdouh Alsharari (PhD student), Mr Hai Tran Minh (Masters Student)
CSIRO	Dr Peter Hunt and Ms Jody McNally (Research Assistant).
DAF (Qld)	Mr Mark Dunlop
Industry	Ben Wells, Guy Hebblewhite, David Refalo, Jason Cauchi, Angelo Sultana

Industry Steering Committee

Rod Jenner, Gary Sansom, Margaret McKenzie, Jorge Ruiz, Pat Blackall, Tim Walker.

Funding

Poultry CRC

Endeavour Foundation (Prof Roy)

UNE PhD student research accounts (value add to project experiments)

13.2 Plain English Compendium Summary

Sub-Project Title:	Methods to quantify and inactivate viruses in poultry litter
Poultry CRC Sub-Project No.:	2.2.3
Researcher:	Prof Stephen Walkden-Brown
Organisation:	University of New England
Phone:	02-6773 5152, 0413 107 973
Fax:	02-6773 3922
Email:	swalkden@une.edu.au
Sub-Project Overview	This project builds on earlier CRC work to better manage the disease transmission risks associated with reusing litter for multiple batches of chickens. The broad aims were to: 1. Develop methods for measuring of virus (viral genome) copy number in litter for 5 key chicken viruses; 2. Investigate factors affecting the temperatures achieved in heaped litter; 3. Define the association between viral genome copy number and virus infectivity (determined by chick exposure and seroconversion) under a wide range of temperature-time conditions in litter; 4. Use the information above to produce practical outcomes for industry including standard recommendations for in-shed pasteurisation of litter by heaping and a decision support tool for optimising litter pasteurisation
Background	Scarcity and price of chicken bedding materials makes reusing litter for multiple broiler batches attractive. One risk associated with this practice is litter-mediated transmission of pathogenic viruses. The most practical method for reducing this risk is heaping of litter to allow natural processes produce pasteurising temperatures. These have been shown to reduce the risk of virus transmission substantially for a number of viruses. However the tools available to both predict the temperatures that will be achieved during litter pasteurisation and the effects of such temperatures on virus inactivation are crude and/or prohibitively expensive.
Research	This project addressed the background problem in the following ways: 1. It developed methods of processing boiler litter to effectively extract viral nucleic acids (DNA or RNA) from it and then enumerate these by real-time quantitative PCR (qPCR) providing absolute quantification of virus copy number per g of litter. The viruses the project worked on were Fowl adenovirus 8 (FAdV), Infectious Bursal Disease virus (IBDV), Infectious laryngotracheitis virus (ILTV), chicken infectious anaemia virus (CAV) and Marek's disease virus (MDV). 2. It worked on farms to define the effects of covering, water addition/moisture content, aeration/turning and heap size and shape on the temperature profiles at different depths in heaped litter. Short pasteurisation times of a week or less, with litter of normal moisture content favour small heaps or windrows,

	without covering, moisture addition or turning. 3. It used this information to develop guidelines and standard operating procedures for litter pasteurisation by heaping, and a spreasheet based decision support tool to predict heap temperatures given various inputs. 4. The project defined the shedding profile of FAdV-8, IBDV, ILTV and CAV in faeces in SPF and commercial broiler chickens, finding important differences, most likely mediated by the presence of maternal antibody directed against the viruses of interest. It discovered that the respiratory virus ILTV is shed in very high amounts in faeces. 5. Two experiments investigated what temperatures for what periods of time were required to inactivate the viruses of interest in litter. They also investigated whether measurement of virus presence by exposing chicks to the litter and measuring seroconversion (bioassay) could be replaced by the simpler measure of determing viral copy number directly in the litter using qPCR. Findings revealed that CAV was highly resistant to the temperatures used in out experiments, but that the other viruses were inactivated by different combinations of temperature and time. It was also clear that qPCR could not replace the bioassay or other measures of virus infectivity, probably because viral nucleic acids continued to be present under conditions that had inactivated the virus.
Implications	The main implications of this project are: 1. Improved tools for decision making about litter pasteurisation. The SOP and guidelines and the temperature model are likely to result in improved pasteurisation practices when litter is reused resulting in an improved biosecurity situation 2. Improved tools for research. Methods for directly measuring viral genome copy in difficult materials such as litter and faeces have been developed. 3. Improved understanding of the shedding profiles of the 5 viruses under test and their persistence under various conditions will improved biosecurity decision making in the industry. 4. High faecal shedding of ILTV is a novel finding with important implications for understanding the epidemiology of this disease. 5. qPCR is not an adequate replacement for the chick bioassay to assess the infective status of virus contaminated litter. Alternative replacement methods should be developed.
Publications	**Achari, R., Islam, A., Renz, K., Hunt, P., Arzey, K. & Walkden-Brown, S (2014).** Monitoring of fowl adenoviruses using environmental samp *2nd World Veterinary Poultry Association Asia Conference on "Tes and Monitoring"*, pp. 78-84. Bangkok: World Veterinary Poultry Association Asia. **Alsharari, M., Islam, A. F. M. F., Walkden-Brown, S. W. & Renz, K. G. (2** Tissue distribution and shedding profiles of Chicken Anaemia Virus specific pathogen-free and commercial broiler chickens. *Proc Aust Sci Symp* **25**, (In press). **Alsharari, M. M. L. (2014).** Tissue Distribution and Shedding Profile of Chi Anaemia Virus in Antibody-Negative Leghorn and Commercial Broi Chickens. In *Animal Science*, p. 71. University of New England: University of New England.

Islam, A., Alsharari, M., Achari, R., Jayasundara, K., Renz, K., Burgess Walkden-Brown, S. (2014). Methods to detect viable viral pathoge poultry litter and implications for disease monitoring. In *2nd World Veterinary Poultry Association Asia Conference on "Testing and Monitoring"*, pp. 78-84. Bangkok: World Veterinary Poultry Associa Asia.

Islam, A. F. M. F., Mahmoud, M., Burgess, S. K., Renz, K. G. & Walkde Brown, S. W. (2013). Effect of litter size, shape, covering and turni litter pasteurization during in-shed partial composting. In *Proceedir the 62nd Western Poultry Disease Conference*, pp. 65-68. Sacram CA, USA.

Islam, A. M. F. M., Walkden-Brown, S. W. & Wells, B. (2013). Litter pasteurization: effects of cover and moisture level on the temperatu the litter heap. *Proc Aust Poult Sci Symp* **24**, 179-182.

Jayasundara, J. M. K. G. K., Walkden-Brown, S. W., Katz, M. E., Renz, Islam, A. F. M. F., McNally, J. & Hunt, P. W. (2015). Infectious bu disease antibody levels and viral load in bursa, faeces, litter and du following infection of commercial broiler chickens at 0 and 14 days age. *Proc Aust Poult Sci Symp* **25**, (In press).

Jayasundara, K., Islam, A., Walkden-Brown, S., Katz, M., Renz, K., Bur S., McNally, J. & Hunt, P. (2014). Determination of shedding profi infectious bursal disease virus in faeces of infected SPF chickens u qRT-PCR and association with levels in host tissues, dust and litte *2nd World Veterinary Poultry Association Asia Conference on "Tes and Monitoring"*. Bangkok: World Veterinary Poultry Association As

Mahmoud, M. A. (2013). The Effect of Shape, Size, Covering and Turning Litter Temperature During In-shed Litter Pasteurization. In *Animal Science*, p. 97. University of New England: University of New Engla

Roy, P., Islam, A., Burgess, S. & Walkden-Brown, S. (2014). qPCR dete of infectious laryngotracheitis virus in tissues, faeces, dust and litte Infection. In *10th International Symposium on Marek's Disease and Herpesviruses*, p. 30. Edited by H. Cheng. East Lansing, Michigan, Michigan State University and USDA-ARS Avian Disease and Onc Laboratory.

Roy, P., Islam, A. F. M. F., Burgess, S. K., Hunt, P. W., McNally, J. & S.W.Walkden-Brown (2015). Real time PCR quantification of Infe Laryngotracheitis virus in chicken tissues, faeces, isolator-dust and bedding material over 28 days following infection reveals high leve faeces and dust. *Journal of General Virology*, (Submitted).

Walkden-Brown, S., Groves, P., Islam, A. & Renz, K. (2014). Monitoring pathogens in poultry dust. In *2nd World Veterinary Poultry Associa Asia Conference on "Testing and Monitoring"*, pp. 66-71. Bangkok: Veterinary Poultry Association Asia.

Walkden-Brown, S. & Islam, A. (2014). How do I treat my litter between batches. In *Poultry Information Exchange (PIX) 2014 Litter Manag Workshop*, pp. 67-77. Edited by R. Jenner. Gold Coast Convention Centre: Poultry Information Exchange (www.pix.org.au).

Walkden-Brown, S. W., Hunt, P. W., McNally, J., Cressman, M. D., Burg S. K. & Islam, A. F. M. F. (2013). Development of methods for rec and quantitation of viral nucleic acids from broiler litter. *Proc Aust P Sci Symp* **24**, 171-174.

Walkden-Brown, S. W., Islam, A. F., Wells, B. A. & Burgess, S. K. (201 shed heaping of broiler litter to inactivate poultry pathogens before In *24th World's Poultry Congress*, pp. 307-310. Salvador, Brazil, 5- August 2012: World's Poultrty Science Association.

13.2.1 References

Adair, BM, Fitzgerald, SD (2008) Group 1 - Adenovirus Infections. In 'Diseases of Poultry.' (Eds YM Saif, AM Fadly, JR Glisson, LR McDougald, LK Nolan, DA Swayne.) (Blackwell Publishing Professional: Ames, Iowa)

Bagust, T, Jones, R, Guy, J (2000) Avian infectious laryngotracheitis. *Revue scientifique et technique (International Office of Epizootics)* **19**, 483-492.

Barker, K, Coufal, C, Purswell, J, Davis, J, Parker, H, Kidd, M, McDaniel, C, Kiess, A (2011) In-house windrowing of a commercial broiler farm during the summer months and its effect on litter composition. *The Journal of Applied Poultry Research* **20**, 168-180.

Beach, J (1931) A bacteriological study of Infectious Laryngotracheitis of chickens. *The Journal of experimental medicine* **54**, 801-808.

Beffa, T, Blanc, M, Marilley, L, Fischer, JL, Lyon, P-F, Aragno, M (1996) Taxonomic and metabolic microbial diversity during composting. In 'The Science of Composting.' pp. 149-161. (Springer:

Blake, PA, Islam, AMFM, Underwood, GJ, Walkden-Brown, SW (2005) Measurement of the effect of storage temperature and duration on the in vivo infectivity of Marek's disease virus in feather dust. *Proc. Aust. Poult. Sci. Symp.* **17**, 109-112.

Bohm, R (2007) Chapter 9 Pathogenic agents. In 'Compost Science and Technology.' (Eds LF Diaz, Md Bertoldi, W Bidlingmaier, E Stentiford.) Vol. Waste Management Series Volume 8 pp. 177-200. (Elsevier:

Brodie, H, Donald, J, Conner, D, Tucker, J, Harkin, H (1994) Field evaluation of mini-composting of poultry carcasses. *Poultry Science* **73**, 41.

Brodie, HL, Carr, LE, Condon, P (2000) A comparison of static pile and turned windrow methods for poultry litter compost production. *Compost Science & Utilization* **8**, 178-189.

Burge, WD (1983) Fate of pathogens. *Land treatment of hazardous wastes* 253-262.

Burge, WD, Cramer, WN, Kawata, K (1983) Effect of heat on virus inactivation by ammonia. *Applied and Environmental Microbiology* **46**, 446-451.

Callison, SA, Riblet, SM, Oldoni, Sun, S, Zavala, G, Williams, S, Resurreccion, RS, Spackman, E, Garcia, M (2007) Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. *Journal of Virological Methods* **139**, 31-38.

Calnek, BW, Hitchner, SB (1973) Survival and disinfection of Marek's disease virus and the effectiveness of filters in preventing airborne dissemination. *Poult Sci* **52**, 35-43.

Carr, LE, Wheaton, FW, Douglass, LW (1990) Empirical models to determine ammonia concentrations from broiler chicken litter. *Transactions of the American Society of Agricultural Engineers* **33**, 60-65.

Carrozza, JH, Fredrickson, TN, Prince, RP, Luginbuhl, RE (1973) Role of desquamated epithelial cells in transmission of Marek's disease. *Avian Dis* **17**, 767-81.

Chen, Z, Jiang, X (2014) Microbiological safety of chicken litter or chicken litter-based organic fertilizers: a review. *Agriculture* **4**, 1-29.

Chinivasagam, HN (2009) Re-use of chicken litter across broiler cycles – managing the food-borne pathogen risk. Final Report of Poultry CRC Project 05-16. Australian Poultry CRC, Armidale.

Coufal, CD, Chavez, C, Niemeyer, PR, Carey, JB (2006) Measurement of broiler litter production rates and nutrient content using recycled litter. *Poultry Science* **85**, 398-403.

Cramer, WN, Burge, WD, Kawata, K (1983) Kinetics of virus inactivation by ammonia. *Applied and Environmental Microbiology* **45**, 760-765.

Davidson, I, Skoda, I (2005) The impact of feathers use on the detection and study of DNA viral pathogens in commercial poultry. *World's Poultry Science Journal* **61**, 407-417.

East, IJ (2007) Adoption of biosecurity practices in the Australian poultry industries. *Australian Veterinary Journal* **85**, 107–112

Elving, J, Emmoth, E, Albihn, A, Vinnerås, B, Ottoson, J (2012) Composting for avian influenza virus elimination. *Applied and Environmental Microbiology* **78**, 3280-3285.

EPA, 2012. Chapter 5. Pathogen and Vector Attraction Reduction Requirements. A Plain English Guide to the EPA Part 503 Biosolids Rule. . US EPA (United States Environmental Protection Agency), 503: 21.

Eterradossi, N, Saif, YM (2008) Infectious Bursal Disease. In 'Diseases of Poultry.' (Ed. AMF Y.M. Saif, J.R. Glisson, L.R. McDougald, L.K Nolan, and D.E. Swayne.) pp. 185-206. (Iowa State University Press: Ames Iowa)

Fahey, K, Bagust, T, York, J (1983) Laryngotracheitis herpesvirus infection in the chicken: the role of humoral antibody in immunity to a graded challenge infection. *Avian Pathology* **12**, 505-514.

Giambrone, JJ, Fagbohun, O, Macklin, KS (2008) Management Practices to Reduce Infectious Laryngotracheitis Virus in Poultry Litter. *Journal of Applied Poultry Research* **17**, 64-68.

Grgic, H, Philippe, C, Ojkic, D, Nagy, É (2006) Study of vertical transmission of fowl adenoviruses. *Canadian journal of veterinary research* **70**, 230.
Grgić, H, Yang, D-H, Nagy, É (2011) Pathogenicity and complete genome sequence of a fowl adenovirus serotype 8 isolate. *Virus Research* **156**, 91-97.
Griffiths, RI, Whiteley, AS, O'Donnell, AG, Bailey, MJ (2000) Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. *Appl. Environ. Microbiol.* **66**, 5488-5491.
Groves, PJ (2002) Reuse of litter – are there disease /food safety implications? In ' Poultry Information Exchange, 14-16 Apr 2002, Surfers Paradise, QLD, Australia. pp. 107-110.
Guan, J, Chan, M, Grenier, C, Wilkie, DC, Brooks, BW, Spencer, JL (2009) Survival of Avian Influenza and Newcastle Disease Viruses in Compost and at Ambient Temperatures Based on Virus Isolation and Real-Time Reverse Transcriptase PCR. *Avian Diseases* **53**, 26-33.
Guan, J, Chan, M, Ma, B, Grenier, C, Wilkie, D, Pasick, J, Brooks, B, Spencer, J (2008) Development of methods for detection and quantification of avian influenza and Newcastle disease viruses in compost by real-time reverse transcription polymerase chain reaction and virus isolation. *Poultry Science* **87**, 838.
Günes, A, Marek, A, Grafl, B, Berger, E, Hess, M (2012) Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). *Journal of Virological Methods* **183**, 147-153.
Haug, RT (1993a) Chapter 5. Kinetics of heat inactivation. In 'The practical handbook of compost engineering ' pp. 161-203. (Lewis Publishers: Boca Raton)
Haug, RT (1993b) 'The practical handbook of compost engineering.' (Lewis Publishers: Boca Raton)
Hoop, R (1992) Persistence and vertical transmission of chicken anaemia agent in experimentally infected laying hens. *Avian Pathology* **21**, 493-501.
Hurt, RA, Qiu, X, Wu, L, Roh, Y, Palumbo, AV, Tiedje, JM, Zhou, J (2001) Simultaneous Recovery of RNA and DNA from Soils and Sediments. *Appl. Environ. Microbiol.* **67**, 4495-4503.
Ignjatovic, J, Sapats, S (2002) Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. *Australian Veterinary Journal* **80**, 689-694.
Islam, A, Cheetham, BF, Mahony, TJ, Young, PL, Walkden-Brown, SW (2006) Absolute quantification of Marek's disease virus and herpesvirus of turkeys in PBL, feather tip and dust samples using real-time PCR. *Journal of Virological Methods* **132**, 127-134.
Islam, A, Harrision, B, Cheetham, BF, Mahony, TJ, Young, PL, Walkden-Brown, SW (2004) Differential amplification and quantitation of Marek's Disease viruses using real-time polymerase chain reaction. *Journal of Virological Methods* **119**, 103-113.
Islam, A, Walkden-Brown, SW (2007) Quantitative profiling of the shedding rate of the three Marek's disease virus (MDV) serotypes reveals that challenge with virulent MDV markedly increases shedding of vaccinal viruses. *Journal of General Virology* **88**, 2121-2128.
Islam, A, Walkden-Brown, SW, Groves, PJ, Wells, B (2013a) Development of a chick bioassay for determination of infectivity of viral pathogens in poultry litter. *Australian Veterinary Journal* **91**, 65-71.
Islam, AFMF, Burgess, SK, Easey, P, Wells, B, Walkden-Brown, SW (2010) Inactivation of viruses and coccidia in broiler litter following heaping or windrowing at the end of the batch. In 'Australian Poultry Science Symposium. Sydney'. (Ed. P Selle) Volume 21 pp. 118-121.
Islam, AFMF, Mahmoud, M, Burgess, SK, Renz, KG, Walkden-Brown, SW (2013b) Effect of litter size, shape, covering and turning on litter pasteurization during in-shed partial composting. In 'Proceedings of the 62nd Western Poultry Disease Conference. Sacramento, CA, USA', 24-27 March, 2013. Volume 62 pp. 65-68.
Islam, AFMF, Walkden-Brown, SW (2010) Multiple Batch Litter Reuse Systems For Broiler Chickens: Review And Risk Assessment. Australian Poultry CRC.
Islam, AFMF, Walkden-Brown, SW, Groves, PJ, Underwood, GJ (2008) Kinetics of Marek's disease virus (MDV) infection in broiler chickens 1: effect of varying vaccination to challenge interval on vaccinal protection and load of MDV and herpesvirus of turkey in the spleen and feather dander over time. *Avian Pathology* **37**, 225-235.
Islam, T, Renz, KG, Ralapanawe, S, Walkden-Brown, SW (2013c) Viral kinetics, shedding profile and transmission of serotype 1 Marek's disease vaccine Rispens/CVI988 in maternal antibody-free chickens. *Avian Diseases* **57**, 454–463.
Islam, T, Walkden-Brown, SW, Renz, KG, Fakhrul Islam, AFM, Ralapanawe, S (2014) Replication kinetics and shedding of very virulent Marek's disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. *Veterinary Microbiology* **173**, 208-223.
Jordan, F, Evanson, HM, Bennett, JM (1967) The survival of the virus of infectious laryngotracheitis. *Zentralblatt für Veterinärmedizin Reihe B* **14**, 135-150.

Kuske, CR, Banton, KL, Adorada, DL, Stark, PC, Hill, KK, Jackson, PJ (1998) Small-Scale DNA Sample Preparation Method for Field PCR Detection of Microbial Cells and Spores in Soil. *Appl. Environ. Microbiol.* **64**, 2463-2472.

Kwak, WS, Huh, JW, McCaskey, TA (2005) Effect of processing time on enteric bacteria survival and on temperature and chemical composition of broiler poultry litter processed by two methods. *Bioresource Technology* **96**, 1529-1536.

Lavergne, TK (2008) In-house pasteurization of broiler litter. In 'Proceedings of the XXIII World's Poultry Congress. Brisbane, Australia', 30 June - 4 July. (World Poultry Science Association:

Lavergne, TK, Stephens, MF, Schellinger, D, Carney, WA (2006) In-house Pasteurization of Broiler Litter. Louisiana State University Agricultural Centre http://www.lsuagcenter.com.

Lu, H, Castro, AE, Pennick, K, Liu, J, Yang, Q, Dunn, P, Weinstock, D, Henzler, D (2003a) Survival of Avian Influenza Virus H7N2 in SPF Chickens and Their Environments. *Avian Diseases* **47**, 1015-1021.

Lu, J, Sanchez, S, Hofacre, C, Maurer, JJ, Harmon, BG, Lee, MD (2003b) Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. *Applied and Environmental Microbiology* **69**, 901-908.

Macklin, KS, Hess, JB, Bilgili, SF (2008) In-House Windrow Composting and Its Effects on Foodborne Pathogens. *Journal of Applied Poultry Research* **17**, 121-127.

Macklin, KS, Hess, JB, Bilgili, SF, Norton, RA (2006) Effects of in-house composting of litter on bacterial levels. *Journal of Applied Poultry Research* **15**, 531-537.

McGahan, E, Bielefeld, N, Wiedemann, S, Keane, O (2014) National Environmental Management System for the Meat Chicken Industry Version 2. Final Report of RIRDC Project No PRJ-005765. Publication No. 14/100. Rural Industries Research and Development Corporation, Barton ACT 2600.

Ophel-Keller, K, McKay, A, Hartley, D, Herdina, Curran, J (2008) Development of a routine DNA-based testing service for soilborne diseases in Australia. *Australasian Plant Pathology* **37**, 243-253.

Park, G, Diez-Gonzalez, F (2003) Utilization of carbonate and ammonia-based treatments to eliminate Escherichia coli O157: H7 and Salmonella Typhimurium DT104 from cattle manure. *Journal of Applied Microbiology* **94**, 675-685.

Ralapanawe, S, Renz, K, Burgess, S, Walkden-Brown, S (2015) Field studies on the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus. *Australian Veterinary Journal* (Subm 13-1-2015).

Reece, F, Bates, B, Lott, B (1979) Ammonia control in broiler houses. *Poultry Science* **58**, 754-755.

Runge, GA, Blackall, PJ, Casey, KD (2007) Chicken Litter. Issues Associated with Sourcing and Use. Rural Industries Research and Development Corporation, Barton, ACT.

Schat, KA, van Santen, VL (2008) Chicken Infectious Anemia. In 'Diseases of Poultry.' (Ed. AMF Y.M. Saif, J.R. Glisson, L.R. McDougald, L.K Nolan, and D.E. Swayne.) pp. 211-235. (Iowa State University Press: Ames Iowa)

Schmidt, A, Davis, J, Purswell, J, Fan, Z, Kiess, A (2013) Spatial variability of heating profiles in windrowed poultry litter. *The Journal of Applied Poultry Research* **22**, 319-328.

Schwab, K, De Leon, R, Sobsey, M (1995) Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR. *Appl. Environ. Microbiol.* **61**, 531-537.

Seddon, HR, Hart, L (1936) Infectivity Experiments With the Virus of Laryngotracheitis of Fowls*. *Australian Veterinary Journal* **12**, 13-16.

Silva, ME, Lemos, LT, Cunha-Queda, AC, Nunes, OC (2009) Co-composting of poultry manure with low quantities of carbon-rich materials. *Waste Management & Research* **27**, 119-128.

Sobsey, MD, Meschke, JS (2003) Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. *Report for the World Health Organization, Geneva, Switzerland* 70.

Standards Australia, 2012. Australian Standard 4454-2012 Composts, soil conditioners and mulches AS 4454-2012 Standards Australia,, Sydney Australia. AS 4454-2012 74.

Steer, PA, Kirkpatrick, NC, O'Rourke, D, Noormohammadi, AH (2009) Classification of fowl adenovirus serotypes by use of high-resolution melting-curve analysis of the hexon gene region. *Journal of Clinical Microbiology* **47**, 311-321.

Takase, K, Nonaka, F, Fukuda, T, Yamada, S (1982) Recovery of virus from feces and tissues of chickens infected with cell-culture-adapted infectious bursal disease virus. *The Japanese Journal of Veterinary Science* **44**, 207-211.

Tiquia, SM, Tam, NFY (2002) Characterization and composting of poultry litter in forced-aeration piles. *Process Biochemistry* **37**, 869-880.

Walkden-Brown, S, Islam, A (2010) Temperature inactivation of viral pathogens in litter. Final Report of Poultry CRC Project 09-34. Australian Poultry CRC, Armidale.

Walkden-Brown, S, Islam, A, Dunlop, M, Wells, B (2010a) Optimising methods for multiple batch litter use by broilers. Final Report of Poultry CRC Project 06-15. Australian Poultry CRC, Armidale.

Walkden-Brown, SW, Islam, AFMF, Van Den Heuvel, A, Cressman, MD, Redding, MR (2013) Effects of various additives to reused broiler litter on ammonia production, chicken welfare and performance. *Proc. Aust. Poult. Sci. Symp.* **24**, 175-178.

Walkden-Brown, SW, Islam, AFMF, Wells, B, Burgess, SK (2010b) 'Litter re-use: techniques to effectively destroy viral pathogens, Proceedings of the 2010 Poultry Information Exchange, 23-25 May, Gold Coast, Queensland.'. (Queensland Department of Primary Industries:

Walkden-Brown, SW, Tannock, GA, Groves, PJ (2007) Improved diagnostics and disease surveillance through the application of molecular biotechnology. Final Report of Poultry CRC Project 03-17. Australian Poultry CRC, Armidale.

Wang, L-G, Ma, J, Xue, C-Y, Wang, W, Guo, C, Chen, F, Qin, J-P, Huang, N-H, Bi, Y-Z, Cao, Y-C (2013) Dynamic distribution and tissue tropism of infectious laryngotracheitis virus in experimentally infected chickens. *Archives of Virology* **158**, 659-666.

Wilkinson, K, Harapas, D, Tee, E, Tomkins, B, Premier, R (2003) Strategies for the safe use of poultry litter in food crop production. Final Report, Horticulture Australia, Project Number VG01049. Horticulture Australia Ltd., Sydney.

Woese, C (1960) Thermal inactivation of animal viruses. *Annals of the New York Academy of Sciences* **81**, 741-750.

World Health Organisation (2004) Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products. *WHO Technical Report, Series*

Yuasa, N, Taniguchi, T, Imada, T, Hihara, H (1982) Distribution of chicken anemia agent (CAA) and detection of neutralizing antibody in chicks experimentally inoculated with CAA. *National Institute of Animal Health Quarterly* **23**, 78-81.

Zelnik, V, Harlin, O, Fehler, F, Kaspers, B, Gobel, TW, Nair, VK, Osterrieder, N (2004) An enzyme-linked immunosorbent assay (ELISA) for detection of Marek's disease virus-specific antibodies and its application in an experimental vaccine trial. *Journal of Veterinary Medicine Series B. Infectious Diseases and Veterinary Public Health* **51**, 61-67.

Zhang, T, Wang, B., Ling, Z., Li, Y., Gao, X., Tian, G., Zhang, J., Han, L., and Zhang, L. (2009) Development of real-time quantitative RT-PCR assay for detection of CAV in chickens. *Chinese Journal of Animal Quarantine* **26**, 58-60.

Zhao, Y, Aarnink, A, Cambra-Lopez, M, Fabri, T (2013) Viral shedding and emission of airborne infectious bursal disease virus from a broiler room. *British Poultry Science* **54**, 87-95.