Muresk Institute

The Fate of Human Enteric Pathogens Following the Land Application of Biosolids in Agriculture

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

Signature ……

Karen R Schwarz

Date: 14 March 2012

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ABSTRACT

A research project was undertaken to study the effect of biosolids on the decay times of enteric pathogens in the soil. This is the most comprehensive study in Australia where the persistence of enteric microorganisms in land-applied biosolids, particularly on broadacre grain farms in Australia, has been studied.

Enteric pathogens such as faecal bacteria and viruses are present in biosolids, and when applied to land, these disease-causing microorganisms are at risk of being transmitted to humans following contact. The main aim of this research project was to examine the decay times of *Escherichia coli* (an indicator of enteric bacterial pathogens), *Salmonella enterica* (a representative of human pathogenic bacteria), bacteriophage MS2 (surrogate virus) and adenovirus (a representative human pathogenic virus). Agricultural soil from two farming properties in Western Australia and South Australia was selected for testing the inactivation of these enteric microorganisms over the growing season of a cereal crop. To do this, soil, biosolids and human enteric microroganisms were inoculated into sentinel chambers and inserted into the soil in the field. Chambers were sampled at regular intervals across the duration of the experiment and pathogen numbers were plotted over time. The decay times (T_{90}) were then calculated based on the slope of decay to determine the estimated time for a one-$\log_{10}$ removal to occur.

The key findings from the soil (field) experiments were that a) very low numbers of bacteria and bacteriophage (MS2) were detectable in the soil by harvest time since the microorganisms decayed rapidly over the growing season of the crop and b) that the decay times for *E. coli, S. enterica* and MS2 were shorter in the biosolids-amended soil compared with the unamended soil. Results indicated that the application of biosolids to the soil may have actually increased the inactivation processes of the enteric microorganisms in the soil. Further findings were that enteric microorganism numbers, particularly bacteria, were significantly correlated with the changes in soil moisture and bacteriophage MS2 was significantly correlated with changes in soil temperature. For industry, this means that while the application of biosolids may introduce harmful pathogens to the field, the pathogens (in biosolids-

amended soils) are adequately reduced over time. In addition, the climatic conditions as typical for Australia with dry hot summers, generally do not favour the survival of enteric pathogens.

A glasshouse experiment was conducted to validate the methodology for the quantification and enumeration of enteric microorganisms from soil and biosolids-amended soil. The resulting methods were a combination of procedures and processes from several sources that proved successful to improve the recovery of microorganisms from manure, biosolids or soil samples. The data from this experiment highlighted the difficulty faced when fitting a linear line of regression to the observed data points in order to calculate the time taken for the reduction of microorgainsms or the decay times (T_{90} values) from the reciprocal of the slope. Because of this, statistical models that take curvature into account with more terms such as quadratic and cubic were examined. The quadratic model was observed to provide the best fit, therefore was considered the most suitable for use for the field (soil) data.

A phyllosphere experiment was conducted to determine the decay times of enteric microorganisms on the leaves, spikelets (grain heads) and grains of wheat. This was important where fodder crops are grown for livestock feed in biosolids-amended paddocks. The concern was that pathogenic contaminants would transfer from the soil to the plant and be of risk at consumption. A key finding from the present study was that enteric microorganisms were detectable for longer in the soil (6 to 7 months) than the plant leaves (less than 1 month) therefore enteric pathogens on plant leaves would be of most risk to livestock where crops such as hay or lucerne are grown. Where withholding periods are maintained the risk of pathogen ingestion was considered to be low. Given favourable weather conditions for hay and silage production, the time from cutting to baling is approximately 1 week and because of this, the risks to livestock from pathogens is also considered low. Although the bacteria and virus examined in this research survived for several months on wheat grains (i.e. the time for a one-$\log_{10}$ removal (T_{90}) for bacteria on stored grains was 9 to 12 d), the risks to humans was considered to be low based on the assumption that grains are often milled, ground and baked prior to consumption.

Thresher and dust studies were conducted to compare indigenous bacterial levels at sites where biosolids had been applied, with sites where no biosolids had been applied. A key finding was that indigenous heterotrophic bacteria and enterococci numbers were higher at the biosolids-amended harvesting site than the unamended site. In addition, the highest numbers of bacteria (and inoculated microorganisms) was found on the chaff, indicating that this region could be sampled for the testing of any pathogenic microorganisms potentially present in dust samples. Results demonstrated that the process of threshing significantly reduced microorganism numbers on matured wheat plants. For industry this means that the risk of transferring human enteric pathgoens (bioaerosols) to humans at harvest time is low where crops have been previously applied with biosolids (particularly if field workers remain inside vehicles in sealed cabs of harvesters, trucks and utes or use dust protection while the harvester is in operation). In addition, the high summer temperatures, dry conditions and low humidity in the field at harvest time do not favour the prolonged survival of bioaerosols.

This study provides scientific data on the survival patterns of enteric bacteria and viruses across the growing season of wheat when introduced into agricultural soil from land-applied biosolids. The practical application of the results to cereal production enables key stakeholders to consider the areas of risk across the supply chain of grain production to contribute towards consumer safety and public protection. It was concluded that pathogens from biosolids are of greatest risk to humans directly involved with the handling of biosolids following dispatch from the wastewater treatment plant since microbial contamination levels are highest during this time. In addition, the Australian climate is not suited to prolonged survival of enteric pathogens outside of the host, particularly from spring to summer where soil moisture declines and soil temperatures increase. The pathways to ingestion are low where withholding periods are maintained and correct management procedures are followed such as the incorporation of biosolids with the soil within the appropriate timeframe. Therefore, the main pathway for the transmission of disease-causing pathogens to humans may be more prevalent where poor hygiene practices occur.

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ABBREVIATIONS

The following abbreviations are used throughout this thesis:

ACM	Australian Collection of Microorganisms
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CFU	colony forming units
d	day (s)
DNA	deoxyribonucleic acid
DS	dry solids
DW	dry weight
EPA	Environmental Protection Agency
t	Tonnes
h	hour (s)
M	Molarity
Min	Minutes
mL	milliliter (s)
MPN	most-probable-number
mth	Month
MWCO	Molecular Weight Cut Off
NBRP	National Biosolids Research Program
NLBAR	Nitrogen Limited Biosolids Application Rate
P-Buffer	Phosphate buffer
PCR	Polymerase chain reaction
pdu	PCR-detectable units
pfu	plaque forming units
QLD	Queensland
RNase	Ribonuclease
rpm	revolutions per minute
SA	South Australia
WA	Western Australia
Y	Year

CHAPTER 1 GENERAL INTRODUCTION

1.1. Research problem

An important aspect of environmental health science is to limit the occurrence of environmentally-transmitted infectious diseases through addressing the fate, exposure, levels, prevalence and health outcomes relating to human pathogens in soil, air, water and food crops. With this in mind, the use of biosolids as a soil fertiliser requires close attention (Guzman *et al.* 2007) because human enteric pathogens are present in biosolids and they may result in infectious diseases being transmitted to humans or livestock through the environment. For this reason, the decay patterns and persistence of human enteric pathogens (or microorganisms) in broadacre cropping needed to be examined from seeding time to harvest in order to determine how long such microorganisms are expected to persist in agricultural soil amended with biosolids.

1.2. Research background

Biosolids are a secondary product of wastewater treatment processes originating from wastewater sludge (Spinosa and Vesilind 2001; Liang *et al.* 2003). They are termed 'biosolids' once they have been treated and processed and can be used as a useful resource (i.e. fertiliser) for soil conditioning (Evanylo 1999; Sidhu 2000); however, biosolids contain contaminants such as heavy metals, toxic organic chemicals and pathogens (LeBlanc *et al.* 2008). Of particular concern in this study is the presence of enteric pathogens which may be of harm to humans and livestock. Having undergone several treatment processes, the pathogenic contaminants found in the sludge are somewhat reduced but not completely eliminated. The study of the pathogens in biosolids has been recognised as a priority for research over other contaminants, since they have a more immediate potential impact on human (and livestock) health, whereas chemical contaminants are more likely to have negative effects after long term soil accumulation, sometimes several decades.

Land application is one of the main avenues for biosolids management in Australia. Of the 360,000 dry tonnes (t) of biosolids produced annually, the majority of biosolids in Australia are used for agriculture. In New South Wales over 65,000 dry t are applied to agricultural land, in Western Australia over 20,000 dry t are applied in the central wheatbelt and in South Australia over 20,000 dry t are stockpiled before being applied to agricultural land. However, in Queensland and New Zealand over 40,000 dry t are placed in landfill each year, and in Victoria the majority of biosolids produced are stockpiled (approx. 60,000 dry t). For the purpose of this research, sites in Western Australia and South Australia were selected as the locations for the field experiments since biosolids are currently being applied to land as a fertiliser to dryland broad acre crops such as canola, cereals and oilseeds in both regions (LeBlanc *et al.* 2008).

Under the current legislation, select bacteria such as thermotolerant coliforms are used to indicate the level of pathogenic contaminants, which are then used to grade the biosolids into a category of acceptance or suitability for release to land. Since this grading system is based on one or two indicator bacteria, the persistence and survivability of any other enteric microorganisms present in biosolids is unknown and, in particular, there is little information about the decay times of these pathogenic microorganisms in biosolids-amended soils in Australia in the field after incorporation. Hence, biosolids are currently being applied to land without comprehensive scientific data to indicate the levels of risk that may be present to the public from pathogenic (enteric) contaminants.

1.3. Research aim

The aim of this research was to determine the decay patterns (or T_{90} decay times) of human enteric microorganisms – *Escherichia coli, Salmonella enterica,* bacteriophage MS2 and adenovirus – across the growing season of wheat where biosolids are applied to agricultural soil. Determining these decay times is important in relation to the protection of public health, particularly where withholding periods are applied to restrict access to biosolids application sites and, more importantly, where appled to ‘sensitive crops’ are planted and harvested (i.e. edible crops that come into contact with the soil and may be consumed raw).

1.4. Research objectives

Specific research objectives were as follows:

- To find a method suitable for the monitoring of the decay of enteric microorganisms from soil and biosolids-amended soil in the field over the growing season of wheat (approx. 6 months);
- To test the methodology to ensure that the environmental conditions outside the microcosms were in equilibrium with the internal environment (of the sample chamber), and to derive initial decay times (for *S. enterica* and MS2) in a pot experiment;
- To determine the most appropriate model for non-linear (broken-stick) type decay patterns (linear, quadratic or cubic) as are common with environmental microorganism decay, in order to obtain decay times (T_{90});
- To examine the decay patterns of *E. coli* (indicator bacteria), *S. enterica* (pathogenic bacteria), bacteriophage MS2 (surrogate virus) and human adenovirus (pathogenic virus) in biosolids-amended agricultural soil;
- To observe any correlation of microorganism decay with changes in soil temperature and soil moisture in the field;
- To determine any effect of microorganism type, soil type or site on decay patterns;
- To examine the effect of plant location (i.e. spikelet, leaves) on microorganism decay (on wheat plants at the flowering stage);
- To examine the effect of microorganism type on the decay patterns of enteric microorganisms on the phyllosphere of wheat;
- To examine the effect of threshing on enteric microorganisms inoculated onto mature wheat plants; in addition, to determine where the microorganisms are being deposited through this process (i.e. chaff, thresher drum, grains);
- To determine the presence/absence and numbers of bacteria - heterotrophic, *E. coli* and enterococci – in a mature wheat crop at harvest in the field, previously applied with biosolids; and
- To test for the presence and levels of bacteria in wheat dust during harvesting operations in a wheat crop where biosolids have been applied.

The enteric microorganisms selected for this study (i.e. *E. coli*, *S. enterica*, bacteriophage MS2 and adenovirus) are representative of the bacteria and viruses found in biosolids. *E. coli* is commonly used as an indicator organism to monitor inactivation of faecal bacteria in wastewater (Sidhu *et al.* 2008). *E. coli* bacteria are found in the gastrointestinal tract of all warm-blooded animals and are usually harmless, however several strains can cause gastroenteritis and when pathogenic, produce a toxin which can cause damage to the kidneys or even be life-threatening (Pepper *et al.* 2006). *S. enterica* bacteria are pathogenic to humans (Pepper *et al.* 2006) and are from a large group of more than 2400 Salmonellae serotypes. They are the main foodborne pathogens that commonly cause bacterial gastroenteritis (www.aihw.gov.au). Bacteriophage MS2 is used as a surrogate virus for enteric viruses (Sidhu *et al.* 2008) and is commonly used as a study microorganism for inactivation in wastewater. Adenovirus is pathogenic and is one of the most common enteric viruses found in wastewater (Pepper *et al.* 2006; Sidhu *et al.* 2008) therefore it may be transmitted to humans from recreational and drinking water. Adenovirus 40 have been detected in anaerobically-digested biosolids and can cause diarrhoea and respiratory infections, particularly in children (Pepper *et al.* 2006).

Most published literature, specific to land-applied biosolids, document the use of the indicator bacteria *E. coli* (Crute 2004; Horswell *et al.* 2007; Lang *et al.* 2007; Lang and Smith 2007; Pourcher *et al.* 2007), enterococci (Crute 2004; Pourcher *et al.* 2007) and the surrogate virus F-specific RNA bacteriophage (Crute 2004; Lang *et al.* 2007). The most common pathogen studied is *Salmonella* spp. (Eamens *et al.* 2006; Horswell *et al.* 2007; Lang *et al.* 2007; Eamens and Waldron 2008; Horswell *et al.* 2010) with some work conducted on *Clostridium perfringens* (Eamens *et al.* 2006; Pourcher *et al.* 2007). Sorber and Moore (1987) referred to different sludge-amended soil studies where inactivation (T_{90} values) for faecal coliforms, faecal streptococci, total coliform bacteria, *Ascaris ova* and *Toxocara* ova were examined; however, no references for these studies were provided. Very few studies have used pathogenic viruses such as adenovirus, and for this reason the present study contributes new information to this field. In addition, industry (i.e. WQRA) requested that dust experiments be conducted to examine potential bioaerosol levels in the environment where biosolids have been land-applied, particularly where dust is generated.

1.5. Research benefits

The main focus of this research was to compare the enteric pathogen decay patterns in the soil where biosolids had been applied, with sites where no biosolids had been applied (unamended). Since grain crops are often grown following biosolids application, the risk pathway from the production of wheat to harvest was used. This included examining the decay times of the study bacteria and virus on the phyllosphere of wheat in the event that enteric pathogens could transfer from the soil onto the plant and be transmitted to livestock or humans (i.e. via grain, hay crops or silage).

To date, no published data is available on the transfer of enteric pathogens onto wheat plants from biosolids-amended soil or the inactivation times of such pathogens from parts of cereal plants. Therefore, the research reported in this thesis is of utmost importance for Australian grain and hay export markets, particularly where biosolids have been used as fertiliser. Very few studies have examined the decay times of such pathogenic contaminants, particularly under Australian conditions. Where studies have been carried out (i.e. Crute 2004; Eamens *et al.* 1996; 2006; 2008), insufficient information as a whole has been collated therefore there are still gaps in the knowledge and understanding of the topic is incomplete.

The principal risk was not considered to be the cereals themselves, but from the following: a) any crop that comes into contact with biosolids-amended soil and is consumed uncooked, b) farm workers/biosolids handlers that come into contact with the biosolids-amended soil; and c) contamination with the public immediately after biosolids have been released from the wastewater treatment plant (i.e. at spreading/seeding time).

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CHAPTER 2 LITERATURE REVIEW

2.1. Background

Population growth has lead to increasing volumes of wastewater produced around the world with corresponding larger amounts of sewage sludge. Sewage, primarily from domestic and industrial sources, is treated and processed (Evanylo 1999) using processes such as dewatering and anaerobic digestion to allow the biosolids to be applied to land for beneficial reuse (Spinosa and Vesilind 2001; Liang *et al.* 2003). Once properly treated and of good quality for use on land, the sludge ("solids") is then termed 'biosolids' to distinguish it in public acceptance terms from other sludges (LeBlanc *et al.* 2008). Of the biosolids produced in Australia, the majority is used in agriculture, forestry, land rehabilitation or as landfill (Gale 2007; LeBlanc *et al.* 2008).

Municipal biosolids contain nutrients and are considered a valuable resource in agriculture for use as fertilisers on agricultural land (Epstein 2003; Sanchez *et al.* 2004; LeBlanc *et al.* 2008). Land application of biosolids has been common overseas but was not widely practised in Australia until the past few decades. In Australia and New Zealand, over one million tonnes of wet biosolids are produced per annum amounting to approximately 360,000 dry t. Of this, the most common use of biosolids (in Australia) is land application with substantial quantities being stockpiled before use (LeBlanc *et al.* 2008).

The primary focus for biosolids end use has been to increase their emphasis as a resource rather than a waste product (Isaac and Boothroyd 1996). It is important to justify the use of biosolids on agricultural land both as an avenue for reuse and as a substitute fertiliser. The application of biosolids onto agricultural land introduces substantial organic matter and is a rich source of plant-available nutrients and trace elements (Joshua *et al.* 1998; Epstein 2003; Horan *et al.* 2004; LeBlanc *et al.* 2008). Several studies show benefits to agriculture such as increased crop yields, improved soil fertility, soil conditioning, improved cation exchange, an increase in soil porosity, decreased bulk density and increased soil water-holding capacity (Epstein 1998; Nicholson *et al.* 2005; LeBlanc *et al.* 2008).

Soils in many Australian cropping regions are low in fertility and organic matter, have a low cation exchange capacity and are acidic, therefore biosolids are well suited to these soil types as soil conditioner and soil improvers (Joshua *et al.* 1998; LeBlanc *et al.* 2008). The use of organic wastes in agriculture has been known to increase soil organic matter and benefited agricultural production in countries such as Australia with depleted soils (Hassen *et al.* 1998). There has been renewed interest in applying biosolids to land in an attempt to save costs and to conserve water and nutrient resources (Cameron *et al.* 1997). Therefore, much research has gone into establishing biosolids as a fertiliser and developing its use with minimal damage imposed on the environment (Bruce and Evans 2002). However, some uncertainties remain as to the risks to human and animal health.

Biosolids may contain contaminants that pose a risk to public health and the environment. Even though the recycling of biosolids onto agricultural land is the most practical, economical and environmentally-beneficial management option (Nicholson *et al.* 2005), some authors suggest that the benefits of biosolids outweigh the risks (Bright and Healey 2003) and that these risks should be assessed against the benefits to soil fertility and agricultural productivity (LeBlanc *et al.* 2008). Such risks include enteric pathogens, heavy metals, dioxins, organic contaminants, synthetic hormones, household chemicals and bioaerosols (Epstein 1998; McFarland 2001) all of which have attracted public concern (Mininni and Santori 1987; Joshua *et al.* 1998; Hassen *et al.* 2001).

2.2. Biosolids production and pathogen reduction

The first barrier, in the multi-barrier approach to protection of the public health from the use of biosolids or sludge, is the requirement for sludge to be treated to reduce pathogen numbers (commonly determined using ‘indicator microorganisms’). It is recognised that pathogens do naturally decay in the environment when outside of their host organism, and the barrier strategy takes advantage of this natural behaviour to ensure that pathogen numbers are reduced to background levels before the biosolids are applied where “ready-to-eat crops” are grown. These treatment processes result in the production of primary and secondary wastewater from management processes such as dewatering and stabilisation to reduce pathogenic contaminants (Tables 2-1 and 2-2).

Table 2-1: The most common technologies used for sludge stabilisation

Stabilisation method	Description
Anaerobic digestion	Fermentation by bacteria of organic material in the absence of free oxygen. It is not designed to disinfect biosolids; therefore pathogens can survive in considerable numbers. Mesophilic Anaerobic Digestion (MAD) is used in the production of P2 biosolids.
Aerobic digestion	This process involves aerating sludge in open basins and directly oxidating any biodegradable matter with the production of cellular material.
Composting	Aerobic biological decomposition of organic materials under controlled conditions to a state where composted material can be handled, stored or applied to land without adversely affecting the environment.
Alkaline stabilization	Adding lime to wastewater solids either before or after the wastewater solids are dewatered. Usually applied to agricultural land in liquid or cake form or are land-filled.
Dewatering	This process involves one of several mechanical processes; usually a belt filter press or centrifuges to separate the water from the sludge.
Heat drying	Drying is needed if removal of water through dewatering processes is insufficient. Wet sludge is heated to remove water. Heating of wet sludge evaporates water mechanically. Heat-drying technologies include flash dryers, rotary dryers, spray dryers, multiple-effect evaporators and multiple hearth dryers. This method can be dusty if not formed into pellets.

Source: USEPA 1999.

Table 2-2: Biosolids classifications and related uses

Category	**Description**
P1 - Primary treatment	Very Limited Exposure. Includes disposal practices such as land-filling or limited access mine site rehabilitation. Removal of insoluble particulate materials by settling, screening, addition of alum and other coagulation agents, and other physical procedures.
P2 - Secondary treatment	Limited Exposure. Includes use of biosolids where public access is possible but limited. May include disposal routes such as tree farming, market gardening and landscaping of public spaces. Biological removal of dissolved organic matter (trickling filters, activated sludge, lagoons, extended aeration systems and anaerobic digesters).
P3 - Tertiary treatment	Unrestricted Exposure. For unrestricted marketing of biosolids where the possibility of public exposure is high. Biological removal of inorganic nutrients, chemical removal of inorganic nutrients, virus removal/inactivation and trace chemical removal

Source: Prescott *et al.* 2002; Gibbs & Goen 1995

Sludge is stabilised before it can be applied to land to reduce pathogenic micro-organisms and chemicals that could be a health hazard to humans to 1) reduce offensive odours and 2) to decrease the rate of putrefaction (Spinosa & Vesilind 2001; Switzenbaum et al. 1997). The most common technologies used to stabilise sludge in order to meet regulatory requirements include anaerobic digestion, aerobic digestion, composting, alkaline stabilization and heat drying (Table 2-1 and Figure 2-1) (Epstein 2003; Gerba *et al.* 2002; Sahlstrom *et al.* 2004; Sidhu 2000; Switzenbaum *et al.* 1997). These stabilisation methods determine the classification and potential end-use of the biosolids. The biosolids classifications commonly used (P1, P2 and P3) are derived from USEPA Part 503 Regulations (Table 2-2).

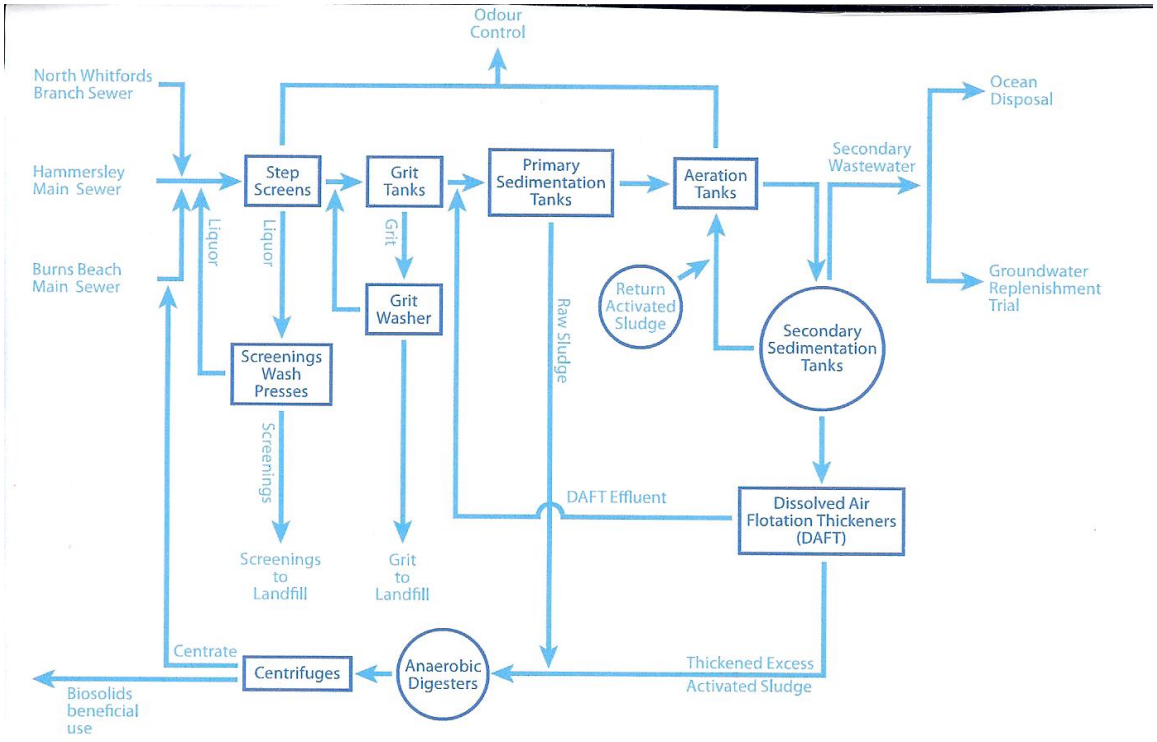


Figure 2-1: Schematic of the Beenyup Wastewater Treatment Plant process, Western Australia (LeBlanc *et al.* 2008).

The greatest short-term risk from untreated excreta, wastewater, septage and wastewater sludge is pathogenic microorganisms that can cause disease (LeBlanc *et al.* 2008). Regulators, producers and users of biosolids operate under set guidelines that have been established to minimise risk with minimum criteria, procedures and approval processes for the treatment and direct application of biosolids (USEPA 1993; DOE 1996; EPA 1997; NSWEPA 2000; ADAS 2001; DEP *et al.* 2002; NZWWA 2003; USEPA 2003; NRMMC 2004; SAEPA 2009; DEP *et al.* 2010). The guidelines vary from state to state for each country according to the different types of biosolids that are produced from individual processing plants; therefore such regulations are referred according to the requirements in each state. Although reduced at stabilisation, pathogens are not completely eliminated (i.e. in conventional, T3 or Class B biosolids) and therefore, such biosolids cannot be completely considered pathogen-free (Bruce and Evans 2002).

2.3. Pathogenic contaminants in biosolids

An enteric pathogen is any virus, bacterium or other agent that lives in the intestinal tract and causes disease (Prescott *et al.* 2002). The pathogen groups associated with biosolids are bacteria, viruses and parasites such as protozoa and helminths (Awad *et al.* 1989; Cameron *et al.* 1997). These pathogen groups are listed in Tables 2-3 and 2-4.

Since biosolids are derived from human faecal material and may contain microbial contaminants a global priority is to provide basic sanitation and this involves the proper treatment and management of excreta, septage and wastewater sludge to reduce the transmission of pathogens. In developed countries this risk is somewhat reduced because of a lower prevalence of diseases amongst the population. These countries also have proper wastewater treatment systems in place and adequate food hygiene practices, unlike many developing countries where disease transmission (particularly from water resources i.e. cholera) is a severe health concern (LeBlanc *et al.* 2008). Pathogens commonly transfer into untreated sludge from human excreta, through the wastewater systems prior to treatment and any remaining microorganisms may be transferred onto agricultural soil through irrigation water or during land application of the biosolids (Figure 2-2).

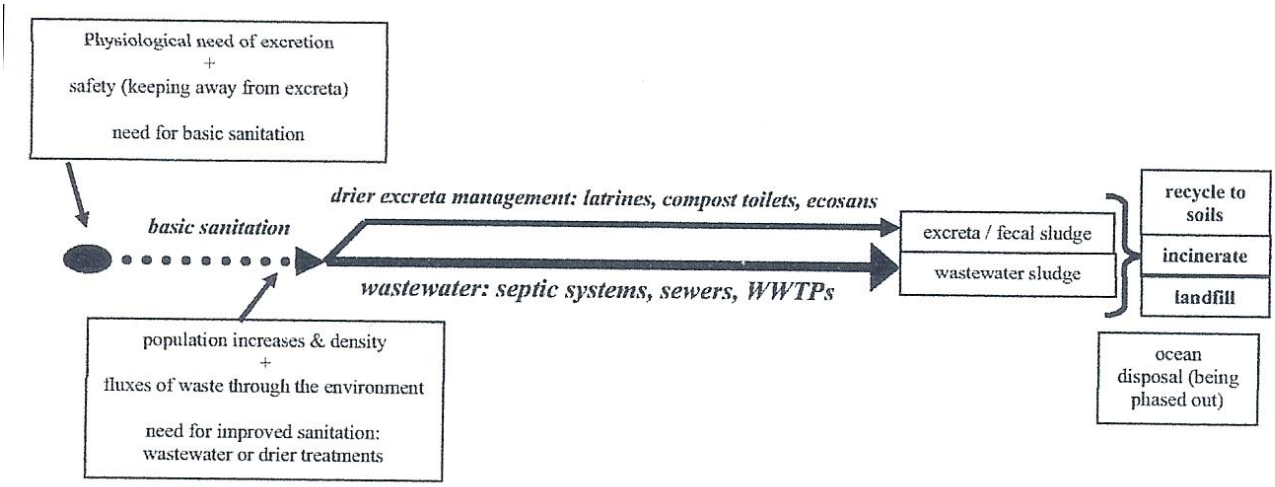


Figure 2-2: The progress of excreta, wastewater sludge and biosolids management (LeBlanc *et al.* 2008).

An awareness as to the potential magnitude of pathogen-related disease dissemination is important. Faeces excreted by a healthy person normally comprise many bacterial species of which there may be 10 thousand to 10 million units per gram (Lewis-Jones and Winkler 1991a). Likewise, faeces excreted by a person with gastrointestinal disorders contain large numbers of the pathogenic microorganisms. Although not all pathogens present in biosolids are infectious (Cliver 1980), it is necessary to treat all faecal coliform microorganisms as indicative of dangerous contamination (Gallagher and Spino 1968). The microbiological properties of sludge will reflect the level of enteric disease within the human population in terms of the numbers and range of pathogens and parasites present. These microorganisms can enter the sewage reservoirs and be of health hazard to the general public if contact occurs (Bruce and Evans 2002; Cliver 1980).

The risk of pathogens to the human food chain is considered low given that pathogen decay along with biosolids loadings are trivial compared with enteric pathogen inputs (in livestock manures), and regrowth only occurs under specific circumstances; however, the impact on the environment is still unknown (Hillman *et al.* 2003). Sahlstrom *et al.* (2004) considered it possible that if biosolids were spread onto agricultural land, the load of pathogens on the environment could increase with time (given the evidence for factors such as regrowth (Sidhu *et al.* 2001)) and thus increase the risk of disease dissemination to people and animals.

Table 2-3: Some of the bacteria and viruses found in biosolids and resulting diseases

Bacteria	**Disease**
Campylobacter jejuni	Gastroenteritis
Clostridium perfringens	Food poisoning, gas gangrene, abdominal pain
Escherichia coli (pathogenic strains)	Gastroenteritis
Listeria monocytogenes	Listeriosis, neonatal sepsis, meningitis
Mycobacterium tuberculosis	Tuberculosis
Salmonellae (approx. 1700 types)	Salmonellosis, gastroenteritis, food poisoning
Salmonella typhi	Typhoid fever
Shigella (four species)	Shigellosis, bacterial dysentery, gastroenteritis
Yersinia sp.	Acute gastroenteritis, diarrhoea, abdominal pain
Vibrio cholerae	Cholera
Virus	**Disease**
Adenovirus (31 types)	Conjunctivitis, respiratory infections, gastroenteritis
Caliciviruses	Epidemic gastroenteritis
Enteroviruses	
Poliovirus	Poliomyelitis
Coxsackievirus	Aseptic meningitis, pneumonia, hepatitis fever
Echovirus	Aseptic meningitis, paralysis, encephalitis
Hepatitis A virus	Infectious hepatitis
Norovirus	Severe gastroenteritis
Reoviruses	Respiratory infections, gastroenteritis
Rotavirus	Gastroenteritis, infant diarrhea

Source: Adapted from Kowal (1985), USEPA (1989) and Epstein (1998).

Table 2-4: Some of the protozoa and helminths in biosolids and resulting diseases

Protozoa	**Disease**
*Balantidium coli**	Balantidiasis, diarrhoea, dysentery
Cryptosporidium	Gastroenteritis
*Entamoeba histolytica**	Amoebic dysentery, amebiasis, acute enteritis
Giardia lamblia	Giardiasis, diarrhoea, weight loss
*Toxoplasma gondii**	Toxoplasmosis
Helminths – Nematodes*	**Disease**
Anclostoma duodenale	Hookworm disease, ancylostomiasis
Ascaris lumbricoides (round worm)	Ascariasis, digestive disturbance, vomiting
Ascaris suum	Fever, respiratory effects, chest pain
Necator americanus	Hookworm disease
Strongyloides stercoarlis (threadworm)	Strongyloidiasis, abdominal pain, diarrhoea
Taenia sp. (tape worm)	Taeniasis, weight loss, abdominal pain, nausea
Trichuris trichiura (whip worm)	Trichuriasis, abdominal pain, diarrhoea, anemia
Toxocara canis (dog roundworm)	Fever, abdominal pain, neurological symptoms

Source: Adapted from Kowal (1985), USEPA (1989) and Epstein (1998).

* *These are generally not an issue in nations with high sanitation such as Australia*

2.4. Public health risk

The potential that pathogens could be of risk to public health and the degree of that risk from faecal pathogens is important in considering the wellbeing and health of a community. One major potential health risk is the contamination of foods grown for human consumption. These pathways are through the consumption of contaminated crops by humans and the transfer of contaminants to animal food products for human consumption (Cameron *et al.* 1997). Nonenteric pathogens can also enter the wastewater system. Abattoirs and funeral homes introduce high levels of nonenteric pathogens (commonly blood-borne pathogens and rare prions) into waste treatment systems. The latter have been linked to Creutzfeldt-Jakob disease and Mad Cow disease or bovine spongiform encephalopathy (BSE). Since experiencing recent health scares from *Listeria, Salmonella, E. coli* O157:H7 and BSE, food retailers have questioned the use of biosolids on agricultural land into the 21st century (Bruce and Evans 2002). This has resulted in the development of the Safe Sludge Matrix thus further extending and clarifying the controls on agriculture utilisation of sludges in relation to pathogens.

The reports of human and animal infection from land-applied biosolids have been low in the United Kingdom since the establishment of the [1]Regulations and Code of Practice. These regulations have reduced environmental damage to human health, animals or crops (Bruce and Evans 2002) from the use of manures and sludges. Continued work has been carried out by research, regulatory and public organisations to find economically feasible and environmentally acceptable means for the use of biosolids (Bruce and Evans 2002; LeBlanc *et al.* 2008).

For disease to occur in the human host, sufficient levels of the pathogen must be ingested (Epstein 1998; Carr 2001) through one of the pathways of transmission (Table 2-6). Dose response levels for a selection of pathogens and reported infective doses for individuals are presented in Table 2-5. Minor levels of pathogenic organisms can cause infection (Hu *et al.* 1996) depending on the characteristics of the host. If diluted in the environment, the probability of infection is greatly reduced (Edmonds and Mayer 1979), and this risk tends towards the background levels.

[1] *Guidelines were established in the 1980's where the EU Sludge Directive 1986 was implemented in the UK in 1989 through the Sludge (Use in Agriculture) Regulations, which was complemented by a non statutory Code of Practice providing further guidance and advice on sludge treatment and land management to protect human health and the environment.*

Table 2-5: Reported infective doses for enteric microorganisms

Pathogen	Infective dose
Bacteria	
Clostridium perfringens	10^6
E. coli	10^4
Salmonella (various species)	10^2
Shigella dysenteriae	10^0 - 10^2
Shigella flexneri	10^2 - 10^9
Vibrio cholerae	10^3
Viruses (human infective dose *HID50)	
Rotavirus	0.9 FFU
Human poliovirus (SM strain)	2 FFU
Human poliovirus (Fox strain)	2 FFU
Human echovirus	17 FFU
Parasites	
Cryptosporidium	10 cysts
Eritamoeba coli	1-10 cysts
Giardia lamblia	1 cyst estimated
Helminths	1 egg

Source: Adapted from Smith *et al.* (2003)

**HID (human infective dose) provided in the range of about 1- 1000 HID50, i.e., about 10^5 - 10^8 pfu (plaque forming units) per dose administered*

FFU – focal forming units

In 2001, infectious diseases were related to approximately 26% of the world's deaths (Ashbolt 2004). Approximately 4 billion cases of diarrhoea per year reported to result in 2.2 million deaths. These incidences, particularly diarrhoea, are commonly excreta-related (Carr 2001). Ashbolt (2004) found that *Campylobacter* strains contributed to more cases of diarrhoea than *Salmonella* spp. and were to be considered one of the world's main causes of gastroenteritis (www.aihw.com.au). In developed countries, 10% of the population have intestinal worms (Ascariasis) resulting in 60,000 deaths per year. Ascariasis is mostly excreta-related and most often caused by exposure to untreated wastewater or foods produced from it (Ashbolt 2004). Hookworm is a major contributor to iron-deficiency anaemia (Loukas *et al.* 2005). Helminthiases are the most common cause of parasitic infections in humans and animals around the world (Nithiuthai *et al.* 2004).

Several factors will determine the transmission of diseases to humans. These include the initial concentration of pathogens in the environment (Eamens *et al.* 1996), the level of pathogens present in wastewater from a community (Carr 2001), the ability of the organism to infect the host, the time period required before the host becomes infected and the organism's ability to survive or multiply in the environment (Carr 2001).

Characteristics of the host will also impact on the occurrence of disease transmission; these include nutritional status, immunity, health status, differential infectivity, age, sex, personal hygiene and food hygiene (Carr 2001; WHO 2003). Infectious agents spread by faecal-oral routes will be affected by the environment, food, poor hygiene, poverty and nutritional status (Ashbolt 2004). Exposure to pathogens can lead to pseudo-infections in the human body. Alternatively, pathogens can attach to a host, multiply and then cause disease (Armon *et al.* 1994). Potential transfer pathways for biosolids contaminants were outlined in Table 2-6. The main pathways associated with public health are via biosolids-amended soil, crops grown using biosolids, livestock produced off biosolids-amended pastures, bioaerosols and water routes.

Table 2-6: Some of the potential pathways of transfer of biosolids-amended contaminants (i.e. chemical and pathogenic) to humans and livestock.

Pathway	Highly exposed individual
Biosolids to human	Food produced from biosolids-amended agricultural land; home garden or residential soil applied with biosolids
Biosolids to soil to human	Water quality criteria; recreational activities; water catchments
Biosolids to crop/pasture to grazing animals	Contact with animal at handling, animal manures or slaughting of livestock
Biosolids to bioaerosol	Tractor operator; biosolids spreading; farmer; farm households

Source: Adapted from Chaney *et al.* (1996)

Pathogens may also be transmitted from animals and other vectors to humans, then from humans to humans. Pathogens can be present in the faeces of healthy animal carriers (Mawdsley *et al.* 1995) and then be transferred to humans through indirect means such as animals or vectors that are found around biosolids stockpile sites (Carr 2001; Smith and Farrell 2003; Koopmans and Duizer 2004). *Salmonella* infections may occur after ingestion of substances such as water, soil and food which have been contaminated by the infected animal's faeces (Lewis-Jones and Winkler 1991b). Note there are many sources of enteric pathogens in the environment (e.g. from livestock, wild animals and birds) with the soil itself as a reservoir for enteric bacteria surviving in the soil microbial community, therefore, the risk from biosolids should be viewed in the context of background risk from all other sources and not just as an isolated source of pathogens.

2.5. Survival times

Understanding survival times is necessary to ensure minimum withholding periods are established for sensitive crops (i.e. those which may be in direct contact with the soil and consumed raw). The survival times of various pathogen-groups in soil or on plants will vary between locations; however, the location itself does not determine the survival times as much as the local conditions such as soil type, temperature, moisture, rainfall and sunlight exposure. This information is necessary to protect biosolids-users and consumers, particularly where the length of the pathogen survival exceeds the growing season of the food crops. Part of the multi-barrier approach (i.e. the second barrier) to protecting human health from pathogen transmission by the management of biosolids is the time taken for the natural decay of pathogens to occur in the environment. From this, the minimal withholding periods are determined and public access to the sites is not permitted (before certain crops can be grown). Tables 2-7, 2-8 and 2-9 provide published survival times of selected bacteria, virus, helminths and protozoa. According to available published data, *E. coli* can survive in the soil for 12 to 267 d, Salmonellae for 15 to 280 d (Table 2-7), poliovirus for 100 d (Table 2-8) and hookworm for 42 to 180 d (Table 2-9).

Table 2-7: Published survival times of bacteria in land application sources

Pathogen and application	Survival times	Sources
Enteric bacteria		
Plants (common maximum)	1 mth	(Kowal 1985; USEPA 1989)
Soil (common maximum)	2 mth	(Kowal 1985; USEPA 1989)
Escherichia coli		
Soil	12-267 d	(Epstein 1998)
Coliforms		
Forest clear cut	162-267 d	(Edmonds and Mayer 1979)
Forest	15 mth	(Edmonds and Mayer 1979)
Grass and clover	6-34 d	(Golueke 1991; Epstein 1998)
Soil surface	38 d	(Epstein 1998)
Vegetables	35 d	(Epstein 1998)
Salmonellae		
Grass and clover	12-42 d	(Epstein 1998)
Soil	15-280 d	(Epstein 1998)
Vegetables and fruit	3-49 d	(Epstein 1998)
Salmonella typhosa		
Soil	29-74 d	(Golueke 1991; Epstein 1998)
Vegetables and fruit	31-68 d	(Golueke 1991; Epstein 1998)

Table 2-8: Published survival times of viruses in land application sources

Pathogen & application	Survival times	Sources
Virus:		
Plants (common maximum)	1 mth	(Kowal 1985; USEPA 1989)
Soil (common maximum)	3 mth	(Kowal 1985; USEPA 1989)
Poliovirus		
Forest ecosystem	28 d	(Edmonds and Mayer 1979)
Radish, lettuce	36 d	(Epstein 1998)
Soil	100 d	(Epstein 1998)

Table 2-9: Published survival times for helminths in land application sources

Pathogen & application	Survival times	Sources
Helminths:		
Plants (common maximum)	1 mth	(Kowal 1985; USEPA 1989)
Soil (common maximum)	2 y	(Kowal 1985; USEPA 1989)
Hookworm		
Soil	42-180 d	(Golueke 1991; Epstein 1998)
Ascaris ova		
Vegetables and fruit	27-35 d	(Epstein 1998)

Minimal, if any, data is available on Giardia cysts and *Cryptosporidium* oocysts survival times (USEPA 1989).

In other literature, *Cryptosporidium* oocysts have been reported to remain viable in moist environments for up to 6 months (Prescott *et al.* 2002). *Salmonella* spp. have been recorded to have persisted for 16 months (~504 d) in sludge applied to land (Dudley *et al.* 1980). However, in most circumstances pathogens below the soil surface are unlikely to survive for more than a year (Cliver 1980). Pathogens are mostly known to survive for longer periods in the soil than on plant surfaces (Epstein 1998) due to higher levels of exposure to environment conditions such as ultraviolet (UV) exposure, increased temperatures and decreased moisture.

In Australia, Eamens *et al.* (2006) studied the prevalence of *E. coli, Clostridium perfringens* and *Salmonella* spp. in soil amended with anaerobically-digested biosolids at Goulburn, New South Wales and found that bacterial numbers were above detection limits for 10 to 17 months. Previous work by Crute (2004) found that *E. coli* and enterococci were detectable for up to 6 months in biosolids-amended soil applied to dryland agricultural land at Toodyay, WA. Survival times are variable depending on a number of factors as is further discussed in Section 2.6.

Internationally, there have only been limited studies on pathogens in soils amended with biosolids. Two examples of these studies include a soil experiment by Lang *et al.* (2007) and a forestry experiment by Horswell *et al.* (2007). Lang *et al.* (2007) measured the survival of *E. coli* in agricultural soil amended with conventionally treated and enhanced-treated biosolids in a temperate environment at Ascot in the Uniteed Kingdom. It was found that *E. coli* (in the conventionally treated biosolids) reached detection limits by 3 months. In New Zealand, Horswell *et al.* (2007) examined the decay rates of *E. coli* and *Salmonella* spp. in sewage sludge applied to young and old pine forests (*Pinus radiata*) and found that *E. coli* numbers returned to background levels after 3 weeks in the spring (with increasing temperatures and decreasing moisture availability), but did not significantly decrease in the autumn/winter until weeks 5 and 13 (with higher moisture levels and lower temperatures).

2.6. Factors influencing survival in the soil

The major factors influencing survival of enteric microorganisms in the soil are summarised in Table 2-10. The survival of pathogens in soil is influenced by soil type (i.e. clay content and organic matter content), temperature, moisture, pH, utilisable organic matter (Ross *et al.* 1991; Lewis-Jones and Winkler 1991b; Epstein 1998; Stevik *et al.* 2003), exposure to ultraviolet light, antagonism, competition and predation from soil microflora (Sorber and Moore 1987), soil nutrients (Estrada *et al.* 2004) and ammonia (Jenkins *et al.* 1998). Survival is also influenced by initial numbers of microorganisms (Eamens *et al.* 1996), frost, concentration of inorganic salts, soil particle size (Lewis-Jones and Winkler 1991b), association with the soil and microorganism type (Pedley *et al.* 2004).

The survival of pathogens in the soil is also affected by the method of biosolids land application and environmental conditions (Epstein 1998). Microorganisms that are protected from sunlight display a slower rate of inactivation (Ross *et al.* 1991). Microorganisms on the outer surfaces of the soil aggregates are exposed to UV light (Lewis *et al.* 2002) and are therefore destroyed through sunlight disinfection, desiccation and higher temperatures (Epstein 1998).

Lower soil temperatures and higher moisture levels have been found to be the most influential environmental parameters to prolonging pathogen survival (Mawdsley *et al.* 1995; Cameron *et al.* 1997; Epstein 1998; Gerba *et al.* 2002; Liang *et al.* 2003). However, in some cases moisture content may be more influential than temperature (Liang *et al.* 2003). A soil moisture content of less than 50% can be detrimental to microorganism survival (Ahmed and Sorenson 1995). This is the minimum level required for a rapid increase in microbial activity. A moisture content of 60-70% will optimise microbial activity (Liang *et al.* 2003).

Once microorganisms have been introduced into the soil they face competition for foods, are exposed to antibiotic materials from other microorganisms and are exposed to predation by other soil microorganisms (Loehr 1974). The application of biosolids introduces nutrients and carbon substrate that stimulate the soil microbial

pool (Cameron *et al.* 1997). Interactions include mutualism, predation, parasitism and competition (Prescott *et al.* 2002). Human and animal pathogens are not well adapted to survival in the soil or at competing with indigenous organisms that are adapted to the environment, thus their existence is threatened and survival is difficult.

Predation and competition activity are greatest at the soil's surface (i.e. top 5 cm) as this region contains the highest levels of oxygen and decomposed matter. Biological controls such as temperature, moisture, pH and organic matter regulate pathogen survival, and thus, biological activity may be slowed through anaerobic conditions and lowered temperatures (Loehr 1974). Protozoa are considered the main predators of bacteria (Stevik *et al.* 2003). Predation can reduce the stress factors caused by the density of microorganisms present, thus allowing prey to increase more rapidly than if the predator were not active (Prescott *et al.* 2002). In a study by Sidhu *et al.* (2001) indigenous microflora in composted biosolids appeared to reduce the potential for bacterial regrowth which may have been attributed to biological control.

Rainy, humid weather may increase bacterial populations through regrowth, thus prolonging survival (Gibbs *et al.* 1995b) for, what is normally, a short period of time. It has been well documented that some bacterial pathogens are capable of regrowth given suitable conditions (Armon *et al.* 1994; Gibbs *et al.* 1995b; Gibbs *et al.* 1997; Sidhu 2000; Gantzer *et al.* 2001; Hassen *et al.* 2001; Sidhu *et al.* 2001; Gerba *et al.* 2002; Pietronave *et al.* 2004). The occurrence of regrowth or repopulation of pathogenic microorganisms needs to be examined as a potential public health threat so that proper management procedures can be established (Ahlstrom 1985). Eamens *et al.* (1996) demonstrated increases in bacterial numbers in warm, wet environmental conditions where periods of maximum air temperatures were recorded in conjunction with recent substantial rainfall. Regrowth can only occur with bacteria though, since viruses, parasite eggs and protozoan cysts cannot grow outside a human or animal host (Lewis-Jones and Winkler 1991a).

Table 2-10: Major factors influencing virus and bacteria survival in soil

Factor	Viruses	Bacteria
Temperature	Persistence longer at low temperatures	Persistence longer at low temperatures
Microbial activity	Inactivation may occur more readily in presence of certain microorganisms, the opposite may also be true, or there may be no effect	Presence of indigenous microorganisms may increase rate of inactivation; possible synergism with some protozoa may reduce inactivation rates
Moisture content	Survival is longer in moist soils and even longer under saturated conditions. However, increased reduction may occur in drying soils.	Most survive longer in moist soils than dry soils
pH	Most are stable over pH range of 3 to 9; however, survival may be prolonged by near neutral pH values.	Most will survive longer at near neutral pH. Increased acidity (i.e. lower pH) may reduce the survivability of bacteria.
Organic matter	Organic matter may prolong survival by competitively binding at air-water interfaces where inactivation can occur	The presence of organic matter may act as a source of nutrients for bacteria, promoting growth and extended survival
Association with soil	Association with soil generally increases survival, although attachment to certain mineral surfaces may cause inactivation	Adsorption onto solid surfaces reduces inactivation rates; the concentration of bacteria on surfaces may be several orders of magnitude higher than the concentration in the aqueous phase

Factor	Viruses	Bacteria
Sunlight inactivation	Viruses are more resistant to sunlight inactivation than bacteria. Adenovirus are resistant to ultra-violet light	Bacteria are more sensitive to sunlight than viruses
Salt species and concentration	Certain cations may prolong survival depending on virus type	Increasing ionic strength of the surrounding medium generally increasing sorption
Bacteria/virus type	Susceptibility to inactivation may vary by different physical, chemical and biological factors	Susceptibility to inactivation may vary by different physical, chemical and biological factors

Source: Adapted from Pedley *et al.* (2004)

2.7. Sampling containers used for soil

Various types of sampling containers have been used to examine the survival of enteric bacteria and viruses in the soil. These include soil corers, microcosms, plastic bags, glass vials, diffusion chambers and sentinel chambers. For the research reported in this thesis, a suitable sample container had to be found to enable the monitoring of enteric pathogens in soil in the field without the loss of microorganisms. For this, some form of chamber was required to equilibrate with the environment so that the microorganisms inside could experience equivalent conditions to the field. This could not be achieved in a sealed plastic or glass vial; both examples of simple microcosms (see section 3.2).

The following examples of the use of sample containers were found. Crute (2004) used sterile corers (syringes with the needle-attachment portion removed) to examine the decay times of *E. coli,* enterococci and bacteriophage MS2. For this study, the sampling was directed towards the biosolids applied to the soil thus collecting the areas where the study microorganisms were considered to be at their highest in number. While this method would have captured any environmental changes in the soil profile (such as soil moisture and temperature) over the duration of the experiment, it would not have enabled the sample contents to be contained, and therefore, did not provide a controlled environment from which to study microorganism decay. Vidovic *et al.* (2007) used polypropylene vials (capped soil microcosms containing 80 mL) to examine the survival of *E. coli* O157:H7 in two soil types amended with bovine manure, under sterile and non-sterile conditions. Hurst *et al.* (1980) used screw-capped (16 mL) glass vials containing sewage effluent-amended soil inoculated with a selection of viruses to measure the effects of various environmental variables on the rate of virus inactivation. Both sample containers, being capped glass vials, would not have allowed for gaseous exchange and moisture changes to flow in and out of the chamber. Since survival can be affected by such changes, particularly moisture, and environmental parameters need to be taken into consideration, this form of sample container was not considered suitable for the present research experiments. Similarly, partially sealed plastic bags were used by Lang and Smith (2007) to examine the fate of *E. coli* in biosolids-amended agricultural soil in relation to soil type and moisture status. Sample bags

were placed into a temperature-controlled incubator and were sampled for each soil type. The plastic bags allowed for gaseous exchange to occur, but would not be suitable in a field experiment to capture moisture osmosis from the external soil environment.

Two studies were found where environmental chambers were used. Toze *et al.* (2010) used Teflon diffusion chambers with mixed cellulose esters (Millipore) with a diameter of 25 mm and membranes (0.025 μm pore size) at either end to examine enteric pathogen decay in groundwater bores. The use of diffusion chambers was selected to allow groundwater and nutrients to cross through the membranes without the loss of inoculated enteric microorganisms, particularly viruses. Jenkins *et al.* (1999) developed a small-volume sentinel chamber (250 mm long x 7 mm internal diameter) to examine the effects of environmental stresses on the survival of *Cryptosporidium parvum* oocysts in soil and animal wastes (Figure 2-3). A 60 μm nylon-mesh filter was placed at the top of the chamber and a 0.45 μm pore-size filter at the base to allow for natural environmental interaction without the leaching of oocysts. Chambers were positioned vertically in animal waste piles and surface soil.

Both types of environmental chambers enabled equilibrium with the external environment without the loss of the study microorganisms. In the current study, the sentinel chambers proved to be suitable environmental chambers in equilibrium with the outer environment (refer to Chapters 4 and 6).

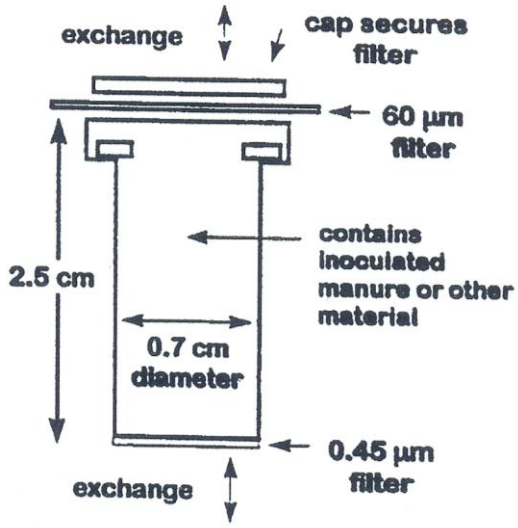


Figure 2-3: Sentinel chamber used by Jenkins *et al.* 1999 in field experiments

2.8. Enteric pathogen survival on the plant phyllosphere

The phyllosphere in microbiological terms is described as the total above-ground surfaces of a plant colonised by a variety of bacteria, yeasts and fungi (Lindow and Brandl 2003). The rhizosphere, or below ground region of a plant where microorganism populations are present, was not examined in this research since the focus of the study was only on edible above-ground plant components. Williams *et al.* (2007) found that the persistence of pathogens (*E. coli* O157:H7) was unaffected at the rhizosphere or root zone. Of particular focus in the present study were the decay times of enteric pathogens from the phyllosphere in relation to biosolids-amended soil where cereal crops (i.e. wheat) are grown.

There are several factors that influence the persistence of enteric bacteria and viruses on edible plant parts. These include the external environment, the survival capabilities of the pathogen, chemical and physical factors, levels of nutrition (carbon and nitrogen) available in the leaves (Mercier and Lindow 2000; Lindow and Brandl 2003; Jablasone *et al.* 2005; Aruscavage *et al.* 2006; Aruscavage *et al.* 2010; Critzer and Doyle 2010), pathogen adaptability (Beuchat 2002), antagonism, sources of contamination (sludge or irrigation water), the toxic compounds released by the plant and other microorganisms and plant leaf health. The survival times of pathogenic microorganisms on plant foliage is primarily influenced by the rapid and extreme fluctuations that occur on the phyllosphere such as temperature (Lindow and Brandl 2003; Choi *et al.* 2004), relative humidity (Crook and Sherwood-Higham 1997; Brandl and Mandrell 2002; Choi *et al.* 2004) and UV radiation (Abdulraheem 1989; Fujioka and Yoneyama 2002).

In addition, competition from other epiphytic microorganisms for moisture (Brown *et al.* 1980; Crook and Sherwood-Higham 1997; Cooley *et al.* 2006) and nutrition (Ibekwe *et al.* 2004) make enteric pathogen survival on leaf surfaces difficult (Mercier and Lindow 2000). Other factors known to influence epiphytic bacterial population sizes on plant leaves are adaptation to stress, reduced pH e.g. to below pH 4.1 (Beuchat 2002; Weinberg *et al.* 2004), the carrying capacity of the leaf (eg. broadleaf vs. grasses) (Lindow and Brandl 2003), leaf texture (Armon *et al.* 1994), washing of the bacteria or viruses off the leaves eg. rainfall (Natvig *et al.* 2002), bio-

film formation on plant tissue (Critzer and Doyle 2010), the location on the leaf (adaxial vs. abaxial) (Zhang *et al.* 2009), leaf ageing (Lindow and Brandl 2003) and the level of leaf damage (Brandl and Mandrell 2002; Aruscavage *et al.* 2008; Barker-Reid *et al.* 2009). Zhang *et al.* (2009) found that *E. coli* O157:H7 populations survived longer on the abaxial (underside) surface than the adaxial (upper) side of plant leaves since the upper side is more exposed to direct sunlight and higher temperatures.

The potential for contamination of salad crops grown in soil enriched with manures mostly depends on the survival capabilities of the pathogens present (Franz *et al.* 2005). Levels of usable water are important for bacterial survival and proliferation on leaf surfaces (Lindow and Brandl 2003), yet a large proportion of bacterial colonies are washed from leaves (Brown *et al.* 1980) or reduced by non-penetrating agents such as UV light (Wilson *et al.* 1999; Sidhu *et al.* 2008). Rainy humid weather may result in increased pathogenic populations and prolonged survival (Brown *et al.* 1980). Solar radiation alters the phyllosphere bacterial community composition (Jacobs and Sundin 2001). Climatic and agricultural determinants such as geographical location, wind, irrigation practices, management practices and the presence of insects, animals and birds can also influence the microbial ecosystem (Beuchat and Ryu 1997; Beuchat 2002).

The plant cuticle contains waxes that make microorganism attachment to plant tissues difficult. For attachment of plant and animal pathogens to occur, this cuticle must be penetrated (Beattie 2002). Leaf lesions can provide protection (for microorganisms) from environmental stress (Brandl and Mandrell 2002). Injured plants can exude nutrients such as proteins and carbohydrates from damaged leaf tissues that can be used by enteric microorganisms present on leaves as a source of nutrition. However, injured plant cells may also release antimicrobial agents that could inhibit microbial populations (Beuchat 2002; Aruscavage *et al.* 2008). The nature of the protective cuticle, tissue pH and presence of antimicrobials dictate the types of plants that are more likely to be affected by microorganisms when damaged (Beuchat 2002). Thick waxy cuticles, which change as leaves age, may interfere with bacterial colonisation by inhibiting leaf surface wetting and diffusion of nutrients (Lindow and Brandl 2003).

Leaves constitute a very large microbial habitat (Lindow and Brandl 2003) and plant micro-biota interactions play a vital role in colonisation or inhibition of enteric pathogens in the phyllosphere of fresh produce (Critzer and Doyle 2010). Plant pathogens may have a synergistic or commensal relationship, such as the incidence of *S. typhimurium* with soft rot bacteria on retail market produce (Wells and Butterfield 1997; Beuchat 2002). Persistence of human pathogens in the phyllosphere may also be more successful where plant pathogens have weakened the plant's defence mechanisms (Aruscavage *et al.* 2006) or where they have combined with human pathogens to reduce the effects of sterilisation (Wilson *et al.* 1999). Competition from human pathogens may increase with warmer temperatures, as in Brandl and Mandrell (2002), where competition from *Salmonella* occurred with two common colonisers of plant leaves (*Pantoea agglomerans* and *Pseudomonas chororaphis*) on cilantro (coriander) leaves. It is thought that the incidence of produce contamination may be reduced by promoting better agricultural practices that encourage the growth of competing bacteria such as *Enterobacter asburiae* (Cooley *et al.* 2006).

The risk that human pathogens may internalise into salad vegetables plants has been reported to be low (Jablasone *et al.* 2005; Zhang *et al.* 2009; Ibekwe *et al.* 2009) and as a result was not discussed in relation to the present study, although it has been demonstrated that *E. coli* O157:H7 is capable of entering the root system of a lettuce plant under extreme conditions (Solomon *et al.* 2002; Horswell *et al.* 2006).

Islam, Morgan *et al.* (2004) suggested that contaminated manure compost and irrigation water played important roles in contaminating soil and vegetables (Solomon *et al.* 2002). It has been well documented that faecal coliform may be dispersed by rain splash, and if so, could bypass physical barriers (Boyer 2008). Alternatively, disease transmission could occur indirectly by aerosols deposited on food, vegetation or clothing surfaces (Abdulraheem 1989). Other possible pre-harvest sources of microorganisms may include water used to apply fungicides and insecticides (Guan *et al.* 2005), insects, inadequately composted manure, wild and domestic animals and human handling. Post-harvest sources may include harvesting equipment, transport containers, dust, rinse water and processing equipment

(Beuchat 2002). No studies could be found on the survival of enteric microorganisms on cereal grains.

2.9. Pathogens in bioaerosols

Bioaerosols are defined as "aerosols comprising particles of biological origin or activity which may affect living things through infectivity, allergenicity, toxicity, pharmacological or other processes" (Hirst 1995). Bioaerosols and exposures to them are influenced by a range of biological, physio-chemical, environmental and management factors. The probability of infection is related to microbial particle size, composition and the concentration of pathogens in the bioaerosols, along with the source, dispersal mechanisms in the air and environmental conditions at the site (Pillai and Ricke 2002). These factors should be considered in association with other factors that impact on the occurrence of disease transmission (such as characteristics of the host and pathways to transmission) as discussed in section 2.3.

Previous studies (using biosolids) have recovered low concentrations of viruses and bacteria from aerosols (Fannin *et al.* 1977) and found that any pathogens in bioaerosols would be well below detection (Dowd *et al.* 2000). Most of the pathogens (i.e. bacteria, viruses, fungi, actinomycetes and biotoxins) commonly occurring in biosolids can be aerosolised except for helminths (Peccia *et al.* 2007). Brooks *et al.* (2005b) collected aerosol samples at loading, unloading, land application and background operations of biosolids. The greatest risk of infection was found to occur during loading operations. Crook and Sherwood-Higham (1997) stated that it was important to measure bioaerosol exposure near the breathing zone of the worker. Carducci *et al.* (2000) investigated the airborne biological hazards for plant workers at urban wastewater treatment plants and found no relationship between temperature, relative humidity or wind and airborne contamination. Fannin *et al.* (1977) found that conditions of lower wind velocity, higher ambient air temperatures and increased distance from bioaerosol emissions reduced airborne coliforms significantly. Mawdsley *et al.* (1995) found that wastes were safest dispersed in conditions of low wind speed, high UV intensity and a relative humidity of between 40-60%.

Microorganisms can die from aerosol shock, that is, once aerosolised they are exposed to a range of environmental decay mechanisms such as relative humidity, temperature and UV radiation (Edmonds and Mayer 1979). Sorber *et al.* (1987) reported most pathogenic bacteria such as *Clostridium* and *Pseudomonas* to be less susceptible to aerosol shock than indicator microorganisms because of the formation of survival mechanisms such as resistant endospores, as in the case of *Clostridium*. Enteroviruses are least affected by aerosol shock compared to other virus types. The viability of aerosolised microorganisms could be influenced by adverse temperatures, dehydration and humidity conditions; dehydration decreases the viability of some bacterial cells.

2.10. Methods used when collecting bioaerosol samples

Bioaerosols samples may be collected using devices that place microorganisms directly onto agar media such as filtration devices and impingers that collect liquid (Crook and Sherwood-Higham 1997). Flow cytometry is also used to determine total numbers of microorganisms in bioaerosol samples. This method characterizes cells according to shape and size. Vital fluorescence staining allows for differentiation between viable and nonviable cells. Immuno-labelled fluorochromes then allow for further characterisation (Crook and Sherwood-Higham 1997).

Delays in transportation between the experimental site and the laboratory may affect the viability of cells. Re-suspension medium may repair damaged cells, restore viability and give a representative sample of the levels of harmful bioaerosols present in the environment. Ingredients such as simple sugars (inositol or trehalose) and osmo-protectants enhance the recovery of stressed cells and may increase the tolerance levels of bacteria to drying (Crook and Sherwood-Higham 1997).

Fannin *et al.* (1977) suggested that coliphages were preferable over coliforms for use as indicators in aerosols as the latter are less stable in an airborne state. Carducci *et al.* (2000) recommended that reoviruses and enteroviruses be used as indicator microorganisms for bioaerosols and that the sampling method represented that of the workplace being examined for the presence of bioaerosols.

2.11. Detection of microbial pathogens

The most conventional ways to detect and enumerate bacteria and viruses from environmental samples is through either cultural or non-cultural methods. Cultural methods are the standard approach and have been adopted by most laboratories. Indicator bacteria and viruses are also used, particularly for water samples, to represent the survival patterns of enteric pathogens. Two of the main reasons that indicators are often preferred over pathogenic microorganisms are 1) to overcome culturing difficulties and 2) because they are safer for human handling.

2.11.1. Cultural methods

Cultural methods are the most common method of detection of microorganisms with the use of agar or broth to supply bacterial cells with the correct nutritional requirements for survival (Baker and Herson 1999) and repression of growth of non study microorganisms. Apart from direct plating onto agar, the three detection methods commonly used are membrane filtration, multiple fermentation tube (commonly known as most-probable-number or MPN) and defined substrate assay such as Colilert® Quanti-tray® 2000 system (WERF 2007). The United States Environmental Protection Agency (EPA) recommends the MPN methods 1680 and 1681 for detection of faecal coliform in biosolids, method 1682 for detection of *Salmonella* and the plaque assay procedure for the detection of viruses in biosolids (USEPA 1989). Most studies on the survival times of enteric pathogens in biosolids have used the MPN method for the detection of bacteria (Eamens *et al.* 2006; Horswell *et al.* 2007; Lang *et al.* 2007; Eamens and Waldron 2008) since most guidelines (internationally) require MPN analysis for pathogen enumeration .

Cultural methods have limitations (Crook and Sherwood-Higham 1997) such as underestimating the total number of cells present due to cell expiry. Some cells are unable to grow in media or at the temperatures used in the laboratory. Some stressed microorganisms are incapable of growth and are therefore undetectable. A theory exists that bacterial cells can enter a viable but non-culturable (VBNC) state where bacteria are still viable but unable to form colonies on growth medium (Baker and Herson 1999). In a study of stockpiled biosolids, where regrowth of faecal coliforms and Salmonellae occurred, Gibbs *et al.* (1997) stated that it should be possible to

detect bacteria in this non-culturable state if the reason for regrowth was due to cells in the viable but non-culturable state converting back to a cultivable form.

Culturing methods may affect the accuracy of results, although this is not commonly a problem for indicators such as *E. coli* since selective agars are highly specific. The problem usually lies with detecting background numbers of pathogens where interference from overgrowth of indigenous flora occurs. Crute (2004) found that the enumeration of cells through culturing processes was hindered by background autochthonous flora that is common when processing samples containing biosolids. This issue can be overcome by inoculating with high numbers of the target pathogen.

2.11.2. Non-cultural methods

In some situations, molecular-based methods may be advantageous over culturing. The most common non-cultural methods used to detect and characterise sequences are staining (immunofluorescence) and molecular methods (nucleic acid sequences) (Baker and Herson 1999). Immunofluorescent methods use immnofluorescent dyes to stain microorganisms being tested so that microbial cells can be counted under an epifluorescent microscope. This method relies on the recognition of antibody molecules. Methods of direct counting by light, fluorescence or scanning microscopy can also be used to calculate the total number of cells in bioaerosol assays. The use of microscopy is limited because it relies on identifying microbial cells by their shape and size alone (Crook and Sherwood-Higham 1997) and is also very time consuming.

Molecular-based methods for analysis such as polymerase chain reaction (PCR) can be beneficial in terms of sensitivity, accuracy and efficiency (Crook and Sherwood-Higham 1997). Molecular techniques also make it easier to explore the diverse range of soil microbes through the nucleic acids present. This process involves culturing or the direct extraction of DNA of specific microorganisms and relating their nucleic acids to known structures in the soil (Prescott *et al.* 2002). There are several issues that can arise with methods such as PCR, particularly from biosolids samples, since PCR is easily inhibited by substances such as proteins, humic acids and fats. This can result in false negative results. Such inhibitors can be reduced by additives such as bovine serum albumin (BSA) (Kreader 1996) or further washing and lysing steps to

purify the samples as much as possible before running PCR. However, the use of further purification steps results in the loss of some DNA. Another disadvantage with the use of PCR is that it will detect both alive and dead cells and thus the 'infectivity' of the detected cells is uncertain. There are many types of commercial kits available now for the extraction of viruses from faecal and water samples (WERF 2007).

Nucleic acid sequences use nucleic acid probes with nucleotide sequences. A gene probe containing deoxyribonucleic acid (DNA) extracted from an environmental sample is used to bind and match specific nucleotide acid sequences to the matching sequence of the selected microorganism (Baker and Herson 1999). The presence of microbiological material can be detected in any sample through the use of gene probes and PCR (Crook and Sherwood-Higham 1997). However, one disadvantage with using nucleic acid sequences is that the number of specifically-matching microorganisms can be too low for them to be drawn towards the probes. The use of PCR is then necessary to amplify these sequences into detectable levels (Baker and Herson 1999).

2.11.3. Indicator bacteria

A common practice in the detection of enteric pathogens is the use of indicator microorganisms as surrogates for pathogenic types (Baker and Herson 1999). Because minimal work has been carried out on pathogens in biosolids, similar indicator microorganisms for water are being used for biosolids. The use of indicators to detect human pathogens in water has long been in question (Koopmans and Duizer 2004). Many sources refer to the use of indicators in water as appropriate to the nature of their ingestion (drinking, recreational activities) but there is very little information on the most appropriate indicators to use for biosolids, given their likely method of ingestion i.e. from the soil (Ashbolt 2004). Despite this, waterborne indicators are still being used to detect pathogens in biosolids. Therefore much work is needed in this area.

The choice of indicators should relate to the pathogenic potential, the range of survival mechanisms across different environments and their association with faecal matter (Eamens *et al.* 2006). It is important to select suitable indicator

microorganisms since most microorganisms of concern are not always present or their numbers are too low to monitor (Horan *et al.* 2004).

Faecal indicator numbers (total coliform, thermotolerant coliform and enterococci) as inoculated into soil or biosolids samples may be higher than those typically found in human faeces (Gauthier and Archibald 2001). Faecal coliform may die off faster than actual bacterial pathogens (Edmonds and Mayer 1979). Hassen *et al.* (2001) found that the use of indicator microorganisms such as coliform was advantageous as opposed to actual pathogens, since indicators were often present at higher numbers than pathogens and indicators were safer to detect. Coliform bacteria have been used as indicators of faecal contamination in water for many years and have been found to give a reasonable indication of probable levels of pathogenic microorganisms present (Gallagher and Spino 1968; Fannin *et al.* 1977).

E. coli is typically used for detection of environmental faecal pollution in water samples (Mawdsley *et al.* 1995) although it is a poor indicator for the presence of parasitic protozoa and viruses in drinking water since both microorganisms survive longer than the indicator bacterium (Stevens *et al.* 2002). Wilkinson *et al.* (2003) found that *Salmonella* and *Campylobacter* were present when the indicator bacteria *E. coli* could only be detected by enrichment. Enterococci are reported to be reliable and successful indicators of faecal pollution because of their ability to grow at high temperatures (45˚C) and high pH (9.6) (Scott *et al.* 2002). They are also generally more resistant to a variety of environmental factors than coliform (Hassen *et al.* 1998). However, faecal streptococci cannot be used as an indicator of faecal bacterial contamination in biosolids as it has a different level of susceptibility to the treatment processes than Salmonellae (Lewis-Jones and Winkler 1991b).

2.11.4. Viral indicators

Because of the many difficulties associated with the culturing of enteric viruses, viral indicators such as bacteriophages are used to indicate viral behaviour in biosolids and wastewater. Bacteriophages are used as surrogate viruses to represent human enteric viruses because they infect bacterial cells and are relatively easily cultured. They are not harmful to humans and have thus been used as a popular indicator for the detection of faecal pollution and for the modelling of viral transport in the

environment (Baker and Herson 1999). Even though similar in structure and behaviour to human pathogenic viruses, bacteriophages cannot be relied upon alone to indicate the presence, nor safety, of human faecal waste at exposure (Koopmans and Duizer 2004).

There can be difficulties in detecting viruses in biosolids-amended soil. The detection and enumeration of bacteriophage MS2 can be difficult due to the viscous and muddy nature of biosolids (Crute 2004). Viruses may bind strongly to the soil and be difficult to recover. Virus survival can be prolonged because of this competitive binding onto organic matter (Pedley *et al.* 2004), soil or biosolids. Bacterial contamination can impair the performance of plaque assays used for the enumeration of bacteriophages (Lasobras *et al.* 1999).

Bacteriophage may be used as an indicator for pathogenic viruses but may not reflect similar survival behaviour. Adenovirus are resistant to UV light (Carducci *et al.* 2000) and therefore are unlike bacteriophage. It has been suggested that bacteriophage could be used as an effective surrogate for norovirus (Dawson *et al.* 2005).

2.12. Future risks from enteric pathogens

2.12.1. Emerging diseases

There is underlying risk that new pathogens may emerge, resulting in new diseases (e.g. *E. coli* O157). Emerging diseases are defined as infections that have reappeared after a decline in incidence, have been present in the population but passed undetected and are new diseases, or are established diseases that have been newly recognised as infectious. Such emerging diseases would normally have been shown to increase within the past two decades and would have the potential to threaten future populations. Emerging pathogens or microbial evolution are also of threat to humans with the increasingly close association between humans and animals, such as the domestication of pets into internal living areas (Ashbolt 2004).

Foodborne infections have also changed considerably over time (Tauxe 2002). Some established pathogens have been controlled or eliminated through technology and some new pathogens have emerged due to changing ecology and adaptation to new technology. Scientific advances such as vaccines, antibiotics, improved sanitation, public health systems, diagnoses and education assist in the control of pathogen-related infectious diseases (Nel and Markotter 2004). Despite this, the infectious diseases of most concern are those emerging, resurging or re-emerging diseases.

Emerging pathogens may develop as a result of microorganisms crossing over from one to another species or non-pathogenic microorganisms transforming into pathogenic microorganisms through mutation, recombination or modification (Nel and Markotter 2004). For example, severe acute respiratory syndrome (SARS) resulted from a small genetic change occurring in a benign Coronavirus (Ashbolt 2004). The disease strains that caused subclinical mastitis in sheep have recently been recognised as being related to a seed-borne disease in rice (Tauxe 2002). This raises the concern that enteric pathogens in biosolids, applied to land where grain crops are grown, could transfer to livestock and humans.

The occurrence of new or re-emerging waterborne pathogens is related to social and environmental change (Ashbolt 2004). Van de Berg *et al.* (2005) stated that new norovirus strains are continuously emerging and that some may be more adept at surviving environmental factors that would normally control such populations. Some of these strains may be more proficient in causing infection to individuals. A major factor contributing to re-emergence of pathogens and the spread of parasitic infections is related to human behaviour, and in particular, poor hygiene (Nithiuthai *et al.* 2004).

The World Health Organisation (2003) implied that despite the degree of uncertainty and lack of information regarding re-emerging pathogens, neither the presence nor the absence of such pathogens should be assumed. Tauxe (2002) stated that we should "expect the unexpected". A more proactive approach by water utilities to constantly improve the quality of biosolids and better communication across the food and distribution sector is important (Bruce and Evans 2002).

The occurrence of re-emerging pathogens will continue to be a public health concern due to global population growth, climate change, ageing population therefore more vulnerable to infection (Tauxe 2002), increased international travel (i.e. infections occur abroad), recreational activities (e.g. water sports), migration, increased urbanisation where sanitation processes are overloaded), closer association with domestic animals (i.e. most modern communities) and changing international food trade patterns that combine new cuisines, tastes and food processing methods (Tauxe 2002; WHO 2003). World population is projected to reach more than 9 billion people by 2050 (currently 7 billion), having increased by one billion people over the last 12 years (Follett *et al.* 2005). Along with the threat of new diseases these increases have resulted in additional generation of waste and the need for improved disposal means. These changes have impacted our environmental systems (Cameron *et al.* 1997) and such changes ultimately affect food safety.

Much research is required into the effects that the future changes to the climate could have on the spread of enteric pathogens, particularly where biosolids are currently applied to land as a beneficial resource. With greater climatic extremes expected, such as flooding or extended dry periods, rapid fluctuations could impact the way we

manage biosolids in the future. Collecting scientific data on the survival patterns of a range of pathogens (such as bacteria, viruses, helminths and protozoa) is the first step towards being able to identify the key areas of risk. Concerns about the potential health risks from pathogens associated with the land application of wastes will continue to occur into the future in the absence of available information on pathogens numbers and the survival of pathogens in biosolids and manures (Gerba and Smith 2005). This is particularly important along the food production supply chain to ensure proper sanitation and reduction of cross-contamination of potentially dangerous diseases.

The development of preventative technologies could possibly reduce future risks of pathogenic transfer. These include animal vaccination against zoonotic foodborne pathogens, treating foods with ionising radiation, composting and other pathogen-reducing treatments for manures and the feeding of non-pathogenic, enteric microorganisms to animals to prevent colonisation of harmful pathogens through competitive exclusion (Tauxe 2002). Livestock herds in developed countries are a major source of enteric pathogens; therefore, it is very unlikely that the risk of pathogen transfer from animals will be eliminated in the foreseeable future.

Therefore, the responsibility of public health is spread across the entire food chain (Tauxe 2002). With the freeing up of food trade laws from developing to developed countries, pathogen guidelines will need to become more stringent and consistent to protect consumers from food crops such as vegetables that have been irrigated with faecally-contaminated water (Ashbolt 2004). The threat of such contaminations should not be overlooked (Kozan *et al.* 2005). Crop production for human consumption should not be permitted in areas where the irrigation water may be a health risk to the consumer (Koopmans and Duizer 2004).

2.13. Gaps in the knowledge

From the search of available literature, several knowledge gaps have been identified in relation to the research to be conducted in this thesis:

- Published data on survival times for enteric pathogens in soil, in particular soil amended with biosolids is relatively sparse. The information available is dated therefore does not generally track decay over the longer term, and the methods used to derive the survival times is often unclear;
- Very few studies have been conducted in Australia on enteric pathogen survival where biosolids are currently used. Australia applies 50-70% of its biosolids to agricultural land yet no data is available specific to the various soil types and climatic conditions for each region;
- No information is available on the decay of enteric pathogens from consumable plant parts or grains that are grown following biosolids application to the soil;
- There is no comparative data on pathogen survival in animal manures which are used extensively as fertiliser throughout Australia and the rest of the world;
- More studies are required on the climatic conditions that influence enteric pathogen survival in biosolids-amended soils;
- There is insufficient information available on the survival times of viruses, helminths and protozoa in biosolids or biosolids-amended with soil; and
- Laboratory methods need to be developed for the quantification of various enteric pathogens from soil amended with biosolids.

2.14. Further research needs

It has been well recognised that there is a lack of information on the survival patterns of enteric pathogens, present in the soil from the application of biosolids to agricultural land (Gerba and Smith 2005; Lang *et al.* 2007; Sidhu and Toze 2009). Microbial risk assessment requires more sophisticated data on the survival and transport of pathogens during the land application of biosolids, to ensure management practices including treatment processes, and cropping and harvesting restrictions are appropriate, to ensure human health is protected from enteric disease where sludge is used in agriculture.

One specific research need, identified in this thesis, was to contribute to the information on numbers and survival patterns of pathogens and indicators in biosolids-amended soil where food crops are produced around the world (Lang *et al.* 2007). In order to do this, the following research was required:

- Development of improved methodology for monitoring and enumerating decay of enteric pathogens in the field over time (eg. across the stages of crop growth);
- Determining the decay times at each stage along the pathways to consumption of a food crop such as from the time biosolids are applied, while the crops are grown and when the plant components are consumed, to identify peak times of risk to consumers;
- Determining the effect that biosolids have on the survival patterns and decay times of enteric pathogens in agricultural soil;
- Establishing the presence or absence of airborne contaminants in dust during grain harvesting operations where biosolids have been recently applied (i.e. 6-7 months prior); and
- Determining the influence of climatic conditions and soil type on enteric pathogen numbers (under Australian conditions).

2.15. Summary

- As increasing volumes of wastewater are produced and, as a result, more biosolids are being produced, sustainable and beneficial ways to use biosolids such as application onto agricultural land is becoming more popular. However, biosolids contain pathogenic contaminants of potential harm to human (and livestock) health and this risk must be understood in order to be correctly managed;
- Pathogens can cause severe illnesses in humans such as gastroenteritis, diarrhoea, dysentery, cholera and meningitis. Regulations are set in place in the form of guidelines to minimise the risk of transmission of pathogens, and thus disease, from land-applied biosolids. Despite this, the survival patterns of such pathogenic contaminants needs to be better understood, particularly in Australian agricultural soils where the majority of biosolids are applied;
- In addition, some of the factors affecting the survival of pathogens in the external environment (i.e. outside the host) need to be examined such as changes to soil temperature and soil moisture levels. This is necessary to identify the factors such as soil type or climate that may prolong pathogen survival (e.g. cold temperatures, wet conditions, soils with high organic matter);
- To enable the survival patterns of pathogens to be examined in the field (where typical broadacre cereal crops are grown), a method needs to be established that enables a more controlled environment, while capturing natural decay processes where biosolids are currently being applied. For this reason, experiments in the glasshouse would not suffice alone since natural changes in soil and air temperature, humidity, soil moisture via rainfall events and natural predation from indigenous microorganisms would not be allowed to occur naturally;
- A form of microcosm needs to be used that acts as an environmental chamber. This needs to allow for gaseous and moisture exchange to occur without the loss of microorganisms;
- Since pathogen decay needs to be examined over the period of time of greatest risk to human health, decay times for bacteria and viruses in the soil needs to be examined over the growing season (approx. 6 months or more) of crops typically grown following the application of biosolids;

- In addition, the decay times for pathogens (or enteric microorganisms) from parts of the wheat phyllosphere needs to be examined. If these microorganisms persist on the leaves and spikelets of wheat, then the risks to human health at consumption would be increased; and
- Following this, should the pathogens survive in the soil into harvest time, the levels of bacteria in the dust at harvest needs to be examined, along with whether the threshing process will result in a more rapid inactivation of the pathogens.
- Since pathogens are difficult to enumerate from biosolids/soil samples (compared to water samples) the use of indicator microorganisms would be a useful research tool, used in conjunction with the study of some pathogenic microorganisms as a comparison. For this reason, the indicator microorganism *E. coli* and surrogate virus bacteriophage MS2 would be useful, to compare with *S. enterica* and human adenovirus. Culturing using selective agars is deemed most suitable for the enumeration of *E. coli*, *S. enterica* and bacteriophage. The molecular-based method of PCR using extraction kits would be most suitable for the detection of adenovirus.

CHAPTER 3 GENERAL MATERIALS AND METHODS

Standard methods of enumeration and quantification of enteric microorganisms (from microbiology laboratory protocols and procedures for water) were used across the entire study to ensure the methodology was robust and able to be replicated. All soil experiments involved the use of sentinel chambers, and general laboratory methods were employed to culture the study microorganisms and to quantify them over the duration of the experiments.

3.1. Experimental strategy

The experimental program used some of the described general methods and materials; other methods as relevant to each experiment are described within each chapter.

The experiments conducted under this research program follow the growing season of wheat in the field, as typical to broadacre cropping conditions. A broad outline of the research program is provided in Table 3-1. The research comprises of an initial experiment in the glasshouse (Chapter 4) to examine the decay times of *S. enterica* and bacteriophage MS2 in soil chambers inserted vertically into soil in pots. Following this, three experiments were conducted in the field in grain growing regions of Western Australia (Moora) and South Australia (Mount Compass) where biosolids are currently applied to land (Chapter 6). These experiments, conducted over approximately 6 months, enabled the decay times of indicator and pathogenic bacteria and viral strains to be derived. A glasshouse experiment was conducted at the flowering stage of wheat (Chapter 7) to examine the decay times of inoculated enteric microorganisms from the spikelets and leaves. At harvest, dust experiments were conducted on-farm in the field in Western Australia to test for the presence/absence of residual bacteria in the soil, chaff, spikelets, grain, leaves and dust where biosolids had been previously applied (Chapter 8). In addition, a thresher experiment was conducted to examine the effect of threshing on the inoculated enteric microorganisms.

Table 3-1: Outline of experimental programs used in the current research project

Experiment	Location	Year/s	Purpose	Study microorganisms
Soil	Glasshouse, Floreat	2006	To test methodology and obtain initial decay times	*S. enterica* and bacteriophage (MS2)
Soil	Field, Moora Mt Compass	2006 2008	To examine the decay times of inoculated enteric microorganisms in the soil of a biosolids-amended wheat crop	*E. coli*, *S. enterica*, MS2 and adenovirus
Phyllosphere	Glasshouse, Floreat	2007	To examine the decay times of inoculated enteric microorganisms on the spikelets and leaves of wheat	*E. coli*, *S. enterica* and MS2
Thresher	Undercover area, Northam	2008	To examine the effect of threshing on inoculated microorganisms from spikelet to dust	*E. coli*, *S. enterica* and MS2
Harvesting	Field, Moora	2008 2009	To determine the presence/absence of bacteria in wheat dust where biosolids have been applied to the soil	*E. coli*, *enterococci* and heterotrophic bacteria

3.2. Sentinel chambers

Sentinel chambers were selected as the tool for studying pathogen decay in soils (see section 2.7). Sampling containers or chambers have two applications: a) to contain sample contents and b) to act as environmental chambers. The sentinel chambers described by Jenkins *et al.* (1999), as similar to the diffusion chamber described in Toze *et al.* (2010), were selected because they provided suitable representation of the external environment (such as changes in soil temperature and soil moisture) while containing the sample contents. The applications for each type are described below:

a) The sample container is to provide an enclosure to hold a sample during controlled environment investigations under laboratory conditions. This is a simple application and can be in the form of a plastic bag, pot or container. The container allows the sample to be exposed to the specific environmental treatment conditions but this exposure is limited. The vessel therefore is simply a means of containing the sample so that the experiment can be carefully performed under controlled conditions i.e. controlled temperatures or moisture levels.
b) The environmental chamber is to enable the test microorganisms inoculated into the matrix in the chamber to be in continuous equilibrium with the external environment, so that they experience the same conditions should they exist in the outside environment. The conditions inside the chamber should adjust with changes in soil moisture levels i.e. following rainfall events or dry period as well as soil temperature changes.

For these reasons, the sentinel chambers were advantageous in providing both a sample container to contain the soil, biosolids and microorganisms, but more importantly, a controlled environmental chamber from which to study microorganism decay in the field. In addition, the chambers avoided leaching and cross-contamination with the environment; reduced random error between samples; simplified sampling procedure; for ease of handling; and the commercial Microsep™ centrifugal devices were easily available.

The sentinel chambers used in the survival experiments (Figure 3-1) were assembled similar to the design used by Jenkins *et al.* (1999). In the present study they were constructed using commercially produced Microsep™ centrifugal devices (35 mm long x 10 mm internal diameter, PALL Life Sciences, New York USA) with a membrane pore size of 300,000 molecular weight cut-off (MWCO) or approximately 0.03 µm encased at one end. A commercially-produced 0.2 µm LidBAC® membrane lid from 2.0 mL Eppendorf® Safe-Lock® (Eppendorf®) was fitted to the opposite end of the chamber and sealed with Petrifilm™. The pore size of the Microsep™ membrane and the Eppendorf lids were sufficiently large to allow exchange of gases and moisture without the loss of bacteria or viruses from the chambers.

Following assembly of the chambers with the calculated proportions of soil, biosolids and microorganisms (Figure 3-2), the chambers were then fitted at the top with the membrane lid, sealed at the sides with tape (as described above) and randomly placed into the soil (at depth <10 cm to simulate the placement of biosolids in the field) in a vertical orientation to allow for gaseous and moisture exchange into and out of the chambers, as would naturally occur in the soil profile.

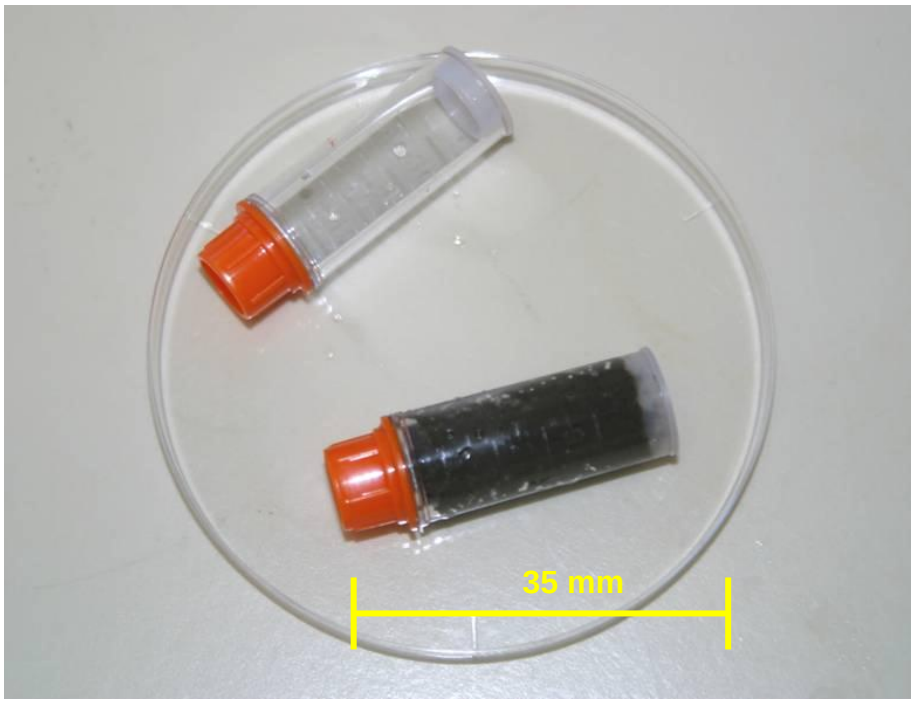


Figure 3-1: (Top left) A commercial 3.5 mL Microsep™ centrifugal device (Pall Life Sciences) and; (bottom right) a sentinel chamber (35 mm x 10 mm) with the sample contents soil, biosolids and seeded microorganisms

Figure 3-2: Filling the chambers with soil, biosolids and cultured microorganisms (CSIRO Microbiology Laboratory, WA)

Figure 3-3: Pink tags used to mark the location of each sentinel chamber under the soil

In the field, flags or pink marker tags were placed in the soil above each of the chambers to mark their location under the soil (Figure 3-3). At sampling, the required number of chambers was randomly selected from the appropriate plot by individually removing them from the soil with a trowel and placing them into labelled plastic bags. Each bag containing the correct number of chambers from each plot was placed immediately on ice and transported to the laboratory for processing within 24 h.

The disadvantages of the sentinel chambers were: the sample size was limited to the available Microsep™ device and therefore quite small (~3 to 5 g); the flow of moisture and oxygen in and out of the chamber may have been slightly delayed or slower than what would have normally occurred across the soil profile; and the use of individual chambers for each sample (i.e. in destructive-style sampling) meant that the decay rates were not derived from the same place (i.e. as would have occurred with a lysimeters-type container).

Figure 3-4 demonstrates that soil moisture content (%) inside the chambers followed similar patterns to the soil moisture outside the chambers (in the topsoil at 0-10 cm in the field) although the soil moisture levels over time were generally more stable inside the chambers. The soil moisture probes outside the chambers (tensiometer readings measuring centibars) followed similar patterns to the moisture changes inside the chambers in the topsoil. In addition, Figure 3-5 shows that microorganism decay patterns followed closely to the changes in soil moisture over the duration of the experiment thus indicating that the chambers were successfully equilibrated with the external environment. More information regarding the chambers is provided in Chapter 6.

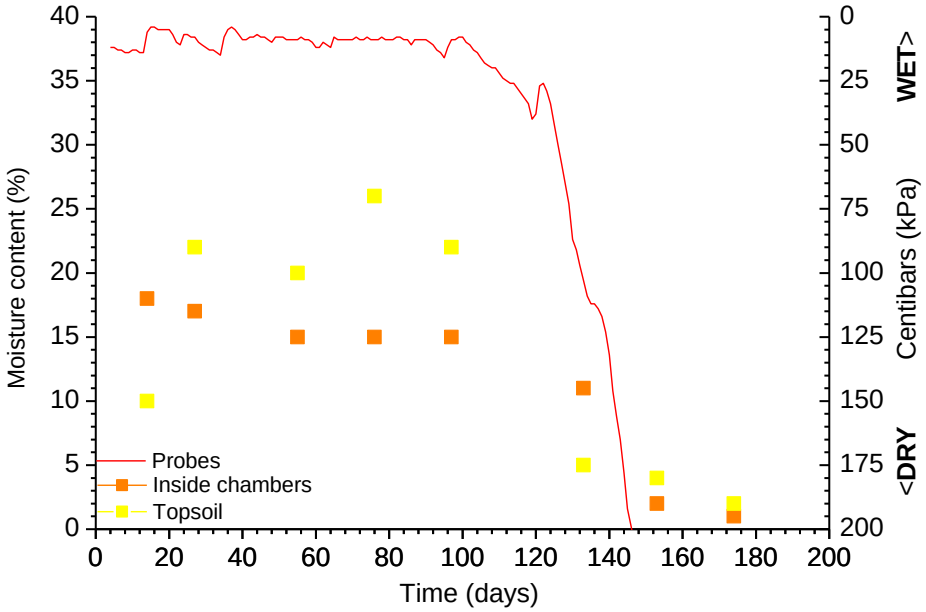


Figure 3-4: Comparison of soil moisture changes inside the sentinel chambers, in the topsoil (0-10 cm) and from the soil moisture probes (kPa) in the unamended soil at Site B, Moora 2008.

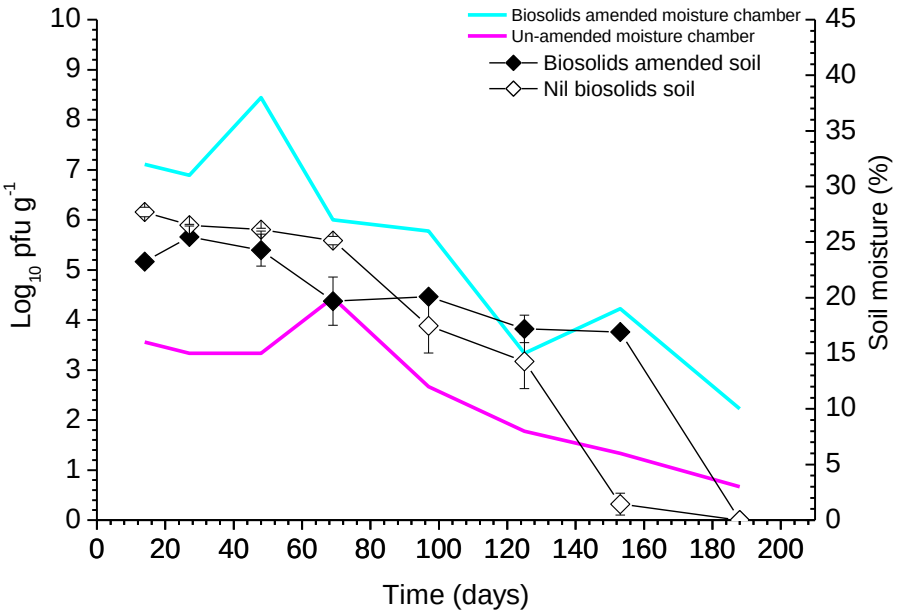


Figure 3-5: Changes in MS2 numbers inside the chambers in association with the soil moisture changes inside the chambers at Site B, Moora 2008.

3.3. Microorganism cultures

The microorganisms used in the experiments were *Escherichia coli* (ACM 11775) commonly used as an indicator of enteric bacterial pathogens; *Salmonella enterica* serovar *typhimurium* (ATCC 13311) as a representative human pathogenic bacteria; bacteriophage MS2 (ATCC 15597-B1) as a surrogate virus; and adenovirus serotype 41 as a representative human pathogenic virus. Note the biosolids used in the experiments was not sterilised (autoclaved) therefore any environmental strains present (such as *E. coli, Salmonella* or adenovirus) would have been enumerated across the duration of the experiment (using selective agars or PCR) along with the laboratory-cultured strains.

E. coli and *S. enterica* were cultured in 100 mL nutrient broth (Oxoid) and incubated in a shaking platform incubator at 37˚C overnight. The cultures were purified by centrifugation at 5,000 rpm for 10 min and washed twice in sterile P-buffer to remove culture media and then resuspended in P-buffer prior to use.

The bacteriophage MS2 was cultured in tryptone yeast extract broth (Oxoid) with a log phase host culture of *E. coli* HS(pFamp)R (ATCC 700881). The *E. coli* host was grown in 100 mL of tryptone yeast extract broth (Oxoid) containing 1 mL each of calcium chloride ($CaCl_2.2H_2O$) solution, magnesium-sulphate ($MgSO_4$) solution and antibiotics (ampicillin and nalidixic acid) in a shaking incubator at 37˚C overnight. To produce the MS2 culture, an exponential culture was made up from the overnight bacteriophage *E. coli* HS(pFamp)R culture by seeding 1 mL of MS2 stock into the new host *E. coli* log culture and incubating in a shaking incubator at 37˚C for 4-5 h. To the exponential culture, 100μL of MS2 stock was added and placed in the static incubator at 37ºC overnight. Crude MS2 culture was purified by centrifugation at 6,000 rpm for 10 min and passed through a 0.2 μm membrane to remove bacterial cells. Purified bacteriophage MS2 culture was stored at 4˚C. The MS2 and bacteria cultures were then acclimatised in sterile P-buffer overnight at room temperature prior to inoculation. The final microbial numbers in the suspensions for each culture type are provided for each individual experiment within the appropriate chapter.

Adenovirus serotype 41 was sourced from the Pathology Centre, WA. The virus was cultured in cell lines (African Green monkey kidney cells) and then harvested from the lawns. The initial number of infective viral particles in the viral suspensions was determined by the Pathology Centre (Perth, WA) through the MPN method in fresh cell culture lawns. The infective titre for the virus suspension was determined to be approximately 10^7 PFU mL $^{-1}$. This stock was stored at -80ºC until required. The detection limit for adenovirus using quantitative PCR was determined by making serial 10-fold dilutions of extracted viral DNA and determining the lowest detectable dilution. The highest detectable dilution in which adenovirus was detected by PCR was 10^{-6} which equated to a detection limit of *ca* 10 or less adenovirus DNA molecules per PCR reaction (based on original MPN titre of 10^7 PFU mL $^{-1}$).

3.4. Microbial quantification

All analyses for the quantification of pathogens were performed in triplicate. All of the bacteria were detected by spread-plating 100 μL of appropriate dilutions (based on the anticipated number of viable bacteria cells present) onto the respective agar plates using sterile glass spreaders. *E. coli* was detected on Chromocult™ coliform agar (Merck), *S. enterica* on xylose lysine deoxychlorate (XLD) agar (BBL), enterococci on Chromocult™ Enterococci Agar (Merck) and heterotrophic bacteria on R2A Agar (Oxoid). Inoculated plates were incubated at 37ºC overnight (28ºC for R2A plates) and then typical colonies were counted. Colony morphology for *E. coli* was deep-purple; for *S. enterica*, black colonies with pink borders and for enterococci, dark-pink colonies. For heterotrophic bacteria, all coloured colonies were counted. Dilutions containing 20-200 colony forming units (cfu) were selected for counting. The detection limit of this methodology was 3 cfu mL^{-1}. The cfu per gram were then calculated from the original weight of the samples processed.

Presumptive *S. enterica* colonies, with clear or yellow surrounds, were confirmed by streaking onto CHROMagar™ Salmonella (BBL). Plates were incubated at 37ºC overnight. Purple-coloured colonies were recorded as positive and any inhibited, blue or colourless colonies were considered negative.

The standard method of enumeration using MPN, as provided under the guidelines (refer to section 2.11.1), along with the recovery step to revive and recover the maximum number of cells was not used in this research project. MPN methodology is generally used for the detection of pathogens which are often present in low numbers. For this study, we considered the use of MPN methodology but it was not used for two reasons. Firstly, the pathogens and indicators were seeded into the biosolids at higher numbers than are found in the environment or in biosolids so that they could be detected easily using spread plate counts. Secondly, the seeded biosolids were sampled at regular intervals in order to determine inactivation rates. From this data collected, a decay slope could be produced. MPN methodology is more labour intensive, with perhaps marginal improvement in quantitative detection of microbial numbers. In addition, the rate of inactivation (which was the objective) could be measured accurately enough, provided that the same methodology was used across the sampling events.

The quantification of F-specific bacteriophage MS2 was carried out using a modification of the standard double layer agar method (Havelaar and Hogeboom 1984) with tryptone-yeast extract medium (TYG) containing: tryptone (Oxoid, England) 10.0 g L^{-1}; yeast extract (Oxoid, England) 1.0 g L^{-1} and sodium chloride (NaCl) solution (BDH, Australia) 8.0 g L^{-1}. The basal medium was sterilised (15 min at 121ºC) and then glucose ($C_6H_{12}O_6$) (BDH, Australia) 500 g L^{-1}; calcium chloride (Merck, Australia) 150 g L^{-1} and magnesium sulphate (BDH, Australia) 150 g L^{-1} were added. Top-layer agar (semi-solid) contained 1% Agar Bacteriological (Oxoid, England) (w/v) and bottom layer agar contained 2% (w/v). The host bacteria, *E. coli* HS(pFamp)R (ATCC 700881) was grown to exponential phase in 100 mL of tryptone yeast extract broth (Oxoid) containing 1 mL each of glucose-calcium chloride solution, magnesium-sulphate solution and antibiotics (ampicillin and nalidixic acid) in a shaking incubator at 37˚C for 3 h. Culture (100 µL) of the exponential phase *E. coli* was seeded into the top-layer agar and poured immediately onto the plates.

MS2 were detected by drop-plating three replicate 10 µL aliquots of appropriate dilutions (based on the anticipated number of infective phage particles present) onto the surface of the double-layer medium inoculated with the *E. coli* host. The method

of spot inoculation or drop-plating was a modification (used in this research) to the referenced pour plate method described by Havelaar and Hogeboom (1984). Inoculated plates were incubated at 37˚C overnight and then typical 1-2 mm clear plaques of MS2 were counted. Clear plaques were counted to determine the average plaque forming units (pfu g^{-1}) after incubation at 37ºC overnight. Dilutions containing 1-30 plaques were selected for counting. The detection limit of this methodology was 30 pfu mL^{-1}.

The quantification of human adenovirus genomes in biosolids was performed by determining the number of PCR detectable copies of genomic DNA using quantitative PCR (examples in Figures 3-5 and 3-6). To extract the adenovirus DNA template, 1 g soil samples from each chamber were weighed, suspended in 5 mL Star Buffer (Roche), vortexed for 2 min and stored overnight at 4ºC. Prior to extraction of the DNA, samples were vortexed again for 2 min, then centrifuged at 2500 rpm for 10 min at 4ºC to remove soil particles. Adenovirus DNA was extracted from the supernatant using a QIAamp DNA Stool Mini (Qiagen) as per manufacturer instructions. To increase the DNA yields, a modification was made to the initial cell lyses step where samples were heated at 90°C for 10 min, rather than 70°C for 5 min. The resulting extract containing template was then stored at -80°C until analysis.

Quantitative PCR reactions for detection of adenovirus DNA were performed on Bio-Rad iQ5 (Bio-Rad Laboratories, California, USA) using iQ Supermix (Bio-Rad). Each 25µL PCR reaction mixture contained 12.5 µL of SuperMix, 120 nM of each adenovirus primer (Heim *et al.* 2003) and 3 µL of template DNA. Bovine serum albumin (BSA) was added to each reaction mixture to a final concentration of 0.2 µg μL^{-1} to relieve PCR inhibition (Kreader 1996). All DNA samples were analysed in triplicate. Thermal cycling conditions for adenovirus DNA detection were undertaken as outlined in Sidhu *et al.* (2010). Briefly, initial incubation at 95ºC for 8 min, then 55 cycles at 95ºC for 30 sec, 55ºC for 20 sec. 72ºC for 20 sec. The final cycle had an extension time of 5 min at 72ºC.

Mean viral copy numbers were calculated from a standard curve generated during the PCR using the iCycler software (Bio-Rad). The standard curve was generated from 100-fold serial dilutions of adenovirus DNA using DNA extracted from the original

washed suspensions. Four dilutions (1:10) of the standard DNA were used to construct the standard curve starting with the initial extracted DNA solution being used as the first dilution (10^0). Aliquots of the same standards were used for all samples and experiments for comparative purposes.

A melt curve analysis was performed after the PCR run to differentiate between actual products and primer dimers, and to eliminate the possibility of false-positive results (Figures 3-7 and 3-8). The melt curve was generated using 80 cycles of 10 s each starting at 55°C and increasing in 0.5°C intervals to a final temperature of 95°C. The T_m for each amplicon was determined using the iQ5 software (Bio-Rad).

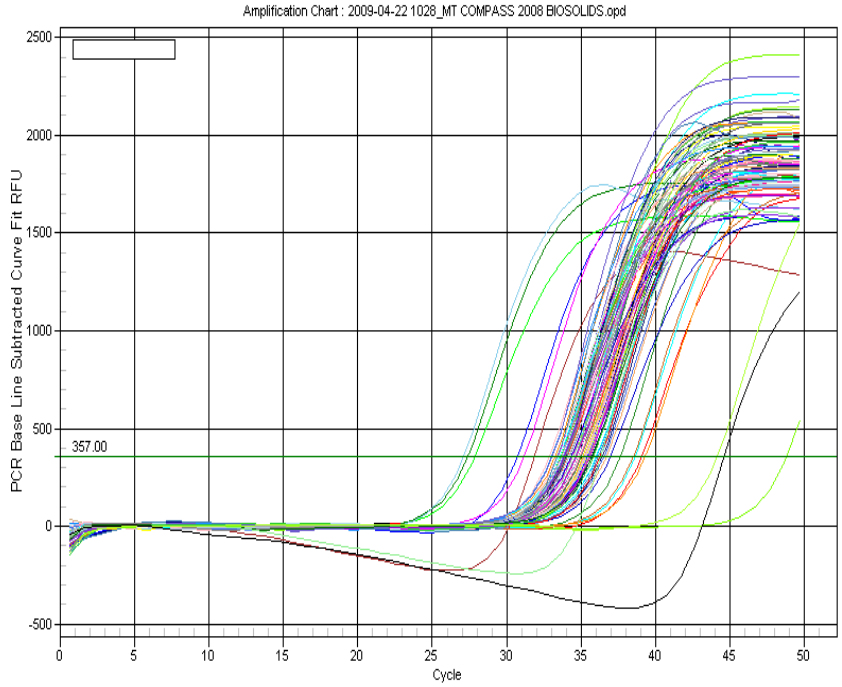


Figure 3-5: Example of qPCR amplification output for adenovirus in biosolids-amended soil

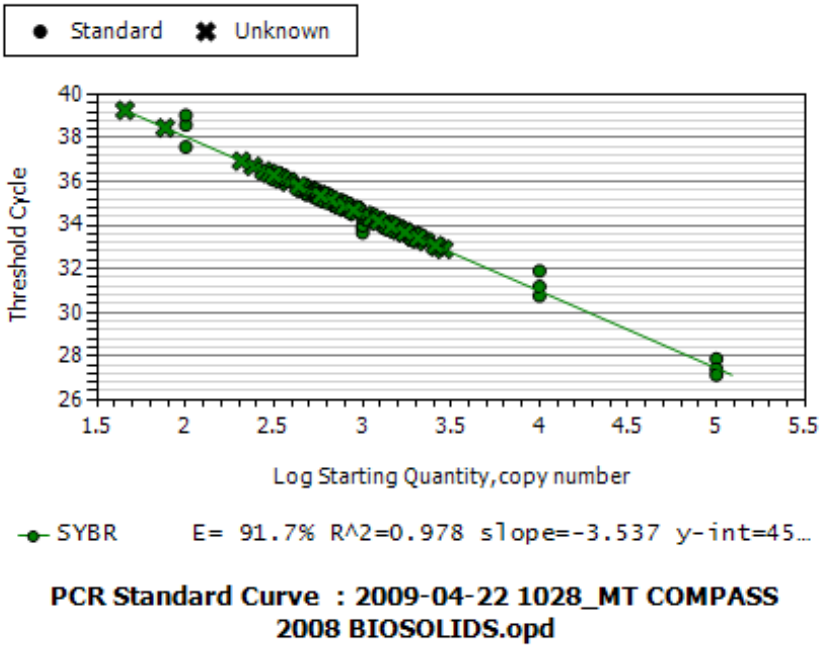


Figure 3-6: Example of PCR Standard Curve for adenovirus in biosolids-amended soil

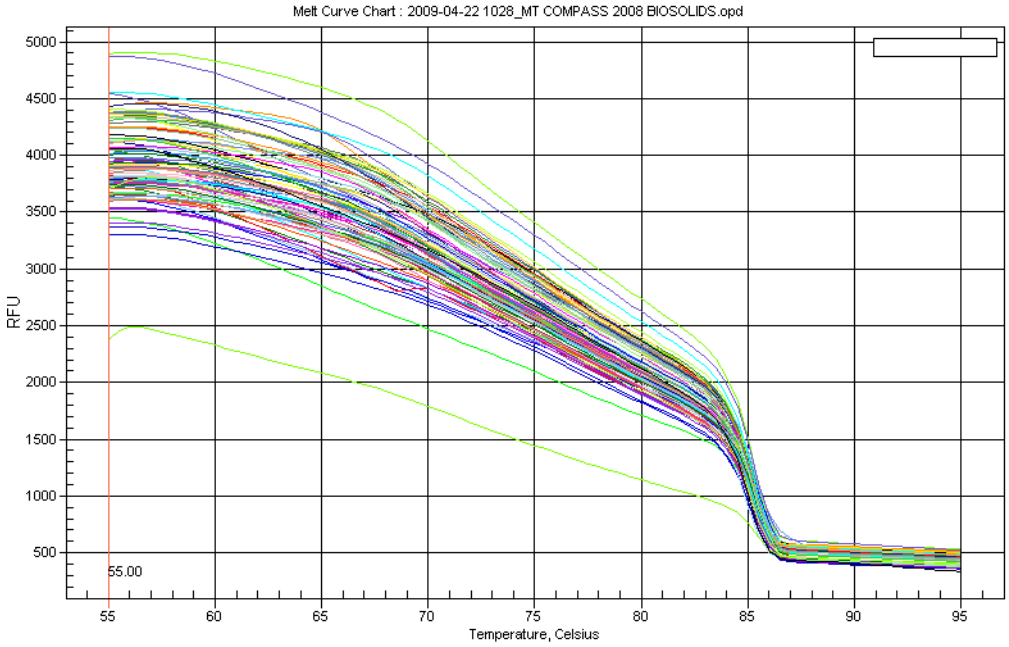


Figure 3-7: Example of PCR melt curve chart for adenovirus in biosolids-amended soil

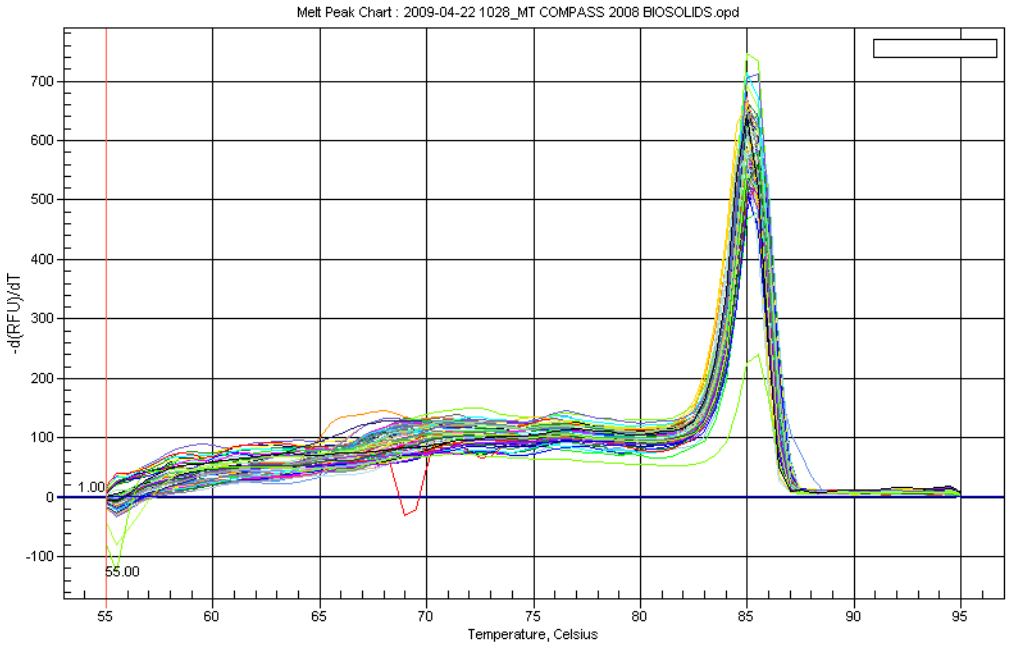


Figure 3-8: Example of PCR melt peak chart for adenovirus in biosolids-amended soil

Along with the internal melt curve testing above, a series of other quality control (QA) and quality assurance (QA) practices were employed. This involved the following measures:

1. The use of separate laboratory areas and equipment for each stage of the process to avoid false positives resulting from carryover contamination of amplified virus particles or viral nucleic acid;
2. The method ensured that if any false positives were identified, the run was discarded and the PCR reactions were re-run;
3. Negative controls (non spiked Rnase/Dnase-free water) and positive controls (virus suspensions) were run with each set of samples and processed through the nucleic acid extraction and enzymatic amplification assays;
4. Blank controls with the same reaction mixture, except for the template, were included with the PCR assays;
5. Inhibition was tested with the viral stock of adenovirus which was diluted by serially diluting 10^{-5} to 10^{-2} in autoclaved biosolids and 100 µL of each dilution was inoculated into 1 g samples in order to test if the biosolids sample was capable of inhibiting the replication of viruses (Schlindwein *et al.* 2010);
6. PCR inhibition was tested with 3 µL of template from the biosolids DNA and 3 µL of adenovirus DNA template to test for any inhibition occurring during PCR reactions. No PCR inhibition was observed with regular testing;
7. Adenovirus recovery in soil and biosolids samples returned mean recovery efficiencies of 56% from the biosolids-amended soil and 55% from the nil-biosolids soil;
8. Non-autoclaved biosolids samples tested positive for human adenovirus. Therefore, the detection of the RNA viruses in this study were not inhibited by the natural levels in the biosolids samples tested.

3.5. Data normalisation

Prior to statistical analysis, pathogen counts were normalised from the raw data in Microsoft ® Excel by transformation into $\log_{10}$ cfu g^{-1} using the log conversion formula (Equation 1). This was done to account for different dilutions, plating volumes, phosphate buffer levels and soil volume used. The pathogen numbers in each replicate for each sampling event were converted to log values so they could be plotted over time and decay times could be determined.

Equation 1:

$$\text{Log}_{10}\ Count = \log_{10}\left[\left(\left(Count * 10^{\wedge\ Dilution} * 10^{\wedge\ volume\ plated}\right) * X\ \text{mL}\right)(1/g) + 1\right] \quad \textbf{(1)}$$

where, *Count* is the number of cfu present between 20-200 per plate; ^{Dilution} is the variable number of the dilution (e.g. 1/1000 = 3, 1/10000 = 4); $^{volume\ plated}$ is a constant amount of sample spread onto each plate (e.g. 10 or 100 µL); *X* mL is the constant amount of P-buffer used to suspend the samples (e.g. 30 mL); 1/g is the variable amount of sample as determined by the net weight (g) of each sample; and the value of 1 was added to non detects (zero values) to take account of the problem of log transformation for the zero observations.

The counts from Time 0 were removed from all field data prior to any statistical analyses as it was observed that some variability in numbers relating to clumping and un-clumping of microorganisms occurred between Time 0 and the first sample event that had a major effect on the reliability of the statistical analysis. Associated standard deviations, trendlines and logarithmic transformations were performed in Origin® 6.1 (OriginLab Corporation 1991-2000).

CHAPTER 4 THE TRIALLING OF THE METHOD TO STUDY THE EFFECT OF BIOSOLIDS ON THE DECAY TIMES OF *S. ENTERICA* AND MS2 IN SOIL

4.1. Introduction

Biosolids applied to agricultural soils contain contaminants, in particular enteric pathogens, which pose a potential risk to human health. Following contact, they can cause rapid illness in infected individuals (Sidhu and Toze 2009). There is a need for research on the survival of enteric pathogens in human waste incorporated with agricultural soil (Gerba *et al.* 2002; Horswell *et al.* 2007; Lang *et al.* 2007). There is not enough scientific data available on the decay times of bacteria and, in particular, viruses in soils and biosolids-amended soils. Some studies have examined enteric bacterial survival in soil, sewage sludge or animal manures under field conditions (Avery *et al.* 2004; Hutchison *et al.* 2004; Holley *et al.* 2006; Horswell *et al.* 2007; Lang *et al.* 2007; Wu *et al.* 2009) and laboratory conditions (Lang and Smith 2007) but there is no standard methodology for monitoring the persistence of enteric pathogens in the field, in agricultural soil or biosolids-amended soil, so that decay times can be obtained. Furthermore, difficulties with methodology are faced by the researcher, such as seeded pathogens being lost through the soil profile via leaching of microorganisms (such as viruses and oocysts) difficult to find in the soil. Therefore, a suitable methodology was required where the sample contents could be contained in some form of chamber while remaining under the influence of environmental changes. Unfortunately, the methodology used to study decay is not the same in every study and so it is difficult to properly compare results. Hence, the validation of the technique was required.

In a few studies, some form of microcosm to contain sample contents such as polypropylene vials (Vidovic *et al.* 2007), screw-capped glass vials (Hurst *et al.* 1980), sealed plastic bags (Lang and Smith 2007) or pots and trays (Chandler and Craven 1980) have been used (refer to Sections 2.7 and 3.2). For groundwater sampling Toze *et al.* (2010) used Teflon diffusion chambers inoculated with enteric

microorganisms with appropriate membrane filters at either end to retain microorganisms. In previous work, Crute (2004) used sterile corers (modified syringes) to sample directly into the field soil. Jenkins *et al.* (1999) used sentinel chambers (25 mm long x 7 mm wide) to examine the environmental stresses on the survival potential of *Cryptosporidium parvum* oocysts in soil and animal wastes. A nylon-mesh filter was placed at the top of the chamber (60 μm) and a porous filter (0.45 μm) was placed at the base, to allow for natural interactions to occur without the leaching of microorganisms and to therefore capture environmental effects. This approach met the following objectives: to provide representative samples of soil, or amended soil, that are consistent and of manageable size for processing and analysis; to prevent environmental contamination from leaching or leakage; to ensure uniformity across replicates in order to reduce random error that occurs when biosolids clump; thus enabling samples to be placed into the environment of choice; and to maintain samples under the influence of natural environmental changes such as rainfall events, soil temperature and sunlight infiltration.

Research presented in this chapter is focused on developing and testing the methodology to be used in the overall study. This method was used to determine the decay times of bacteria and viruses introduced into agricultural soil through the land application of biosolids. The purpose of the study was to compile and then test all methodology, from seeding time (of the cereal crop and the inoculation of cultures) to the enumeration of microorganisms in the laboratory. This involved the development of an easy-to-use environmental sample container by modifying Jenkins *et al.* (1999) sentinel chamber design (designed for animal wastes) which could be used to take measurements of enteric pathogens in soil from the field. The specific objective was to compare the decay times of individual microorganisms in biosolids-amended soil and unamended soil over the timescale of a growing wheat crop (in a pot study) to determine any treatment effect, as well as to compare the decay times across microorganisms.

4.2. Materials and methods

4.2.1. Site description

The study was carried out at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) centre for Land and Water at Floreat, Western Australia (WA). A pot experiment was conducted in glasshouse facility # 48 during a typical WA wheat season from May to November 2006. Soil was collected from a farming property in Moora, WA where biosolids are currently used as a fertiliser across a regular farm cropping program. This was also the site used for the field experiments described in Chapter 6. The soil, collected from the medium slope of a wheat paddock (A26 Silverfox Map, 30º 50'24.07"S, 116º 05'18.37"E), was a gravely-loamy sand soil (Site A, Table 6-1, p. 102).

4.2.2. Pot and sentinel chamber establishment

Soil samples were taken from the topsoil (at depth of 10 cm) to fill the pots and chambers. Six TerraBoxes ™ (Planterra) plastic pots (450 mm length x 150 mm width x 100 mm deep) were established. Three pots for the biosolids-amended treatment were filled with approximately 7.60 kg of soil and the topsoil was amended with approximately 70 g of non-sterile (non-autoclaved) biosolids. The other three pots were filled with 7.65 kg of unamended soil (as the control). Biosolids to soil ratios were based on 1 litre of soil weighing approximately 1.10 kg (refer to Appendices Section 11.4.2).

Mesophilic anaerobically digested biosolids were collected from the Woodman Point wastewater treatment plant, Perth, WA. The biosolids applied to the pots had a 20% total solids content and pH 7.0 (Site A, Table 6-2, p. 103). The rate of application of biosolids in the biosolids-amended pots was 14 g per 675 cm^2 (based on 10 cm depth) which was based on 10 t ha^{-1} dry solids (DS) as equivalent to 1% biosolids to soil. Biosolids dewatered cake are currently applied in WA at rates of approximately 8 t ha^{-1} DS (LeBlanc *et al.* 2008). The biosolids-amended and non-amended pots were cultivated with a sterile spatula and then wheat (*Triticum aestivum cv.* Calingiri) was planted at 10 grains pot^{-1}, 4.5 cm apart and 2.5 cm deep (Figure 4-1). The fertilisers, diammonium phosphate and urea, were applied at 77 mg kg^{-1} and 53 mg kg^{-1}, respectively (refer to Appendices Section 11.4.6).

The microorganisms tested were *S. enterica* and bacteriophage MS2 and each was cultured as described in Section 3.3. The final suspension of *S. enterica* had a final number of approximately 1 x 10^7 cfu mL^{-1}. The final MS2 suspension was determined to have a final plaque count of approximately 1 x 10^8 pfu mL^{-1}.

The sentinel chambers, constructed as described in Section 3.2, were filled with soil (either unamended or mixed with biosolids) and inoculated with a suspension of the washed cultures (*S. enterica* and MS2). Prior to filling the chambers, unamended soil was sieved (<2mm) and then split into two equal portions. One portion (385 g) was amended with biosolids at a rate equivalent to 10 t DS ha^{-1} (assuming an incorporation depth of 10 cm) to a final ratio of 113:1 (3.4 g biosolids to 382 g soil). The other control portion (385 g) was maintained in an unamended condition (refer to Appendices Section 11.4.5). Both portions were seeded with 3.5 mL of washed *S. enterica* and 3.5 mL of bacteriophage suspension to achieve a final number in the soil of approximately 1 x 10^6 cfu g^{-1} of *S. enterica* and 1 x 10^8 pfu g^{-1} of MS2. The amended and unamended soils (~2 to 5 g) were then used to fill the sentinel chambers. A total of 216 chambers (108 with unamended soil and 108 with soil amended with biosolids) were prepared. Each chamber was oriented vertically in the surface soil (to a depth of 10 cm) at the beginning of the experiment (after biosolids had been applied and wheat had been sown). Over the duration of the experiment, pots were watered to gravimetric soil water holding capacity without leaching to maintain constant soil moisture.

Figure 4-1: Pots sown to wheat (in biosolids-amended soil) with chambers placed in soil beneath.

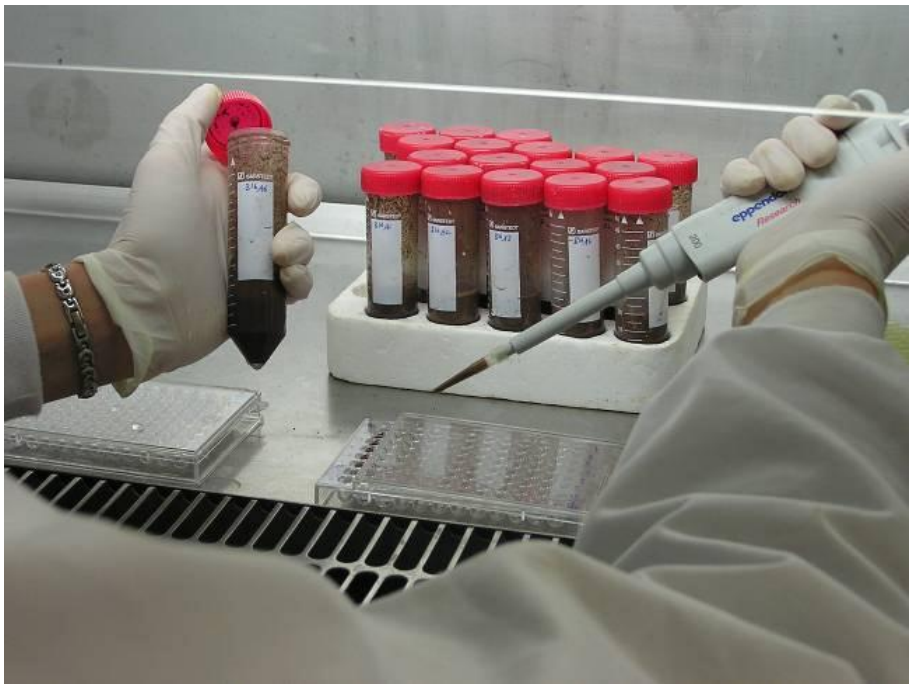


Figure 4-2: Diluting soil samples in phosphate buffer for plating

4.2.3. Glasshouse conditions

Air temperature in the glasshouse was maintained at 17˚C (± 0.25) and relative humidity at 72% (± 0.97) by an air-conditioning unit. A data logger was used to detect any changes from this temperature range.

4.2.4. Sample collection and microbial quantification

The survival experiments comprised: biosolids-amended soil (as the experimental treatment) and soil only (as the control). Sentinel chambers were collected at days 0, 5, 14 and 19. Sampling frequency was then reduced to approximately fortnightly and monthly intervals (days 34, 54, 76, 104, 133, 175 and 202) to a maximum of 7 months, or until the experimental microorganisms fell below detection limits. At each sampling event, from days 0 to 34, three chambers from three pots in both treatments were randomly selected (9 chambers per treatment). However, from day 54 to 202 (as chamber numbers decreased, but pathogens were still able to be detected) only one chamber from each block was sampled (three chambers per treatment), since only a limited number of chambers had been able to fit on the surface area of the pot. All samples were transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA for processing within 8 h.

Sample contents (~3 g) in each chamber were transferred into pre-weighed sterile polypropylene tubes (Sarstedt) and net weights were obtained. Earlier experimentation had been carried out (in this research) to determine the best sample size (1, 2.5 or 10 g) in proportion to the level of phosphate (P) buffer (9, 22.5 or 45 mL) for optimal recovery of *S. enterica*. It was found that a higher rate of recovery of microorganisms could be obtained where 1:9 proportions were used. P-buffer (pH 7.2) was added (30 mL), and the samples were vortexed for 2 min, left to settle, then vortexed again for 1 min (Keegan *et al.* 2009). A portion of the resulting supernatant above the soil was then collected and serial 10-fold dilutions were made in P-buffer (Figure 4-2). The quantification of pathogens was performed as described in Section 3.4. The cfu g^{-1} and pfu g^{-1} were calculated on a per dry soil weight basis from the original weight of the soil sampled inside the sentinel chamber.

4.3. Data analysis

Prior to statistical analysis, pathogen counts were normalised from the raw data as described in Section 3.5.

4.3.1. Statistical analysis

All analyses were performed using SAS package version 9.1 (SAS Institute, 2005).

4.3.1.1 Analysis of variation sources for decay of individual microorganisms

The mixed effect model analysis of variance (ANOVA) was used to identify significant variation sources affecting final pathogen counts ($\log_{10}$ *Count*) in individual experiments. The variation sources included the fixed effects (treatment, linear term of a covariate - sampling date, and their interactions) and random effects (block nested within the treatment and then the chamber nested within the block). The following equation was used (Equation 2):

Equation 2

$$Log_{10}Y_{ijkln} = \mu + T_i + S_j + (TS)_{ij} + C_l(TB)_{ik} + e_{ijkln} \quad \textbf{(2)}$$

where, $Log_{10}Y_{ijkl}$ is the observation of the nth individual; μ is the overall mean; T_i is the fixed effect of the treatment (i =1, 2 corresponding to biosolids and nil, respectively); S_j is a covariate (regression coefficient) representing the fixed effect of the sampling date (j= 5, 14 …175); $(TS)_{ij}$ is the interaction between the ith treatment by the kth sampling date, representing the specific decay rate for either treatment; $C_l(TB)_{ik}$ is the random effect of the lth chamber (l=1, 2, 3) nested within the ith treatment and the kth block; and e_{ijkln} is the residual error of the nth individual.

The variance and covariance estimates for random and fixed effects, and the least-square effects of all the fixed factor comparisons were then produced. The regression coefficient of sampling date was used as the pathogen inactivation rate, or decay rate, where the time to a one $\log_{10}$ reduction of numbers (T_{90} value) was calculated using Equation 5 in Section 5.3.1 (p. 85) based on the decay slope (sdate). Based on the decay rate, the predicted model for individual pathogen survival patterns was also established for either ‘nil’ or ‘biosolids’ treatment.

4.3.1.2 Comparison of decay rates across all microorganisms

A mixed model was formulated to examine the general decay patterns of treatments by comparing decay effects within trial across individual microorganisms as follows (Equation 3):

Equation 3

$$Log_{10}Y_{ijklmn} = \mu + O_m + T_i + S_j + (OT)_{im} + (OS)_{jm} + (OTS)_{mij} + C_l\,(OTB)_{mik} + e_{ijklmn} \quad \textbf{(3)}$$

where, $Log_{10}Y_{ijklmn}$ is the observation of the *n*th individual; μ is the overall mean; O*m* is the fixed effect of the organism (*m* = *Salmonella*, phage); T_i is the fixed effect of the treatment (*i* =1, 2 corresponding to biosolids and nil, respectively); S_j is the fixed effect of the sampling date (*j*=0, 5, …202); $(OT)_{mi}$ is the interaction between the *m*th organism by the *i*th treatment; $(TS)_{ij}$ is the interaction effects of the *i*th treatment by the *j*th sampling date; $(OTS)_{mij}$ is the interaction effects of the *m*th organism by the *i*th treatment by the *j*th sampling date; $C_l\,(OTB)_{mik}$ is the random effects of the *l*th chamber (*l*=1, 2, 3) nested within the *m*th organism, the *i*th treatment and the *k*th block; and e_{ijklmn} is the residual error of the *n*th individual.

4.4. Results

4.4.1. Survival patterns of individual microorganisms

Changes in *S. enterica* and MS2 numbers in the sentinel chambers in the biosolids-amended soil and unamended soil are presented in Figure 4-3 and the decay times of these microorganisms are presented in Table 4-1. Further statistical results are available in the Appendices Section 11.4. The error bars in the following figures represent the standard deviation between the means of three replicates.

Over the first 54 d, *S. enterica* numbers decreased more than four-$\log_{10}$ and fell below detection in the biosolids-amended soil (Figure 4-3a). At day 76, *S. enterica* in both soil types was able to be detected (>1 $\log_{10}$ cfu g^{-1} dry weight soil (dw)) but fell below detection again in the biosolids-amended soil at day 133. There was no significant difference (P=0.97) between the *S. enterica* numbers in the biosolids-amended soil compared with the nil-biosolids soil since only 1% biosolids was used. The expected time for a one $\log_{10}$ reduction (T_{90}) of *S. enterica* to occur was 25 d in both soil types (Table 4-1) with no significant difference (P=0.99) between decay times.

Bacteriophage MS2 decay patterns were slower than the bacteria (*S. enterica)* and an approximate two-$\log_{10}$ loss occurred over the first 131 d of the experiment (Figure 4-3b). After day 131, the decay rate was observed to increase such that MS2 was unable to be detected in both soil types. There was no significant difference (P=0.24) in the bacteriophage numbers in the biosolids-amended soil compared with the nil-biosolids soil and no significant difference (P=0.41) between decay rates in both soil types. The estimated T_{90} decay times were 29 d in the biosolids-amended soil and 31 d in the nil-biosolids soil (Table 4-1).

When comparing the two microorganisms, using a linear decay rate, there was no significant difference (P=0.73) between treatments and no significant difference (P=0.67) between the decay rates in both treatments.

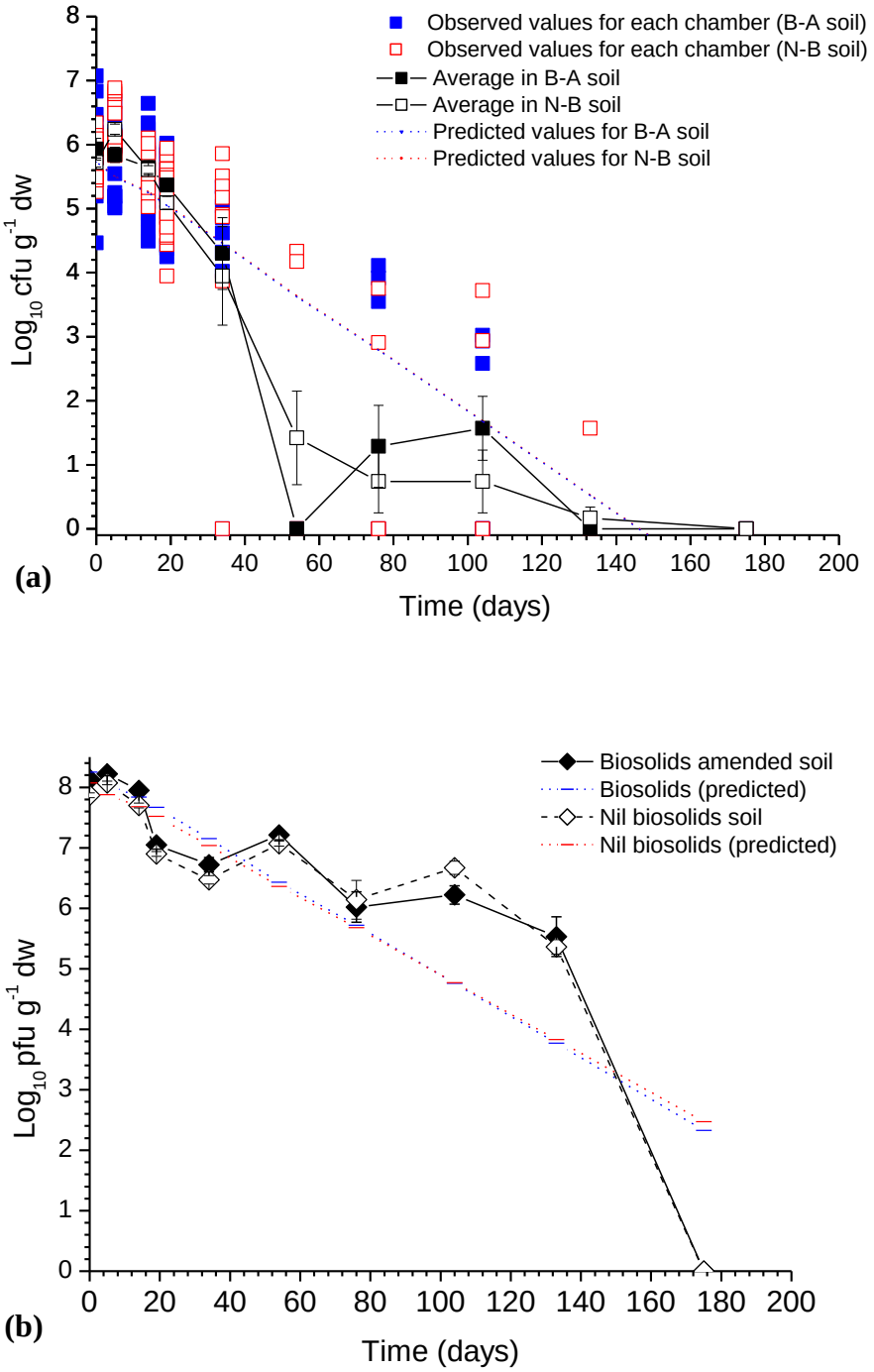


Figure 4-3: Decay of (a) *S. enterica* and (b) MS2 numbers in biosolids-amended (B-A) soil and nil-biosolids (N-B) soil with average, predicted observed values and standard error bars shown.

Table 4-1: Time for a one-log_{10} reduction (T_{90}) of *S. enterica* and MS2 to occur in biosolids-amended and unamended soil.

	T_{90} *times (days)*	
Treatment	***S. enterica***	**Bacteriophage MS2**
Biosolids-amended soil	25	29
Nil-biosolids soil	25	31

NB: The standard deviations on individual sampling events are provided in the figures.

4.5. Discussion

The main purpose of this experiment was to act as a 'trial run', to validate all methodology (i.e. the chambers, laboratory protocol and procedures, inoculation, timing of seeding and biosolids application rate) and to collect some initial data on the decay times of *S. enterica* and MS2.

4.5.1. Decay times

In this experiment, the decay times of a bacteria and a surrogate virus were studied in soil inside soil microcosms (sentinel chambers). The experiment was established to determine the rate of inactivation of the enteric microorganisms in biosolids-amended soil and unamended soils over the growing season of wheat in the form of an initial study.

The estimated time required for one $\log_{10}$ reduction of microorganisms (T^{90}) to occur was 25 d for *S. enterica* in both soil treatment types. Reduction times were slower for bacteriophage MS2 with T_{90} values of 29 d in the biosolids-amended soil and 31 d in the nil-biosolids soil. In a similar study, Crute (2004) found shorter decay times of less than 9 d for *E. coli* and enterococci seeded into biosolids-amended soil (8 and 16 t DS ha^{-1}) and nil-biosolids soil in the glasshouse. Decay times in this study were less than 14 d for MS2. However, Crute (2004) sterilised the biosolids in an autoclave before seeding them with microorganisms and incorporating them into the soil and this may have affected decay times, possibly due to the lack of predation that would have otherwise occurred between inoculated microorganisms and indigenous or other pathogens present in the biosolids. Soil corers were used to sample the soil rather than chambers and thus the microorganisms may have been reduced through leaching rather than as a result of inactivation.

Holley *et al.* (2006) reported similar reduction times (time (DRT) for 90% reduction of *Salmonella* viability) across a simulated winter-summer period in Canada. Decimal reduction times were 16 to 21 d in hog manure-amended clay soil and 4 to 5 d in unamended soil. In loamy sand soils, decay was faster with reduction times of 16 to 17 d (manure-amended) and 0.4 to 3 d (unamended). Holley *et al.* (2006) stored

soils in containers covered with perforated lids under temperature-controlled conditions to simulate the seasons. *Salmonella* was quantified using direct agar plating as similar to the present study. A reduction time of *E. coli* in moist soil amended with dewatered mesophilic anaerobically digested sludge (DMAD) was reported by Lang and Smith (2007) as 20 d in different soil types (sandy loam and silty clay). NB: this was an incubation study using controlled temperatures.

4.5.2. Reliability of sentinel chamber for pathogen survival studies

The monitoring of microorganisms in the environment may be difficult since there are many external conditions that are unable to be controlled. The reduction of microorganism numbers may be a result of leaching, washing from rainfall or binding onto soil particles. One of the purposes of this study was to develop a soil microcosm that could contain the microorganisms under examination while still allowing the exchange of gaseous substances and moisture to occur, and to therefore provide a more controlled environment from which to study microorganism inactivation in the field. No studies apart from Jenkins *et al.* (1999) report the use of sentinel chambers to study pathogen inactivation in soil. In particular, no information was available on the decay times of enteric pathogens in chambers containing biosolids-amended agricultural soil.

In the present study, a commercially-available chamber was sourced (i.e. the Microsep™ centrifugal device from PALL Life Sciences, New York, USA) suited to the dimensions and design required to act as a sentinel chamber, as described by Jenkins *et al.* (1999). Earlier experimentation had been carried out using hand-made Teflon diffusion chambers, similar to those used to monitor pathogen inactivation in water samples (Toze *et al.* 2010). However, due to the scale of the experiments designed for the present research, the diffusion chambers were too time-consuming and expensive to construct. The diffusion chambers were also difficult to assemble with soil, biosolids and microorganisms since the soil particles would prevent the screw-thread from sealing properly. This would result in the leaching of inoculated microorganisms from the diffusion chambers.

The sentinel chamber was thus a versatile tool for examining pathogen decay over time because the chambers could be ordered in large quantities, were sterile, easy to assemble at any preferred laboratory (using the soil and manures of choice), transported to the field site, inserted into the soil at the experimental site of choice and retrieved for later testing. The sealed chambers limited the potential harm to humans or livestock via leaching or at contact. Therefore the chambers resolved the difficulties often encountered with random sample collection (using cores or similar tools into the soil) by reducing the lack of uniformity, cross-contamination and potential for infectivity to those entering biosolids-application sites.

Previously, Crute (2004) used sterile corers to collect soil samples from pots and field but the decay rates reported may have been the result of leaching. Alternatively, the method of random selection of soil may not have located the biosolids-amended soil where the majority of pathogens were present. An advantage of the sentinel chamber was that the entire contents of the sample chambers could be processed so that each sampling event included all of the surviving microorganisms which could potentially be enumerated. In the field soil experiments (Chapter 6), the decay times of *E. coli* inoculated into the chambers were directly compared with *E. coli* numbers outside the chambers (topsoil). It was demonstrated that the patterns of decay was significantly correlated ($P<0.05$) between both sources of *E. coli* at both sites (Moora and Mt Compass) (Figures 6-20 and 6-21, p. 126). This shows that the chambers were suitable environmental microcosms for monitoring microorganism decay patterns.

The conditions inside the sentinel chambers were expected to be in equilibrium with the external environment, as rapid moisture and gaseous exchange across the membranes can occur. From the field climatic data (Chapter 6) it can be seen that the moisture content inside the chambers responded according to the changes in moisture across the duration of the season (Figures 6-10, 6-11, 6-14 and 6-15). These changes were significantly correlated in the topsoil at Site B ($P=0.001$) and in the chambers, thus indicating that moisture and gaseous exchange did occur within the chambers.

Jenkins *et al.* (1999) found that factors other than temperature affected *Cryptosporidium* oocyst survival, and that the chambers exposed the oocysts to ambient environmental stresses. In the present study, a wheat crop was sown in pots to represent the environment where biosolids are applied. This was done because shading from the plant could influence soil temperature, and moisture uptake by the plant could reduce soil moisture levels and thus influence pathogen decay. Similarly, biosolids were applied to the topsoil, even if not directly sampled into, to represent the environment where biosolids are applied to cereal crops. The influence of environmental factors (on the seeded microorganisms inside the chambers) was expected to be similar to the soil in the pots and thus the sentinel chambers were designed to capture any changes to the external environment.

4.5.3. The development of the methodology

The methodology used to process the soil and biosolids samples was based on the microbiology protocol and procedures commonly used by CSIRO Land and Water for water samples. Some earlier work had been conducted to enable soil and biosolids samples to be processed in this manner (Crute 2004); however, further experimentation was required to reduce background flora and improve colony visibility on plates of isolation media. This included laboratory experimentation to determine the levels of P-buffer to add to samples for optimal microorganism recovery, heat treatments, antibiotics, centrifugation, optimal sample size, testing of chambers and the use of CHROMagar to confirm *S. enterica* colonies. The resulting methodology described in Chapter 3 was a combination of successful procedures along with processes that other researchers have found to improve microorganism recovery from manure, biosolids and soil samples.

As previously discussed, other studies commonly use MPN's (based on international standards and guidelines), sampled directly into the soil or amended soils (biosolids or animal manure) along with enrichment steps, so that the length of time that enteric microorganisms can be detected in the soil is determined. The differences between the methods used in the present study and the methods used by other researchers are as follows:

- The inoculation of indicator and pathogens (for higher starting numbers) was used;
- The microorganisms were cultured on selective agars (for quantification rather than presence or absence);
- The chambers were used to contain all the inoculated and environmental microorganisms;
- Biosolids were used rather than animal manures;
- The methods were designed for field experimentation rather than just for laboratory or glasshouse experimentation;
- Decay slopes were used to calculate T_{90} decay values; and
- The decay times were estimated using a quadratic model rather than a linear model.

The following benefits of the methodology were realised:

- This method was suited to high numbers of microorganisms, i.e. in particular immediately following release of the biosolids from the wastewater treatment plant;
- The method was suited to processing large volumes of samples from several experimental/testing sites and therefore compilation and comparison of data from several locations was made possible;
- The use of selective agar reduced the formation of indigenous and presumptive colonies, thus enabling the study microorganisms to be determined without further time-consuming identification steps (e.g. dark violet colonies for *E. coli*);

The following disadvantages of the method were acknowledged:

- More variation in standard error occurred where microorganism numbers were at lower numbers i.e. below 10^1;
- Direct plating (without an enrichment step) may not result in all available colonies growing on the plates e.g. viable but non-culturable cells; however, since this method quantifies microorganisms using the relevant dilution (e.g. 10^1, 10^2 or 10^3) the corresponding logarithm is determined as accurately as possible within the appropriate range (e.g. 1-100, 101-999, 1000-9999 etc.);
- The quantification of microorganisms from individual sentinel chambers did not enable decay slopes to be developed from the same chamber across the duration of the experiment. In order to achieve this, other sample types such as lysimeters should be trialled.

The testing of the overall methodology involved determining the best sampling frequency based on the rate of decline of seeded microorganisms in the soil. This was important when later designing the field experiments so that samples from two locations could be processed at the same laboratory (for uniformity of method). Experimentation was also carried out with the inoculant levels to be added to the soil and biosolids. This was done to provide high enough numbers so that decay slopes could be analysed over a timescale of >6 months. The application rate of biosolids was equivalent to what would normally be applied in the field. Results from this initial experiment demonstrated that the rate of 1% used was not high enough to show any treatment effect that the biosolids may have had on the survival times of the enteric pathogens in the soil.

4.5.4. Need for appropriate statistical model to determine decay times

When plotting the data from this initial experiment, it was realised that the fit of linear regression lines to the observed data had several limitations. Figure 4-3a demonstrates the limitations associated with the linear model by showing the poor goodness-of-fit that can occur when trying to place a linear line-of-fit through the observed data points. The plotting of *S. enterica* numbers in the soil over time resulted in a regression line that moved towards the X-axis a lot earlier than was represented by the scatter points. The occurrence of zero numbers around day 54 resulted in drawing down of the average and thus the decay patterns of the

microorganism may not have been properly represented. Similarly, the general pattern of decay of bacteriophage (Figure 4-3b) was consistently steady to day 133, but the decay rate then increased to day 175 where no bacteriophage were detected. The linear regression used was influenced by this change in decay rate such that it overestimated decay up to day 133 and underestimated the decay from days 133 to 175. This demonstrates the importance of using the correct model to provide an accurate fit of data. It is probable that the use of non-linear models to solve the equations would provide a better line-of-fit. Currently, linear regression (using a broken-stick) is commonly used to derive decay times (T_{90}) but as demonstrated, it is not suited to non-linear data. Ideally, a model using quadratic and cubic equations would provide a better goodness-of-fit and this alternative has been examined in Chapter 5.

4.5.5. The treatment effect of adding biosolids to soil

Under glasshouse conditions, the addition of biosolids to the soil at a rate equivalent to what is applied in the field had no significant impact on the decay times of study microorganisms. This study was undertaken to gain initial data on the effect of adding biosolids to soil on the survival times of enteric pathogens. This was important since one of the main concerns when applying biosolids to agricultural soil is the risk that enteric pathogens will transfer from the soil to humans or livestock and cause disease. It was expected that the addition of biosolids to soil would increase the persistence of the microorganisms introduced into the soil since biosolids are thought to provide a protective effect for microorganisms in the soil (Eamens *et al.* 2006); however, the rate of biosolids to soil, although equivalent to what is used in the field (1%), was not high enough to influence the decay patterns.

In a similar experiment conducted in a glasshouse, decay times were reported to be longer using a biosolids application rate of 16 t DS ha^{-1} for *E. coli* (T_{90}=5 d), enterococci (T_{90}=7 d) and MS2 (T_{90}=30 and then 4 d) compared with an application rate of 8 t DS ha^{-1} where T_{90} decay times were 2 and then 13 d for *E. coli*, 5 d for enterococci and 11 d for MS2 (Crute 2004; Crute *et al.* 2005); however, this difference was not significant. Holley *et al.* (2006) reported that manure application enhanced the survival of *Salmonella* in soil. Lang and Smith (2007) found that higher removal rates of *E. coli* occurred in sludge-amended soil, although this was clearly

related to moist soils as their removal rates were significantly reduced in amended soils that were air-dried. Holley *et al.* (2006) also found that *Salmonella* survived better where the soil moisture content was higher. In previously published literature (Crute *et al.* 2005; Holley *et al.* 2006; Lang *et al.* 2007; Lang and Smith 2007), the decline of microorganisms over time was attributed to the effects of temperature, soil type, moisture and the addition of sludge (Holley *et al.* 2006; Lang and Smith 2007). The reduction of pathogens in sludge-amended soils has also been related to soil biota (Lang and Smith 2007) and the input of organic substrate from sludge, stimulating the activity of predatory and competing soil flora (Lang *et al.* 2007). More work is required to determine the effect of different per cent solids of sludge on decay times in the soil.

4.6. Conclusions

The findings from this chapter are summarised below:

- The experiment reported in this chapter was designed to validate all methodology processes for robustness for their ability to be replicated;
- Standard microbiology protocols and procedures commonly used for water samples were adapted for use on soil and biosolids samples;
- The addition of biosolids to the soil, at a rate equivalent to what would be applied in the field (i.e. 1%), did not result in an increased persistence of the enteric microorganisms tested;
- The decay time (T_{90}) of *S. enterica* in both soil types was 25 d. MS2 had slightly longer decay times of 29 d in the biosolids-amended soil and 31 d in the unamended soil;
- The sentinel chambers were determined to be suitable microcosms to contain sample contents without the loss of microorganisms from the soil profile. In addition, they reduced the risk of contamination, particularly where harmful pathogens may be used;
- The chambers were easy to assemble, sample and process and therefore would be suited for use in the field. Other benefits were the commercial availability of the chambers (Microsep™ centrifugal devices) and the filters (Eppendorf® Safe-Lock®) thus overcoming other chamber deficiencies;
- The resulting methodology was a successful combination of methods to suit biosolids and soil testing, and the experiment demonstrated that the methods were reliable;
- The data plotted in this chapter demonstrated the limitations associated with fitting linear regression lines to microbial numbers observed over time. For this reason the use of more terms, such as quadratic or cubic, has been explored in Chapter 5.

CHAPTER 5 THREE STATISTICAL MODELS TO ESTIMATE THE DECAY TIMES OF ENTERIC MICROORGANISMS

5.1. Introduction

The decay times of enteric pathogens in water and biosolids have been commonly estimated using a simple linear equation (Chandler and Craven 1980; Gordon and Toze 2003; Holley *et al.* 2006; Sidhu *et al.* 2008). T_{90} values, also known as decimal decay rates or decimal reduction times (DRT), are estimated by the linear regression analysis from the reciprocal of the slope using $\log_{10}$ values plotted against time. It is common that the survival patterns of microorganisms are rarely completely linear when plotted across a scale of time (Sidhu *et al.* 2008). This usually means that the goodness-of-fit of the linear regression lines are often poor and R-square values (indicating the power of a model fitting) are so low (eg. $R^2<0.65$) that no meaningful trend line can be fitted to the results (Hutchison *et al.* 2004). In order to place a better line-of-fit to the data, often two or three lines are required (commonly known as the broken-stick model) thus resulting in more than one T_{90} value or decay time. Therefore, there is potential for developing a more accurate statistical method for estimating T_{90} values and this was explored in this chapter.

The purpose of the research reported in this chapter was to compare the powers of three statistical models (linear, quadratic and cubic models) in estimation of decay times. The specific objective was to compare the output from these models in order to select the most practical and accurate solution for use in the field studies. Output data comparisons included ANOVA outcomes, the R-squared values, the accuracy of the predicted values to correctly represent the plotted data ($\log_{10}$ values), and the variation of decay times across the three models, as well as the practicality and efficiency of using the models to derive the desired outputs.

5.2. Materials and methods

5.2.1. Source of data

The input data used for the purpose of this chapter was derived from the data reported in Chapter 6 from *E. coli* numbers quantified in the biosolids-amended soil collected at Site A, Moora WA in 2006 (Section 6.2.1, p. 99). The data from this site, already normalised as described in Section 3.5, was selected for illustrative purposes only.

5.3. Statistical analysis

The least-square effects of all the fixed factor comparisons were produced and the regression coefficients of sampling date (linear, quadratic and cubic terms) within each treatment were used as the indication of pathogen inactivation rate or decay time. Based on the decay times and intercept of the final model, the predicted equation for individual pathogen survival patterns was established for either 'biosolids' or 'nil' treatment. Decay times (T_{90} d), or time for a one $\log_{10}$ reduction of microorganism numbers to occur, were then estimated by solving the linear, quadratic and cubic equations. All analyses were performed using SAS version 9.1 (SAS Institute, 2005).

5.3.1. The linear model

The generalised linear model analysis of variance (ANOVA) was conducted using SAS (version 9.1) to identify significant variation sources affecting final pathogen counts ($\log_{10}$ *Count*) in individual experiments. These variation sources included the fixed effects of trial, treatment, a linear term of a covariate – sampling date, the interactions between sampling date with trial and treatment. The statistical linear model can be written simply as $\gamma = \alpha + b\chi$ or the following (Equation 4):

Equation 4

$$Log_{10}Y_{ijkln} = \mu + T_i + S_j + (TS)_{ij} + (TB)_{ik} + C_l\,(TB)_{ik} + e_{ijkln} \quad \textbf{(4)}$$

where, $Log_{10}Y_{ijkln}$ is the observation of the *n*th individual; μ is the overall mean; T*i* is the effect of the treatment (*i* =1, 2 corresponding to biosolids and nil, respectively); S_j is a regression coefficient referring to the linear decay effect of the sampling date (*j*=14, 28 …181); $(TS)_{ij}$ is the interaction between the treatment by the *j*th sampling date (also a regression coefficient) representing the specific decay rate for either treatment; $(TB)_{ik}$ is the effect of the *k*th block (*k*=A, B, C) nested within the treatment; $C_l\,(TB)_{ik}$ is the effect of the *l*th chamber (*l*=1, 2, 3) nested within the *i*th treatment and the *k*th block; and e_{ijkln} is the residual error of the *n*th individual.

Based on the regression coefficients of corresponding terms in the above linear model (S and TS), where sample date was involved for either experiment, the time to a one $\log_{10}$ reduction (d) of numbers (T_{90} value) was calculated based on the decay slope (sdate) according to the following formula (Equation 5):

Equation 5

$$\chi = 1/\,b \quad \textbf{(5)}$$

where, χ is the expected number of days for one log reduction of microorganisms to occur; *b* is the derived decay rate for either treatment (i.e. "nil-biosolids" or "biosolids-amended") from the linear model. The decay times were calculated in Microsoft ® Excel.

5.3.2. The quadratic model

The quadratic model was applied to study non-linear curvature of the relationship between sampling timeline and final pathogen count. The difference between a linear model and a quadratic model was that the squared terms of the regression coefficient of the sampling date were added to the quadratic model. The statistical quadratic model can be written simply as $\gamma = \alpha + b\chi + c\chi^2$ or by the following equation (Equation 6):

Equation 6

$$Log_{10}Y_{ijkln} = \mu + T_i + S_j + (TS)_{ij} + S^2_j + (TS^2)_{ij} + S^3_j + (TS^3)_{ij} + (TB)_{ik} + C_l\,(TB)_{ik} + e_{ijkln} \quad \textbf{(6)}$$

Most of the variables in the quadratic model were the same as in the linear model, except S^2 (the quadratic terms of the sampling date), $(TS^2)_{ij}$ (the interaction between the *i*th treatment by the quadratic terms of the *j*th sampling date), S^3_j (the interaction between the linear and quadratic term of the *j*th sampling date) and $(TS^3)_{ij}$ (the interaction between the *i*th treatment and the interaction between the linear and quadratic term of the *j*th sampling date (decay rate).

Based on the regression coefficients of corresponding terms in the quadratic model, the time taken for a one $\log_{10}$ reduction (T_{90} days) of pathogen numbers was estimated with the following formulae (Equation 7):

Equation 7:

$$c\chi^3 + b\chi^2 + a\chi + 1 = 0 \quad \textbf{(7)}$$

where, χ is the expected number of days for one log reduction of microorganisms to occur; *a*, *b* and *c* were the least square effects of cubic, quadratic and linear decay rates from the quadratic model. These were derived from the ANOVA results where the variables "sdate*treatment", "sdate*sdate*treatment" and "sdate*sdate*sdate*treatment" were fitted for each individual treatment (i.e. "biosolids" or "nil"). The decay times (χ) were determined using the following website for cubic solvent: http://www.1728.com/cubic.htm.

5.3.3. The cubic model

The cubic model aimed to further improve the pathogen count variation explained by various factors in the experiments. This included higher order of the linear decay rate in the linear model by following the basic idea of mathematic Taylor's theorem. The cubic statistical model can be written simply as $\gamma = \alpha + b\chi + c\chi^2 + d\ \chi^3$ or by the following equation (Equation 8):

Equation 8

$$Log_{10}Y_{ijkln} = \mu + T_i + S_j + (TS)_{ij} + S^2_j + (TS^2)_{ij} + S^3_j + (TS^3)_{ij} + S^4_j + (TS^4)_{ij} + S^5_j + (TS^5)_{ij} + S^6_j + (TS^6)_{ij} + (TB)_{ik} + C_l\,(TB)_{ik} + e_{ijkln} \quad \textbf{(8)}$$

The cubic model was similar to the quadratic model except higher orders of regression variables (inactivation time) and their interactions with other fixed effects were fitted. These included S^4_j (the quartic terms of the sampling date), and the interactions with other variables such as $(TS^4)_{ij}$ (the interaction between the *i*th treatment by the quartic terms of the *j*th sampling date), S^5_j (the quintic terms of the sampling date), $(TS^5)_{ij}$ (the interaction between the *i*th treatment by the quintic terms of the *j*th sampling date), S^6_j (the sextic terms of the sampling date) and $(TS^6)_{ij}$ (the interaction between the *i*th treatment by the sextic terms of the *j*th sampling date).

For the cubic model, the time taken for a one $\log_{10}$ reduction (days) of initial pathogen count was derived by solving the following quintic equation (Equation 9):

Equation 9:

$$a\chi^5 + b\chi^4 + c\,\chi^3 + d\,\chi^2 + e\,\chi + 1 = 0 \quad \textbf{(9)}$$

where, χ is the expected number of days for one log reduction of microorganisms (T_{90}) to occur; the regression coefficients *a, b, c, d* and *e* are the corresponding quintic, quartic, cubic, quadratic and linear decay rates respectively for either "nil" or "biosolids" treatment. The estimated values of these terms were extracted from the least square effects of fixed effects tables of ANOVA for "sdate", "sdate*sdate", "sdate*sdate*sdate", "sdate*sdate*sdate*sdate" and "sdate*sdate*sdate*sdate*sdate" for the individual treatment (i.e. "biosolids" or

"nil"). The value 1 corresponds to the required one log reduction of microorganisms. The following website was used to determine the χ values http://www.freewebs.com/brianjs/ultimateequationsolver.htm.

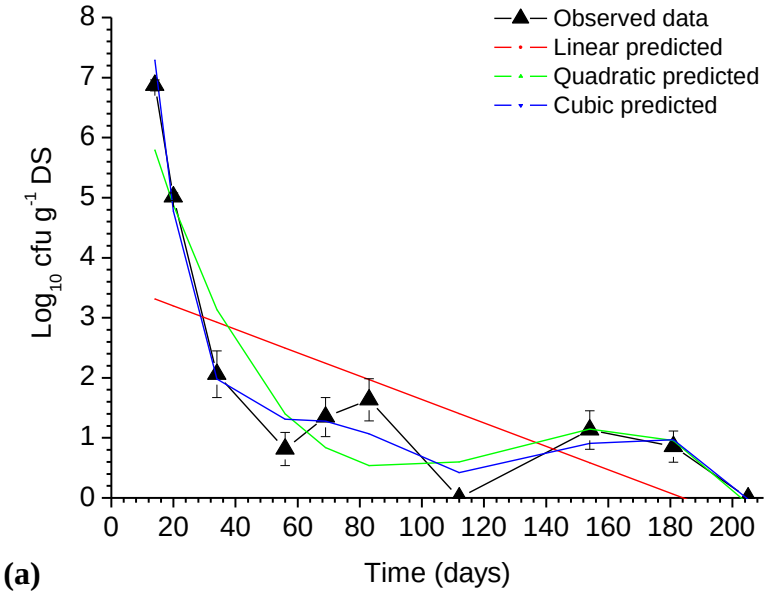
5.4. Results

5.4.1. Comparison of ANOVA model fits

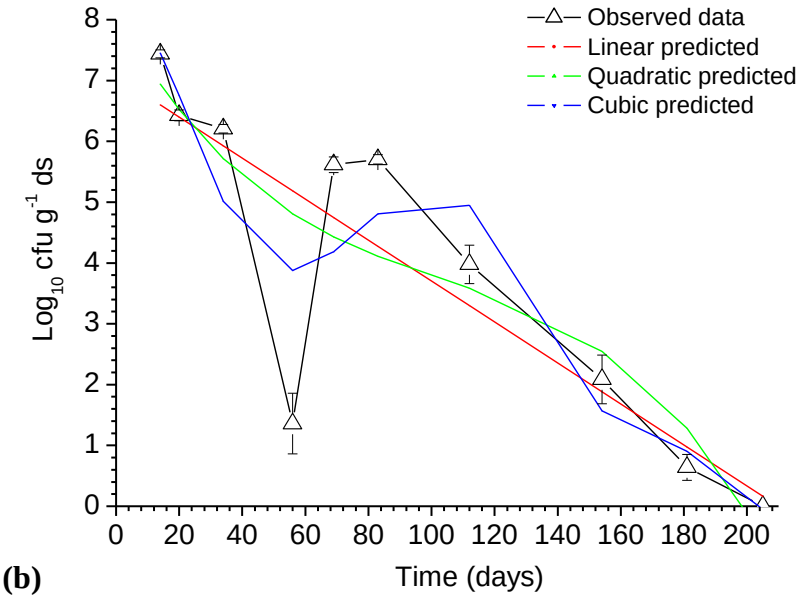
The predicted pathogen values for the linear, quadratic and cubic models plotted against the observed sample data ($\log_{10}$ *Count*) are presented for biosolids-amended soil and nil-biosolids soil in Figure 5-1. The error bars in the following figures represent the standard deviation between the means of three replicates.

An increased improvement of fit from the linear model to the cubic model can be seen. The linear predicted values passed in a straight line through the middle of the observed data points and the ANOVA model had an R-square value (goodness-of-fit) of 57% (Table 5-1). However, the linear model for the biosolids-amended soil grossly underestimated the observed values at the first data point and overestimated the following values (apart from at day 80) from days 30 to 150 (Figure 5-1a). The quadratic predicted values followed more closely the same trend as the observed data and the model had a better goodness-of-fit of 66% than the linear model (Table 5-2). The cubic predicted values closely followed the direction of the observed data and the model had the best R-square value of 72% (Table 5-3).

The treatment effect varied across the three models. Using the linear and cubic models, the difference between the sample data in the biosolids-amended soil compared with the nil-biosolids soil (treatment effect) was highly significant ($P<0.001$), however using the quadratic model of ANOVA there was no significant difference ($P=0.65$) between treatments. In all models, the block and chamber differences had no significant effects on final pathogen counts.



(a)



(b)

Figure 5-1: Predicted values from the linear, quadratic and cubic models plotted against observed sample data (with SE bars) in (a) biosolids-amended soil and (b) nil-biosolids soil

Table 5-1: ANOVA for fixed effects on pathogen count (Log numbers) using linear model.

Source	df	Type III SS	Mean Square	F Value	Pr > F
Treatment (± biosolids)	1	491.58	491.58	140.03	<0.001
Sdate[1]	1	1507.23	1507.23	429.34	<0.001
Sdate*treatment	1	107.98	107.98	30.76	<0.001
Block[2] (treatment)	4	6.22	1.55	0.44	0.778
Chamber[3] (block*treatm)	12	32.29	2.69	0.77	0.685
Error	504	1769.34	3.51		
Corrected total	523	4136.14	2114.55		
R-square	0.57				

[1] Sdate refers to sampling date
[2] Block is for each plot replicate (i.e. A, B or C)
[3] Chamber refers to each individual sample collected

Table 5-2: ANOVA for fixed effects on pathogen count (Log numbers) using the quadratic model.

Source	df	Type III SS	Mean Square	F Value	Pr > F
Treatment (± biosolids)	1	0.58	0.58	0.21	0.6479
Sdate	1	363.43	363.43	130.95	<0.001
Sdate*treatment	1	55.18	55.18	19.88	<0.001
Sdate*sdate	1	205.10	205.10	73.90	<0.001
Sdate*sdate*treatment	1	36.01	36.01	12.97	0.003
Sdate*sdate*sdate	1	167.43	167.43	60.32	<0.001
Sdate*sdate*sdate*treatment	1	17.96	17.96	6.47	0.011
Block (treatment)	4	4.97	1.24	0.45	0.774
Chamber (block*treatment)	12	33.73	2.81	1.01	0.435
Error	500	1387.71	2.78		
Corrected total	523	4136.14	852.51		
R-square value	0.66				

Sdate*sdate = S^2 Sdate*sdate*sdate = S^3

Table 5-3: ANOVA for fixed effects on pathogen count (Log numbers) using the cubic model.

Source	df	Type III SS	Mean Square	F Value	Pr > F
Treatment (± biosolids)	1	38.34	38.34	16.25	<0.001
Sdate	1	32.81	32.81	13.91	0.002
Sdate*treatment	1	50.70	50.70	21.49	<0.001
Sdate*sdate	1	7.69	7.69	3.26	0.072
Sdate*sdate*treatment	1	49.05	49.05	20.79	<0.001
Sdate*sdate*sdate	1	1.04	1.04	0.44	0.507
Sdate*sdate*sdate*treatment	1	48.69	48.69	20.64	<0.001
Sdate*sdate*sdate*sdate	1	0.01	0.01	0.00	0.951
Sdate*sdate*sdate*sdate*treatment	1	47.22	47.22	20.01	<0.001
Sdate*sdate*sdate*sdate*sdate	1	0.66	0.66	0.28	0.596
Sdate*sdate*sdate*sdate*sdate*treatment	1	44.50	44.50	18.86	<0.001
Sdate*sdate*sdate*sdate*sdate*sdate	1	1.68	1.68	0.71	0.399
Sdate*sdate*sdate*sdate*sdate*sdate*treatment	1	41.15	41.15	17.44	<0.001
Block (treatment)	4	4.34	1.08	0.46	0.765
Chamber (block*treatment)	12	33.99	2.83	1.20	0.279
Error	494	1165.53	2.36		
Corrected total	523	4136.14	369.83		
R-square	0.72				

Sdate*sdate = S^2 sdate*sdate*sdate*sdate = S^4 sdate*sdate*sdate*sdate*sdate*sdate = S^6
Sdate*sdate*sdate = S^3 sdate*sdate*sdate*sdate*sdate = S^5

5.4.2. Comparison of T_{90} values between models

The decay time estimates from the three statistical models is presented below in Table 5-4. Using the linear model, the estimated decay times (T_{90}) for the sample data in biosolids-amended soil was 51 d and 30 d in the nil-biosolids soil. Using the quadratic model, the decay times were much shorter with 5 d calculated for the biosolids-amended soil and 12 d in the nil-biosolids soil. Using the cubic model, T_{90} decay times were calculated to be 71 d in the biosolids-amended soil and 21 d in the nil-biosolids soil.

Table 5-4: Estimation of decay times (T_{90}) for sample data using linear, quadratic and cubic equation.

Statistical model	Biosolids-amended soil (T_{90} d)	Nil-biosolids soil (T_{90} d)
Linear	51	30
Quadratic	5	12
Cubic	71	21

5.5. Discussion

This chapter examined the use of three statistical models to provide a more accurate representation to analyse the observed data (other than the linear model). Since most studies have used general linear models to estimate T_{90} values, D values or decimal reduction times (DRT) (Gordon and Toze 2003; Hutchison *et al.* 2004; Hutchison *et al.* 2005; Horswell *et al.* 2010; Lang and Smith 2007) it was decided that statistical models with further terms such as quadratic and cubic should be examined and compared, to determine whether the additional models provide a better data fit and thus, more accurately estimated decay times. Sidhu *et al.* (2008) stated that while T_{90}s are useful to compare inactivation rates of enteric pathogens, the decline of pathogens is not always linear. In particular, microorganisms in environmental samples rarely, if ever, follow a linear pattern of decay and therefore plotting a linear regression line to the observed data can misrepresent true data. The effects such as treatment effect, estimated decay times (T_{90}) or other interactions may be incorrectly analysed. The need for accurate analysis of data is important for proper risk management

Commonly, the decay patterns of microorganisms are exponential or follow a 'broken-stick' pattern (i.e. varying rates of decay across the timescale observed). For this reason, the use of linear models is not always ideal and therefore not accurate enough to use for calculating decay times from non-linear data. Hutchison *et al.* (2004) also found that the variation in plotted observed data made the calculation of T_{90}s (D values) impossible because straight-line fitting with an R^2 of >0.65 was not possible for some data sets. For what appears to be a similar reason, Lang *et al.* (2007) used an exponential decay function and the Gompertz equation to describe an asymmetrical sigmoidal decay response in relation to time. From the data collected in this research, it was found that the appropriate model had to be selected for each data set, and that the model had to be refined according to the type of analysis required. Charles *et al.* (2009) used a log-likelihood method to fit decay models to data. From comparing a first-order decay model with a biphasic decay model it was found that significant differences existed between the datasets.

In the present study, the output from the ANOVA for the linear and cubic models detected a highly significant effect (in the *E. coli* numbers) between treatments (Tables 5-1 and 5-3, respectively). However, using the same data, the quadratic model determined that there was no significant effect between treatments (Table 5-2). In scientific research, these outcomes may be crucial and if incorrectly represented because of statistical analysis methods, could affect important results.

The decay times calculated from the three models were different, particularly those from the quadratic model. In the biosolids-amended soil, the decay times (T_{90}) for the linear and cubic models were 51 d and 71 d, respectively. Using the same data, the quadratic model estimated only 5 d. The same pattern occurred in the nil-biosolids soil where the linear and cubic models estimated longer decay times (30 and 21 d) compared with the quadratic model (12 d). Since the fit of the linear regression lines has limitations, and the cubic model returned similar output to the linear model, it was conceded that the quadratic model should be used to calculate decay times. In addition, the cubic model is more complex and time-consuming to use compared with the quadratic model.

Through fitting the higher orders of decay times and their interactions to different treatments and other environmental effects, it can be seen that the cubic and quadratic models returned a much better goodness-of-fit (R^2=0.72 and 0.66, respectively) than the linear model (R^2=0.57). The error variances were much lower in the quadratic and cubic models. A similar conclusion about the quadratic model was also drawn by Stone *et al.* (2009) where a log-quadratic model (the same concept as the quadratic model used in this study) was compared to the commonly used first-order linear model, a biphasic model (assuming a microbial population consists of two subpopulations) and Weibull model. Stone *et al.* (2009) observed that the log-quadratic model provided for a better model fit than the biphasic and Weibull models and best explained the observed microbial counts. However, the R-squared value alone cannot be used to determine the best type of equation to use.

In the present study, it can be seen that the cubic model followed the observed data the closest. However, the predicted pathogen count using the cubic model will also follow the data curvature upwards if the sampling events should go beyond the

observed experiment period (for example in the event of regrowth) as this likely explains the longer decay times. As Stone *et al.* (2009) correctly pointed out regarding the limitations of the log-quadratic model, extreme caution needs to be taken as quadratic and cubic models may be unsuitable for determining the time required to achieve higher log-reduction outside the experiment time ranges.

While some authors and regulators (e.g. food safety) still prefer to use a first-order or linear model, more studies are using nonlinear semi-logarithmic and non-log-linear models such as the biphasic and Weibull models to describe microbial inactivation. Current computing technology has continued to develop, assisting in the ability to refine statistical models to provide better solutions and useful additional information (Stone *et al.* 2009). However, further work is required to develop and test models such as the quadratic model for accuracy, practicality and application for use on microorganism inactivation data derived from environmental samples.

Following comparison of the three statistical models, the quadratic model was preferred for analysis of microorganism data from the field studies in Chapter 6. The reasons for this choice were as follows:

1. The observed data across the experimental period was mostly non-linear;
2. The quadratic model followed the observed data more closely than the linear model, although not as closely as the cubic model (which also has the ability to follow data curvature upwards);
3. Since the quadratic model followed the observed data more closely, the quadratic model better explained the observed microbial counts and thus, the decay response in relation to time;
4. By more accurately representing the observed data, the decay times could be more accurately estimated;
5. The more accurate representation of the observed data was reflected in the improved R-squared values (from linear to quadratic to cubic), although these values alone should not be used to determine the best model to use; and
6. The need to determine the time required to achieve higher log-reduction outside the experimental time range was not required, therefore the cubic model was not considered necessary.

5.6. Conclusions

The following conclusions were made from this chapter:

- The fit of linear regression lines to non-linear datasets showed limitations in the model and resulted in a variation in the output of statistical data;
- The quadratic model showed significant treatment variation compared with the linear and cubic models;
- The use of decay slopes derived from the linear model to determine decay times (T_{90}s) showed several limitations, particularly where microorganism decay patterns were non-linear;
- Despite the ability of the predicted values to closely follow the observed data, the cubic model fitted too closely to the data and this may have resulted in a poor reflection of decay times. This would particularly be an issue if microorganism numbers tested higher at certain sampling times;
- The cubic model was more complex and time-consuming compared to the quadratic model; and
- Fitting the models to the *E. coli* inactivation data showed that the quadratic model best explained the observed microbial counts and thus was the preferred solution for the data collected from the field study (Chapter 6).
- Further work on this topic should be conducted by experienced statistical technicians.

CHAPTER 6 THE EFFECT OF BIOSOLIDS ON THE DECAY TIMES OF *E. COLI, S. ENTERICA*, MS2 AND ADENOVIRUS IN AGRICULTURAL SOIL

6.1. Introduction

The treatment of human waste is a global issue with growing challenges that every country needs to manage well in order to ensure proper sanitation. Wastewater and sludge (solids) containing human excrement pose a health risk unless they are properly managed. Finding suitable end-solutions for the management of this waste is part of the challenge and for some countries has resulted in the sludge being disposed to landfill, used as a source of energy or treated and applied to land. Treated sludge is known as biosolids to distinguish it from raw sludge. Biosolids possess many beneficial qualities when land-applied as a fertiliser and soil conditioner and is therefore considered a valuable resource (LeBlanc *et al.* 2008).

Despite undergoing stabilisation, biosolids may contain residual numbers of enteric pathogens which are potentially harmful to human and livestock health following exposure (LeBlanc *et al.* 2008). There is insufficient scientific data available on the fate of enteric pathogens in sludge or biosolids-amended soils (Sorber and Moore 1987; Gerba and Smith 2005; Horswell *et al.* 2007; Lang *et al.* 2007; Sidhu and Toze 2009). This includes survival in or on the soil following land-application (Gerba and Smith 2005). In addition, most of the available data is restricted to *Salmonella* and indicator bacteria (Sorber and Moore 1987). In particular, very little information is available on other pathogens such as human viruses in biosolids or animal manures.

In this study, the decay of selected enteric bacteria and viruses was monitored in biosolids-amended and unamended agricultural soil at three field sites on two farming properties in the southern region of Australia. The purpose of the study was to investigate the addition of biosolids to soil on the decay times of *E. coli*, *S. enterica*, bacteriophage (MS2) and human adenovirus when introduced to the soil where cereal crops are grown. These four enteric microorganisms were selected based on the availability of earlier research or as examples of common enteric

viruses detected in biosolids (such as adenovirus). The specific objectives investigated were to compare the decay times of individual microorganisms in biosolids-amended soil and unamended soil, and to identify the major drivers that may affect decay, and thus influence the management of land-applied biosolids.

6.2. Materials and methods

6.2.1. Site description and preparation

Three field sites were selected in dry temperate cropping regions of southern Australia to determine the decay patterns of the selected pathogens; Sites A (30º 50'24.07"S, 116º 05'18.37"E) and B (30º 50'9.31"S, 116º 05'44.53"E) were located at Moora, WA, and Site C (35º 21'39.68"S, 138º 32'47.67"E) was located at Mt Compass, SA (Figure 6-1). The field experiments were conducted from May to December over two years, 2006 and 2008; these months being the cereal growing season in both regions. Moora is 175 km north-east of Perth, WA, with an annual rainfall of 500 mm (www.bom.gov.au). Topography was undulating with medium slope and soil type was gravelly-loamy sand. Mt Compass is 69 km south of Adelaide, SA, with an annual rainfall of 800 mm (www.bom.gov.au). Topography was undulating with gentle slope and soil type was a sandy soil. Soil (analysed through the Chemistry Centre, Perth) and biosolids characteristics (analysed through SGS Environmental Services, East Perth) for each site are presented in Tables 6-1 and 6-2, respectively. Soil pH ($CaCl_2$) was between 4 to 5, with a high sand content (87-96%) and low organic carbon (~2%). Trial plots were established for biosolids-amended soil (treatment) and nil-biosolids soil (control). Three replicate plots (each 10 m^2) were established for each treatment (to a total of six plots) using a randomised-block design (*n* = three replications).

In Western Australia, mesophilic anaerobically-digested dewatered biosolids were delivered fresh to the trial sites from Beenyup WWTP, Perth CBD (Figure 6-2). They were typically 20% solids, with high nitrogen content (77,000-78,000 mg kg^{-1}) and neutral pH (7.0-7.9) (Table 6-2). Table 6-3 shows the arithmetic mean contaminant and nutrient concentrations for Beenyup WWTP. The full summary of physico-chemical properties for Beenyup biosolids is available from the Water Corporation, Perth. Biosolids were applied to Site A at 6 t DS ha^{-1} in May 2006 and Site B at 19 t

DS ha^{-1} in May 2008 for Trials 1 and 2, respectively; with three control plots left unamended. Biosolids were incorporated into the top 10 cm of soil using a disc-seeder.

In South Australia, tertiary treated (stockpiled) biosolids were collected from Bolivar sewage treatment plant, Adelaide. Biosolids were typically dry (total solids 66%), low in nitrogen (18,000 mg kg^{-1}) with a pH of 6.7 (Table 6-2). At Mt Compass, biosolids were applied in May 2008 to three of the plots at a rate of 28 t DS ha^{-1}. Biosolids for Site B (Moora 2008) contained 1,200,000 thermotolerant coliforms and Site C (Mt Compass 2008) contained 620 thermotolerant coliforms (Table 6-2) as determined by MPN method via the analysis laboratory. Biosolids were incorporated into the top 10 cm using a rotary hoe. NB: the biosolids from the two locations (WA and SA) underwent different treatment processes and were typical of the biosolids produced for each area, as it was impractical to truck the same biosolids interstate.

Biosolids application at Site A was 1 x nitrogen limited biosolids application rate (NLBAR) according to district practice, however biosolids applications at Sites B and C were 1.5 times the NLBAR (DEP *et al.* 2002) so that the treatment effect of biosolids on pathogen survival could be examined (Crute 2004). The application rates were calculated according to soil nitrogen requirements and biosolids moisture content and therefore were specific to each site.

At Moora, wheat (*Triticum aestivum cv.* Calingiri) was sown to all plots using a disc seeder at 60 kg ha^{-1}, 18 cm row spacing and 2.5 cm depth within 2 h according to common district practice. At Mt Compass, wheat (*Triticum aestivum cv.* Clearfield Janz) was sown at 60 kg ha^{-1}, 25 cm row spacing and 2.5 cm depth within 2 h. The seeding of wheat further incorporated the biosolids with the soil as required under the land application guidelines.

Soils in the treatment and control plots were tested at the beginning of the study (after sludge application) for *E. coli* (i.e. namely site samples). Soil tested from the biosolids-amended plots contained 3 x 10^6 cfu g^{-1} of *E. coli* at Site B and 2 x 10^3 log cfu g^{-1} of *E. coli* at Site C. As part of the background test, no *E. coli* were detected in the nil-biosolids (unamended) control plots at Site B, however *E. coli* numbers were

3 x 10^3 cfu g^{-1} in the nil-biosolids plots at Site C at the beginning of the experiment. It was noted that cattle and native kangaroos had been grazing at the site up to two weeks before the experiment commenced. Faecal pathogens can be introduced into the soil in this manner (Wu *et al.* 2009). The presence of *E. coli* in the topsoil at Site A was not tested therefore this information is not available.

Figure 6-1: Field site locations in Western Australia and South Australia.

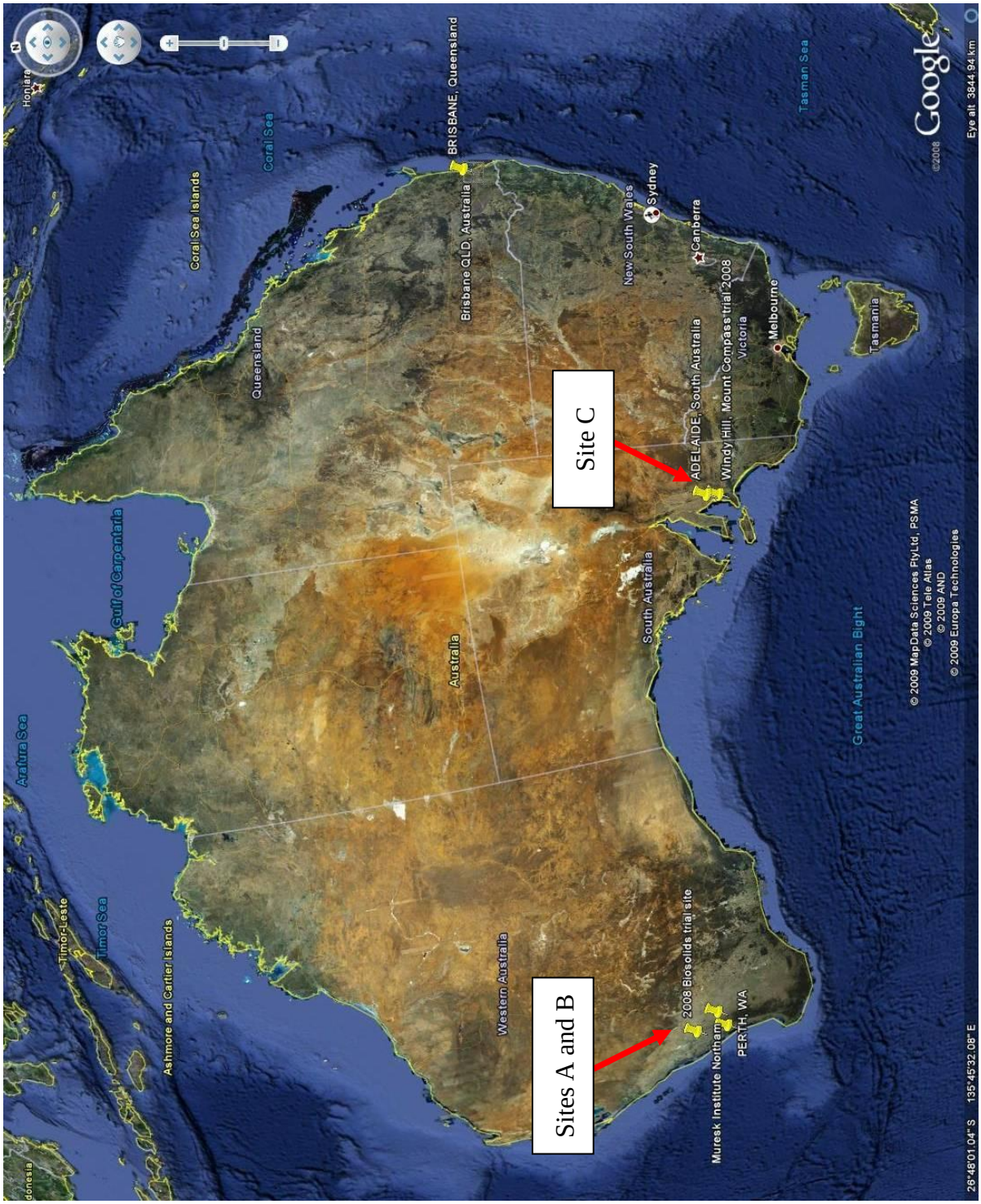


Table 6-1: Soil characteristics from three field experiments

Soil characteristic	Site A	Site B	Site C
pH ($CaCl_2$)	5.3	4.6	4.4
Sand content (%)	-#	87	96
Silt content (%)	-#	5.5	2.5
Clay content (%)	-#	7.5	1.5
Total Nitrogen (%)	0.20	0.14	0.21
Organic Carbon (% W/B)	2.76	1.90	2.54
Cation exchange capacity, NH_4Cl (me %)	8a	6	9
Total Phosphorus (mg kg^{-1})	480	150	140
Phosphorus Retention Index (mL g^{-1})	30	8	-0.7
Aluminum, AmOx (mg kg^{-1})	1800	1	<1
Iron, Fe AmOx (mg kg^{-1})	410	73	63
Exchangeable Calcium (me %)	6.13a	2.65	4.60
Exchangeable Magnesium (me %)	0.68	0.60	1.26
Exchangeable Potassium, K (me %)	0.20a	0.32	0.40
Exchangeable Sodium, Na (me %)	0.09a	0.04	0.10

Source: Chemistry Centre, East Perth, WA (refs: 05A639/1-1 and 08A7/1-5)

 # not determined, but site was within 2 km of Site B in the same paddock.

Table 6-2: Biosolids characteristics used in the three field experiments

Biosolids characteristics	**Site A**	**Site B**	**Site C**
pH units (H_2O)	7.0	7.9	6.7
Total solids (% w/w)	20.0	18.5	65.7
Total Kjeldahl Nitrogen (mg kg^{-1})	78,000	77,000	18,000
Total Phosphorus, P (mg kg^{-1})	21,000	21,000	26,000
Ammoniacal Nitrogen, NH_3-N (mg kg^{-1})	460	13,000	1200
Nitrate, NO_2 (mg kg^{-1})	1.5	<5	9000
Nitrite, NO_3 (mg kg^{-1})	0.1	<5	15
Thermotolerant coliform (CFU $g^{-}1$)	#	1.200,000	620

Source: SGS Environmental Services, Newburn, WA.

 # Results not available

Table 6-3: Mean contaminant and nutrient concentrations for Beenyup biosolids.

Nutrient and contaminant concentrations (mg kg^{-1})	**Site A - 2006 Arithmetic Mean (SD)**	**Site B - 2008 Arithmetic Mean (SD)**
Aluminium	3780.00 (±540.37)	6500.00 (±1564.18)
Arsenic	6.48 (±3.42)	2.25 (±1.27)
Cadmium	1.59 (±0.29)	1.16 (±0.22)
Total Chromium	54.67 (±12.35)	39.57 (±4.64)
Copper	1037.62 (±136.05)	963.85 (±173.76)
Lead	37.29 (±5.46)	25.21 (±3.26)
Mercury	2.99 (±2.09)	1.90 (±0.30)
Molybdenum	12.40 (±6.07)	13.50 (±1.91)
Nickel	26.00 (±3.29)	22.71 (±3.71)
Selenium	4.18 (±1.16)	4.43 (±1.22)
Zinc	701.43 (±178.17)	842.14 (±97.28)

Figure 6-2: Delivery of biosolids from Beenyup Wastewater Treatment plant to the Moora trial site, May 2008.

Figure 6-3: Inserting a sentinel chamber into the soil at Moora, WA

6.2.2. Chamber preparation and inoculation

Sentinel chambers were used in the survival experiments as a tool for monitoring the inactivation of enteric microorganisms in the field. The sentinel chambers were constructed as described in Section 3.2.

To fill the sentinel chambers, unamended soil (collected from each corresponding site) was sieved (<2 mm) and then split into two equal portions. One portion was amended with biosolids to a final ratio of 1:4 biosolids to soil for Site A (20% biosolids to 80% soil) and 1:3 for Sites B and C. Higher than normal biosolids application rates (i.e. than is usually applied in the field) were used so that treatment effect (of adding biosolids to soil) could be examined.

The second control portion was maintained in an unamended condition. Each of the portions was then inoculated with the washed *E. coli, S. enterica*, bacteriophage MS2 and adenovirus cultures. The amended and unamended soils were then used to fill the sentinel chambers. Over 440 chambers were prepared so that destructive sampling could occur throughout the experiment for both treatments: 200 chambers (100 and 100) were prepared to test pathogen decay, 120 chambers (60 and 60) were prepared to test moisture content at each sample event and 120 chambers (60 and 60) were prepared to measure the number of adenovirus genomic copies in the samples. Adenovirus was seeded into separate chambers (from *E. coli, S. enterica* and MS2) due to the different analysis methods (i.e. PCR). The microorganisms tested were *E. coli, S. enterica*, bacteriophage MS2 and adenovirus, cultured as described in Section 3.2. The final bacteria suspensions had a final cell count of more than 1×10^6 cfu mL^{-1} and the bacteriophage MS2 suspension was determined to have a final cell count of more than 1×10^7 pfu mL^{-1}. The titre for adenovirus was 10^7 pfu mL^{-1}.

Once constructed, the chambers were placed in a vertical orientation in the topsoil (at 10 cm depth) in each plot (Figure 6-3). Labelled pink tags were used to mark their location in the soil (Figure 6-4). The biosolids-amended chambers were placed in random positions in the biosolids-amended plots and the nil-biosolids chambers were placed in the nil-biosolids plots. Each of the plots contained 220 sentinel chambers at the start of the experiment.

Figure 6-4: Field tags used to mark the location of sentinel chambers under the soil in the wheat crop at Site B Moora, WA.

Figure 6-5: Humidity and soil moisture data loggers stationed at Site C Mt Compass, SA.

6.2.3. Climatic monitoring

At each site, daily air temperature and relative humidity were recorded every 20 min using a Tinytag Plus 2 (Gemini Data Loggers Ltd, UK). Soil temperature and soil moisture were recorded at hourly intervals using a Watermark Monitor (Irrometer Company Riverside, CA USA) (Figure 6-5). Rainfall was recorded every 20 min with a tipping bucket rain gauge (Davis Instruments Corp, Hayward CA USA) and Tinytag data logger (Gemini Data Loggers Ltd, UK). Soil moisture was manually determined by oven-drying (105ºC for 24 h) soil samples from the field. Moisture probes were set at the same depth as the chambers (i.e. 0-10 cm) for direct comparison.

6.2.4. Sample collection and analysis

Samples were collected at Time 0 and then every second week up until week 4. Sampling frequency was then reduced to monthly intervals up to a maximum of 7 months or until the target microorganisms fell below detection. At each sample event, three chambers were randomly selected from each of the three blocks in each treatment. Topsoil from the biosolids-amended plots, namely 'site samples', was also taken in triplicate (at 10 cm depth) to compare any changes in *E. coli* numbers outside the chambers with the *E. coli* inside the chambers (at Sites B and C in 2008 only). All samples collected from Moora were transported on ice to Floreat, Perth. Samples from Mt Compass were transported on dry ice via overnight courier for processing in Floreat to reduce potential variation that may have occurred across laboratories. All samples were delivered to the CSIRO Microbiology Laboratory, Floreat, WA and were processed within 24 h.

6.2.5. Sample processing and quantification of microorganisms

Sample contents (~2-5 g) from each chamber were transferred into pre-weighed sterile polypropylene tubes (Sarstedt), 30 mL of sterile P-buffer (pH 7.2) was added and the samples vortexed for 2 min, left to settle, then vortexed again for 1 min. A 1 mL sample of the resulting supernatant was then collected without disturbing the pellet. A serial 10-fold dilution according to expected numbers was made in the P-buffer from the supernatant, for the detection of *E. coli, S. enterica* and MS2. The quantification of each of the microorganisms was performed as described in Section

3.4. The samples containing adenovirus were stored at -80ºC and analysed all at the same time (using the same standards) to reduce variance in analysis.

6.3. Data analysis

6.3.1. Data preparation

Prior to statistical analysis, pathogen counts were normalised from the raw data and converted to log values as described in Section 3.5. The counts from Time 0 were also removed from all trials prior to statistical analyses as described in Section 3.5. Associated standard deviations, trendlines and logarithmic transformations were performed in Origin® 6.1 (OriginLab Corporation 1991-2000).

6.3.2. Statistical analysis

6.3.2.1 Analysis of variation sources for decay of individual microorganisms

The quadratic model was used to analyse the data in this chapter. The least-square ANOVA was conducted using SAS (version 9.1) to identify significant sources of variation affecting final pathogen counts ($\log_{10}$ *Count*) within individual experiments. These variation sources comprised the fixed effects (trial, treatment, linear terms of a covariate – sampling date, their interactions with trial and treatment, block and chamber). The statistical model (quadratic) was the same as described in Section 5.3.2 (Equation 6), p. 86.

The least-square effects of all the fixed factor comparisons were then produced. All statistical data is available in the Appendices Section 11.6. The regression coefficients of sampling date (quadratic terms) within each treatment were used as the indication of pathogen inactivation rate or decay time. Based on the decay times and intercept of the final model, the predicted equation for individual pathogen survival patterns was established for either 'biosolids' or 'nil' treatment.

The decay times (T_{90} d) were then estimated by solving the quadratic equations as described in Section 5.3.2 (Equation 7). All analyses were performed using SAS version 9.1 (SAS Institute, 2005).

6.3.2.2 Comparison of decay rates of all microorganisms across sites

A least-square ANOVA was conducted to identify the significant experimental interactions affecting final pathogen counts ($\log_{10}$ *Count*) across sites within individual microorganisms. The statistical model can be written as (Equation 10):

Equation 10

$$Log_{10}Y_{ijklnp} = \mu + T_i + D_p + (TD)_{ip} + S_j + (DS)_{pj} + (TS)_{ij} + (DTS)_{pij} + S^2_j + (DS^2)_{pj} + (TS^2)_{ij} + (DTS^2)_{pij} + S^3_j + (DS^3)_{pj} + (TS^3)_{ij} + (DTS^3)_{pij} + B_k (DT)_{pi} + C_l (DTB)_{pik} + e_{ijklpn} \quad \textbf{(10)}$$

where, $Log_{10}Y_{ijklnp}$ is the observation of the *n*th individual; μ is the overall mean and e_{ijklnp} is the residual error of the *n*th individual. All the other terms and their descriptions are presented in Table 6-3.

Table 6-4: Description of quadratic terms and their interactions (Equation 10).

Symbol	Description
T_i	The fixed effect of the treatment (i =1, 2 corresponding to biosolids and nil, respectively)
Dp	The fixed effect of the pth trial (p='Moora06', 'Moora08', 'Mt Compass08')
$(TD)_{ip}$	The interaction between the ith treatment and the pth trial
S_j	The fixed effect of the jth sampling date (j=14, 28, …205)
$(DS)_{pj}$	The interaction effects of the pth trial by the jth sampling date
$(TS)_{ij}$	The interaction effects of the ith treatment by the jth sampling date
$(DTS)_{pij}$	The interaction effects of the pth trial by the ith treatment by the jth sampling date
S^2_j	The quadratic terms of the jth sampling date
$(DS^2)_{pj}$	The interaction between the pth trial and the quadratic terms of the jth sampling date
$(TS^2)_{ij}$	The interaction between the ith treatment and the quadratic terms of the jth sampling date
$(DTS^2)_{pij}$	The interaction between the pth trial and the ith treatment and the quadratic terms of the jth sampling date
S^3_j	The interaction between the linear and quadratic terms of the jth sampling date (decay rate)
$(DS^3)_{pj}$	The interaction between the pth trial and the linear and quadratic terms of the jth sampling date
$(TS^3)_{ij}$	The interaction between the ith treatment and the linear and quadratic terms of the jth sampling date
$(DTS^3)_{pij}$	The interaction between the pth trial and the ith treatment and the linear and quadratic terms of the jth sampling date
B_k	The effects of the kth block (k=A, B, C) nested within the pth trial and the
$(DT)_{pi}$	ith treatment
C_l	The effects of the lth chamber (l=1, 2, 3) nested within the pth trial, the
$(DTB)_{pik}$	ith treatment and the kth block

6.3.2.3 Climatic effect

To determine the relationships between soil temperature or soil moisture change with individual decay patterns of *E. coli*, *S. enterica*, MS2 and adenovirus in both biosolids-amended soil and unamended soil, the correlations were calculated in Microsoft® Excel using the CORRE function and the significances were tested using Student *t-tests* for each experimental site. The critical *P*-value for the test was set at 0.05.

A one-tailed Student *t-test* was also applied in Excel to determine any significant difference between soil moisture levels in the chambers and in the topsoil (outside chambers).

All statistical data from the analyses of climatic effect are available in the Appendices.

6.4. Results

6.4.1. Environmental conditions

The climatic conditions during the inactivation experiments are summarised in Table 6-4 and climatic patterns for the three sites are shown in Figures 6-6 to 6-15. Average daily air temperatures ranged from 12 to 16ºC across the duration of the experiments (autumn to spring) at all sites with higher average temperatures recorded at Moora than Mt Compass. Minimum daily temperatures ranged from 7 to 10ºC with maximum daily temperatures ranging from 17 to 22ºC. Mean soil temperatures ranged from 12 to 18ºC with lower temperatures recorded at Mt Compass than Moora. Average relative humidity was 62 to 73% and average soil moisture content was 12 to 16% (based on oven-dried manual samples, not centibars). Cumulative rainfall was 262 mm and 275 mm at Moora (Sites A and B, respectively) and 328 mm at Mt Compass (Site C).

As observed from the soil moisture and rainfall conditions in Figures 6-6 and 6-7, Site A had a dry period in June 2006 at the commencement of the growing season, and then another dry period occurred from the end of September into the summer. From the moisture probes in the soil it can be seen that soil moisture in the nil-biosolids plots became drier much earlier than the biosolids-amended plots (Figure 6-7). Soil moisture content for the topsoil and inside the chambers at each sampling event were not taken at Site A, therefore this data is not available.

At Site B, a dry period occurred from August to September 2008 (Figures 6-8 and 6-9) and then again in October. Soil moisture levels from probes, topsoil and chambers in the biosolids-amended soil followed the same pattern as the moisture levels in the nil-biosolids soil (Figure 6-9, 6-10 and 6-11); however, soil moisture in the topsoil was higher at the unamended site (than in the biosolids-amended soil), and the soil moisture in the chambers was higher in the biosolids-amended soil than the unamended soil. Despite this, the changes in soil moisture in the chambers over the growing season were significantly correlated ($P<0.001$) to the soil moisture patterns

in the topsoil (outside the chambers) in the biosolids-amended and the unamended soils (Figures 6-10 and 6-11).

Soil moisture levels, from soil moisture probes taken at Site C (Figures 6-12 and 6-13), remained constant across the growing season of the wheat until October 2008, when conditions became drier up until November. Soil moisture levels from the probes in the biosolids-amended soil followed the same patterns as the moisture levels in the nil-biosolids soil (Figure 6-13). Soil moisture content outside the chambers (topsoil) followed similar patterns to the moisture content inside the chambers in both the biosolids-amended soil (Figure 6-14) and the unamended soil (Figure 6-15); however, these changes were not significantly correlated ($P<0.20$). At all three sites, soil and air temperatures increased from the winter through to the summer.

Table 6-5: Summary of seasonal parameters during field experiments

Seasonal parameter	**Site A** **Moora 2006** Arithmetic mean (SD)	**Site B** **Moora 2008** Arithmetic mean (SD)	**Site C** **Mt Compass 2008** Arithmetic mean (SD)
Mean daily air temperature (˚C)	16 (±6.4)	14 (± 3.6)	12 (± 3.7)
Minimum daily temperature (˚C)	10 (±5.0)	9 (± 3.4)	7 (± 2.7)
Maximum daily temperature (˚C)	22 (±5.8)	21 (± 5.0)	17 (± 5.4)
Mean relative humidity (%)	63 (±12.2)	73 (±14.6)	62 (±22.7)
Cumulative rainfall (mm)	262	275	328
Soil temperature (˚C)	18 (±6.5)	17 (±5.4)	12 (±3.6)
Soil moisture content (%) topsoil	*ND*	12 (±12.0)	16 (±9.7)

ND – Not determined

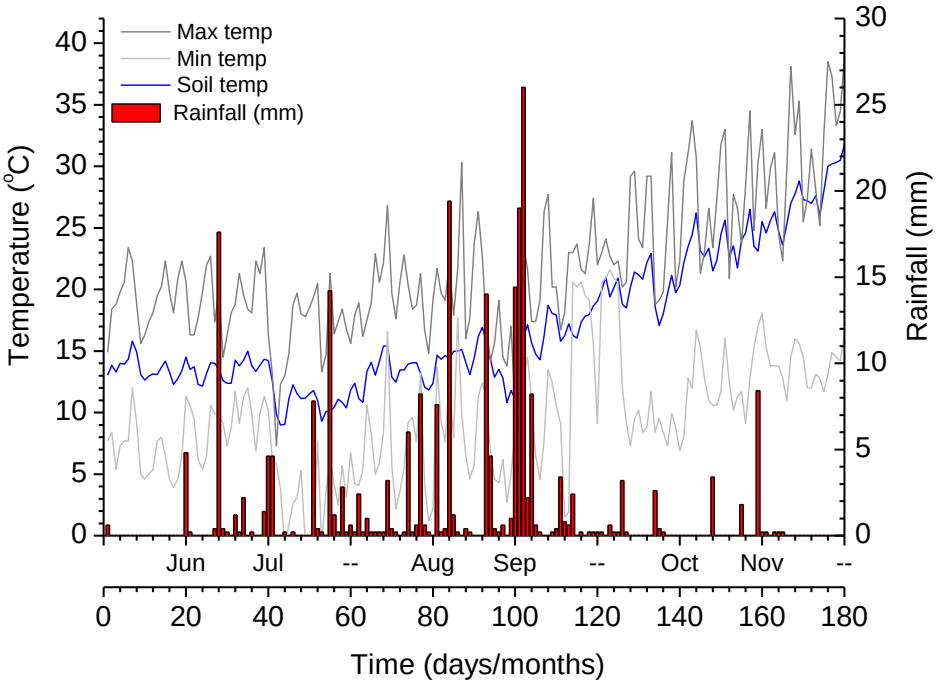


Figure 6-6: [Site A – Moora 2006] Daily rainfall and average soil and minimum and maximum air temperatures.

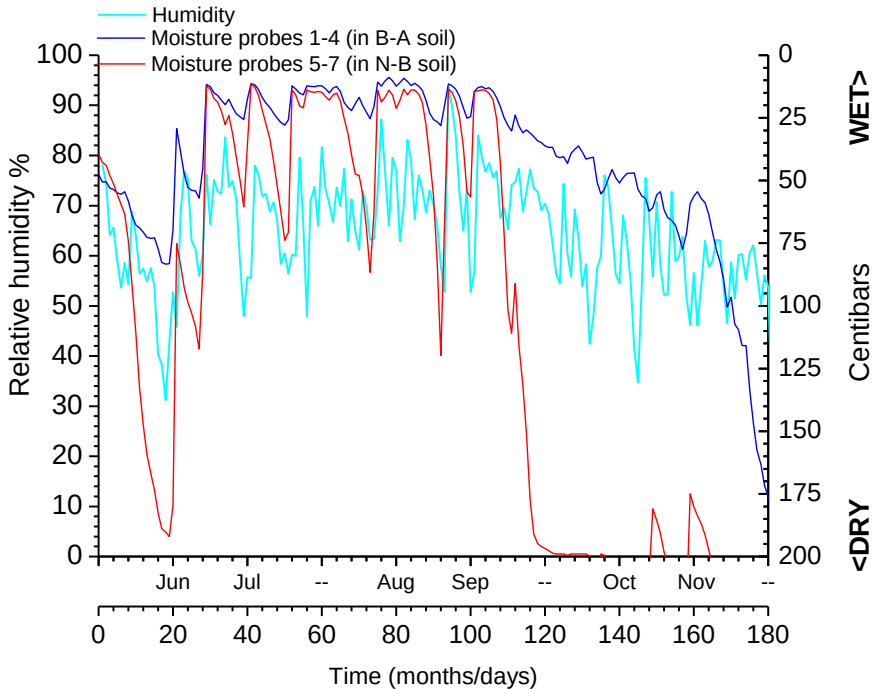


Figure 6-7: [Site A] Mean daily relative humidity (%) and soil moisture levels (kPa) from probes in biosolids-amended (B-A) and nil-biosolids (N-B) soil.

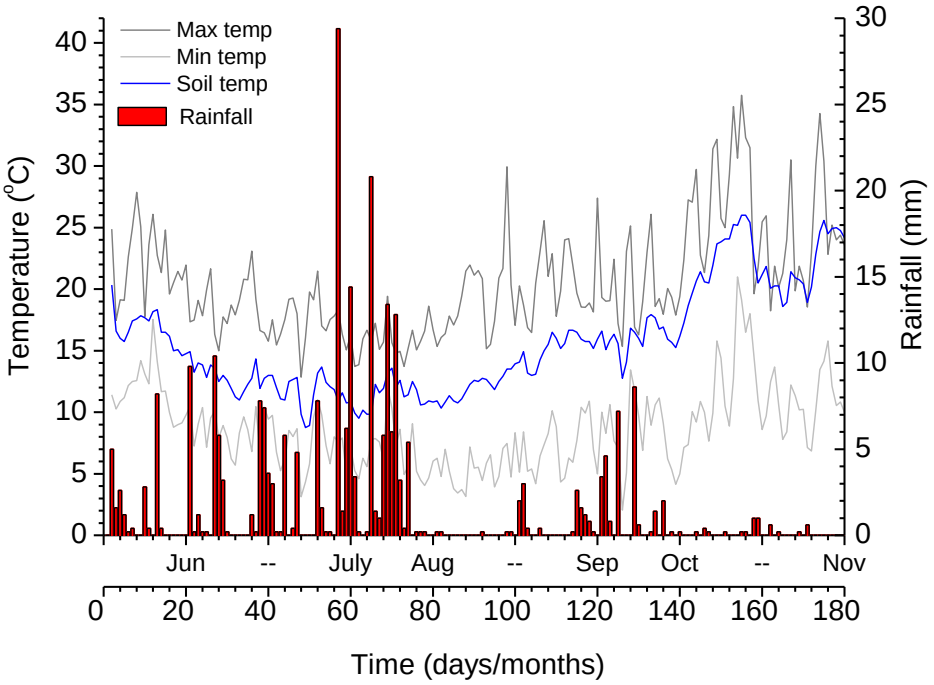


Figure 6-8: [Site B – Moora 2008] Daily rainfall and mean soil and minimum and maximum air temperatures.

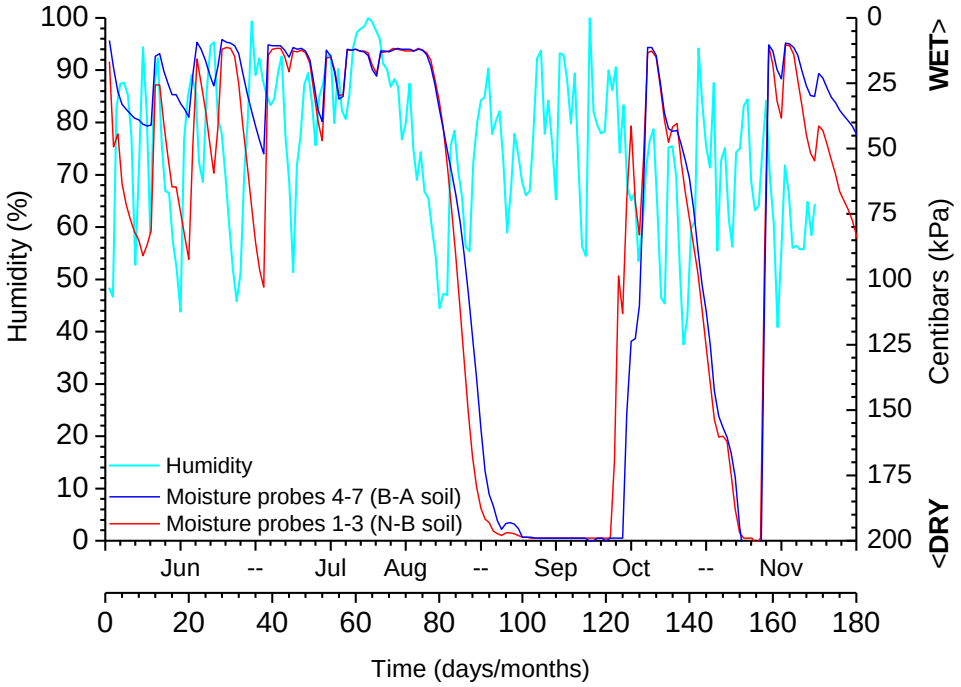


Figure 6-9: [Site B] Mean daily relative humidity (%) and soil moisture levels (kPa) from probes in biosolids-amended (B-A) and nil-biosolids (N-B) soil.

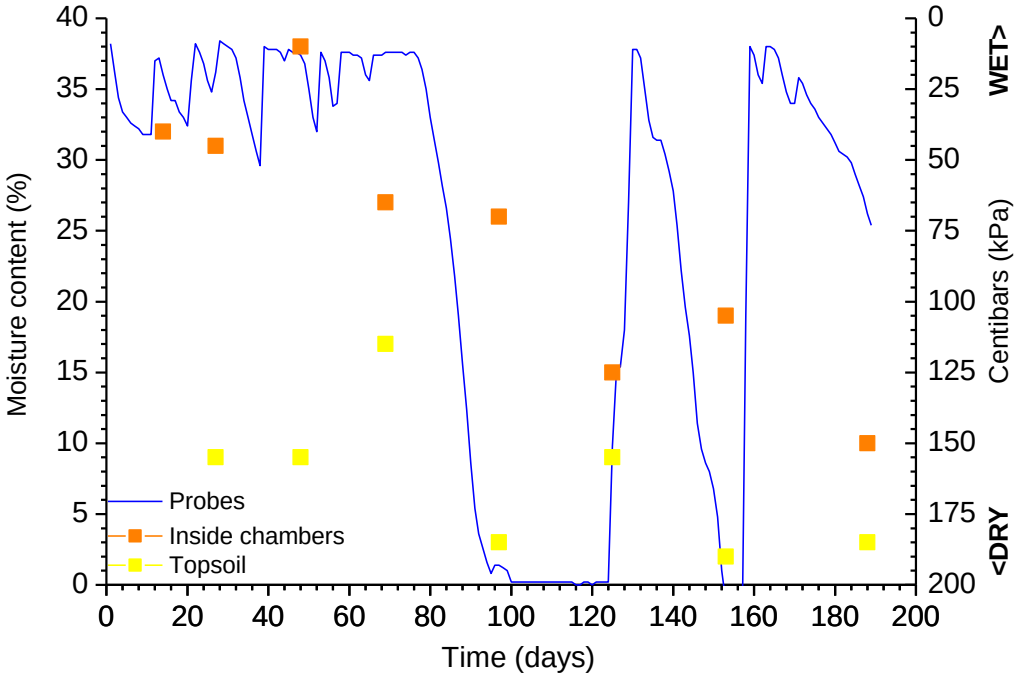


Figure 6-10: [Site B] Comparison of soil moisture recordings from soil probes (kPa), mean topsoil moisture and mean soil moisture inside chambers of the biosolids-amended soil across the duration of the experiment.

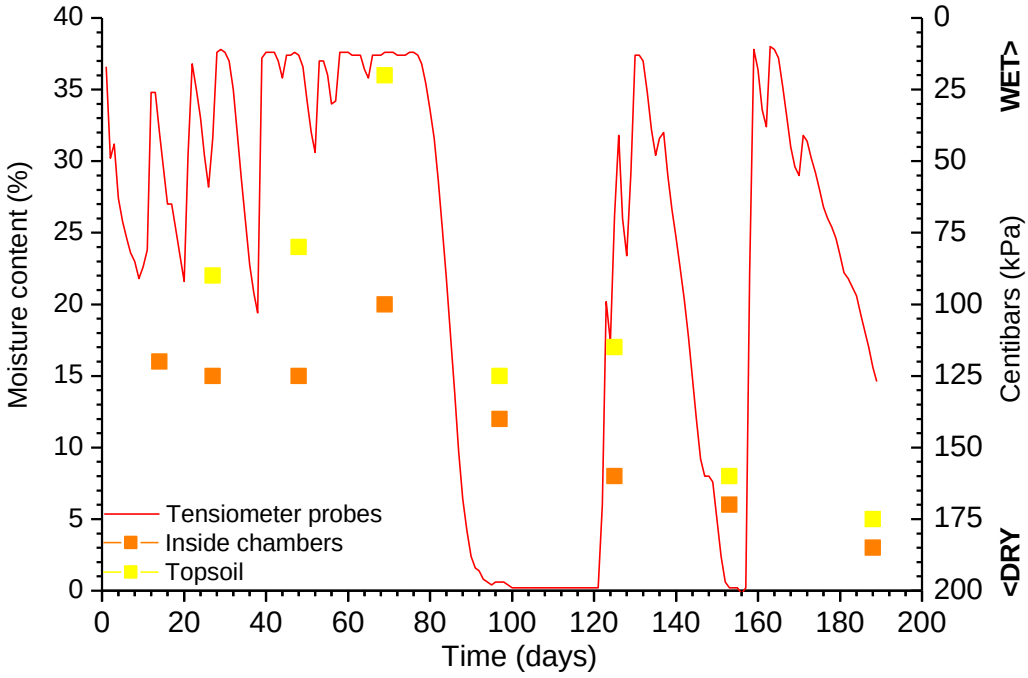


Figure 6-11: [Site B] Comparison of soil moisture recordings from soil probes (kPa), mean topsoil moisture and mean soil moisture inside chambers of the unamended soil across the duration of the experiment.

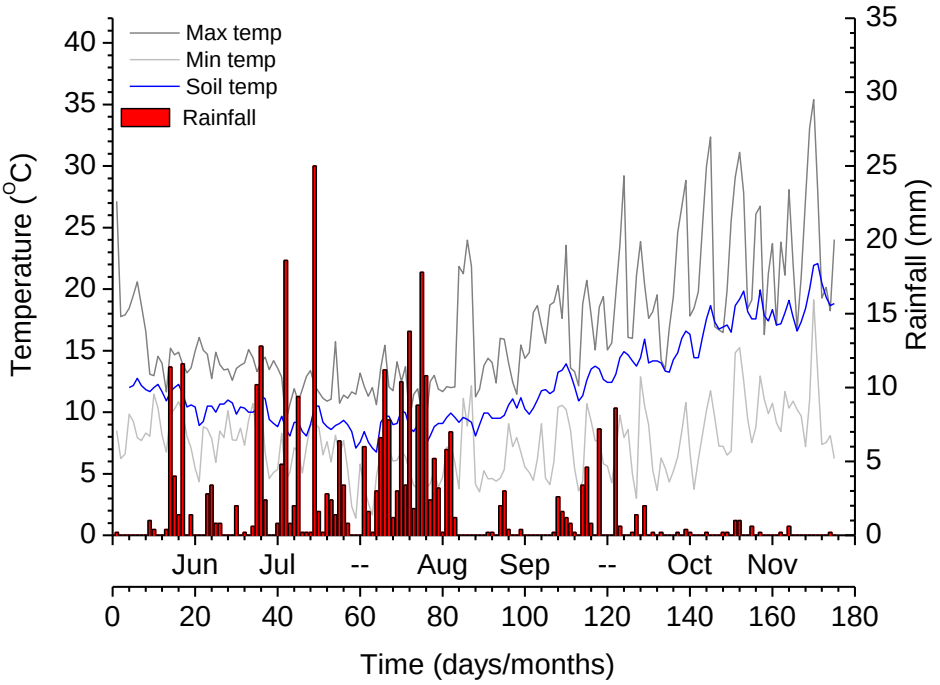


Figure 6-12: [Site C – Mt Compass 2008] Daily rainfall and mean soil and minimum and maximum air temperatures.

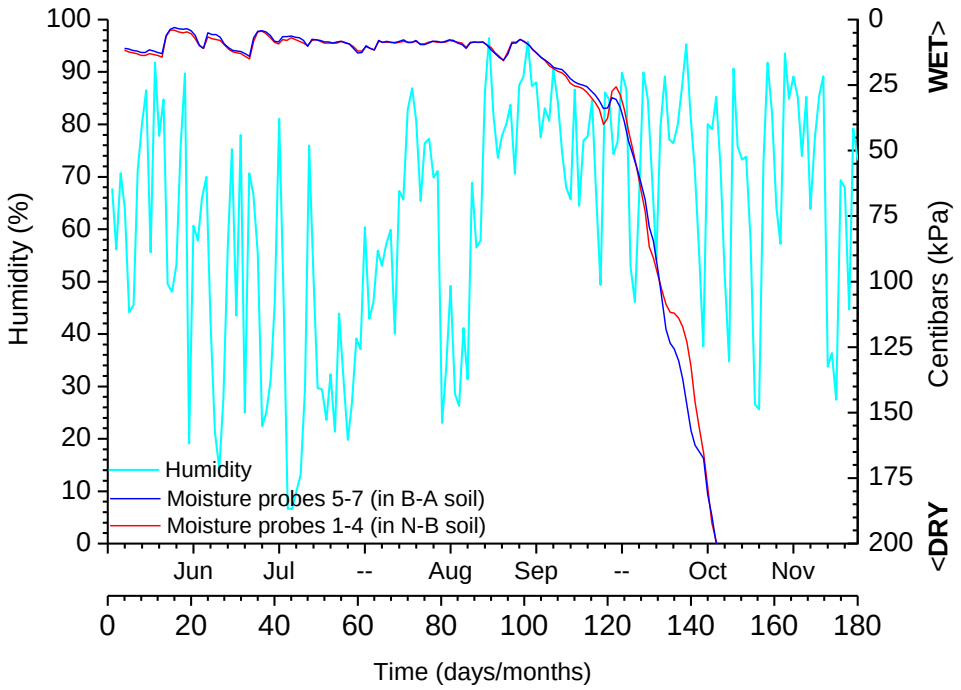


Figure 6-13: [Site C] Mean daily relative humidity (%) and soil moisture levels (kPa) from probes placed in biosolids-amended (B-A) and nil-biosolids (N-B) soil.

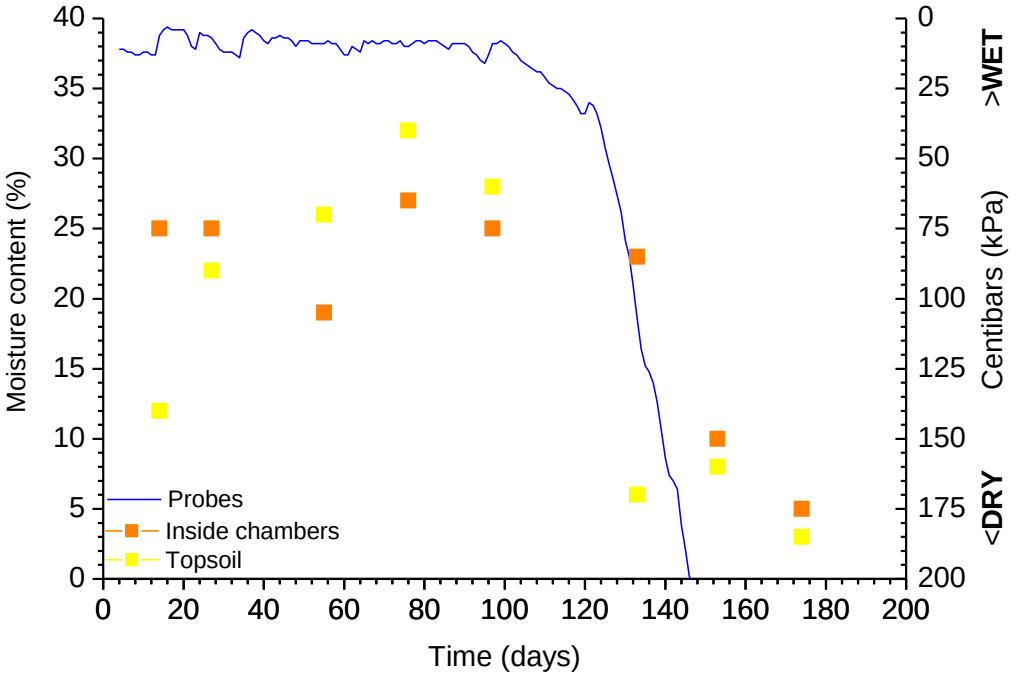


Figure 6-14: [Site C – Mount Compass] Comparison of soil moisture recordings from soil probes (kPa), mean topsoil moisture and mean soil moisture inside chambers of the biosolids-amended soil across the duration of the experiment.

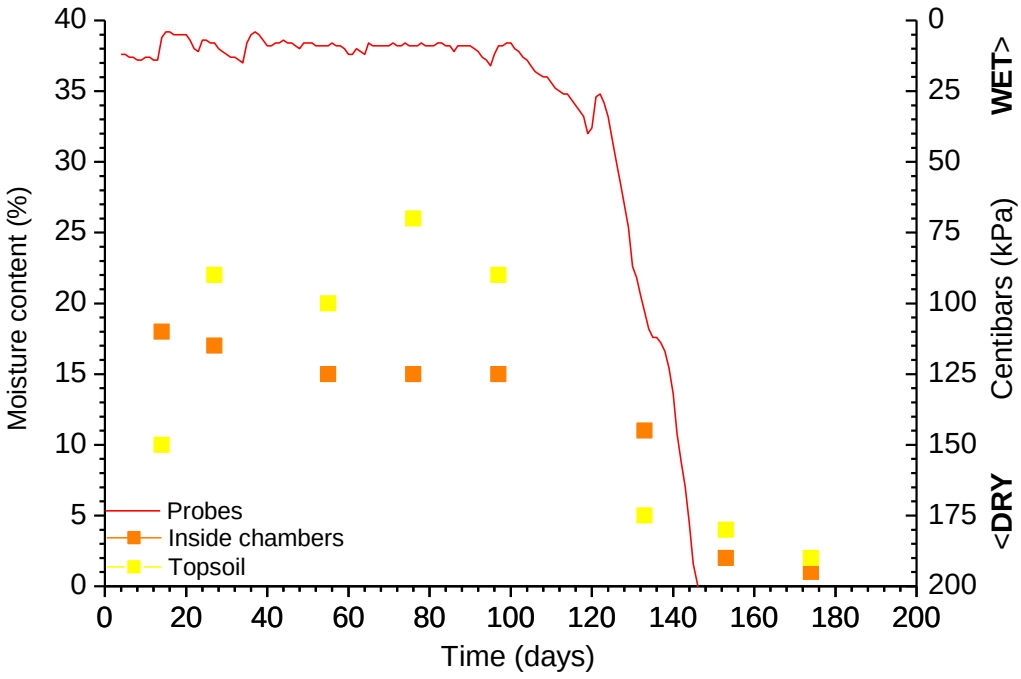


Figure 6-15: [Site C] Comparison of soil moisture recordings from soil probes (kPa), mean topsoil moisture and mean soil moisture inside chambers of the unamended soil across the duration of the experiment.

6.4.2. Enteric microorganism survival in the soil

All of the study microorganisms were observed to decay in the soil; however, the ANOVA results showed that the rate of inactivation was highly significantly ($P < 0.01$) affected by the variations in the microorganism, site location, treatment, related soil type and climatic conditions during the wheat growing season. The plots and the chambers within plots had no significant effects ($P > 0.05$) on the pathogen numbers.

The observed and predicted decay patterns are presented in Figures 6-16 to 6-21. The estimated decay times (T_{90}) for each of the enteric microorganisms tested at all three sites are presented in Table 6-6. Full statistical data is available in the Appendices Section 11.6.

Rapid decay of bacteria in both biosolids amended soil and control soil occurred over the duration of the experiment (Figures 6-16 to 6-18). The numbers of *E. coli* and *S. enterica* fell below 1-$\log_{10}$ by 180 d. The decay times (T_{90}) for *E. coli* were less than 12 d at sites A and C (Table 6-6). Longer decay times occurred at site B (56 d for biosolids amended soil and 83 d for unamended soil). For *S. enterica,* the decay times were less than 25 d at Sites A and B (Moora, WA) but were longer at Site C (Mt Compass, SA, 37 and 57 d for biosolids and control soils respectively). *E. coli* decay times in the biosolids-amended soil, outside the chambers, were 29 d at site B (Moora, WA) and 109 d at site C (Mt Compass, WA). The changes in *E. coli* inside the chambers was significantly correlated ($P<0.05$) to the decay patterns of *E. coli* outside the chambers (topsoil) at sites B and C (Figures 6-20 and 6-21). When the bacteria count was compared across the duration of the experiment, the ANOVA (results) showed that the difference between biosolids-amended soil and unamended soil was highly significant ($P<0.001$) at sites A and B, but not at site C ($P>0.05$).

Viral decay in the soil was less rapid than the bacteria (Figure 6-19). Bacteriophage (MS2) numbers declined only 2 to 3-$\log_{10}$ over the first approximate 120 d of the experiment. Following this, MS2 numbers rapidly fell below 1-$\log_{10}$. Climatic parameters observed during this time showed reduced rainfall events, decreasing soil moisture levels and increasing soil temperatures (above 20ºC) (Figures 6-6 to 6-15).

Decay times (T_{90}) for MS2 were less than 36 d, except at sites A and B (Moora, WA) in the unamended soil where estimated decay times were 108 and 90 d, respectively (Table 6-6). The ANOVA results showed that the difference between treatments in the MS2 counts was highly significant (P<0.001) for all sites and in general the biosolids-amended soil had higher viral numbers than the control soil.

Unlike other microorganisms in the study, inconsistent or limited reduction in adenovirus numbers was observed at the three field sites over the duration of experiment (Figure 6-19). At Site B there was little change in adenovirus count, therefore no T_{90} value was achieved. At Sites A and C there were significant fluctuations in viral numbers, as results multiple decay times (T_{90} values) were observed (Table 6-6). The ANOVA results indicate that the difference between adenovirus numbers in the biosolids-amended soil compared with the unamended soil over time was highly significant (P<0.001) at sites B and C, but not at site A (P=0.82).

A general trend occurred where bacteria and bacteriophage in the biosolids-amended soil (chambers) had shorter decay times than those in the unamended soil (Table 6-6). Soil moisture content in the moisture chambers at Site B and Site C were also higher in the biosolids-amended soils than the unamended soil (Figures 6-10, 6-11, 6-14, 6-15 and Table 6-5) and higher in the chambers than the topsoil (Table 6-5). Soil moisture in the topsoil was only higher at the biosolids-amended site at Site C (compared with the unamended soil).

Table 6-6: Mean soil moisture content (%) in chambers and topsoil (0-10 cm) at Sites B and C.

	Site B		**Site C**	
Mean soil moisture content (%)	Biosolids-amended	Unamended	Biosolids-amended	Unamended
Chambers	25 (±3)	12 (±2)	20 (±3)	12 (±2)
Topsoil (0-10cm)	7 (±2)	7 (±2)	17 (±4)	14 (±3)

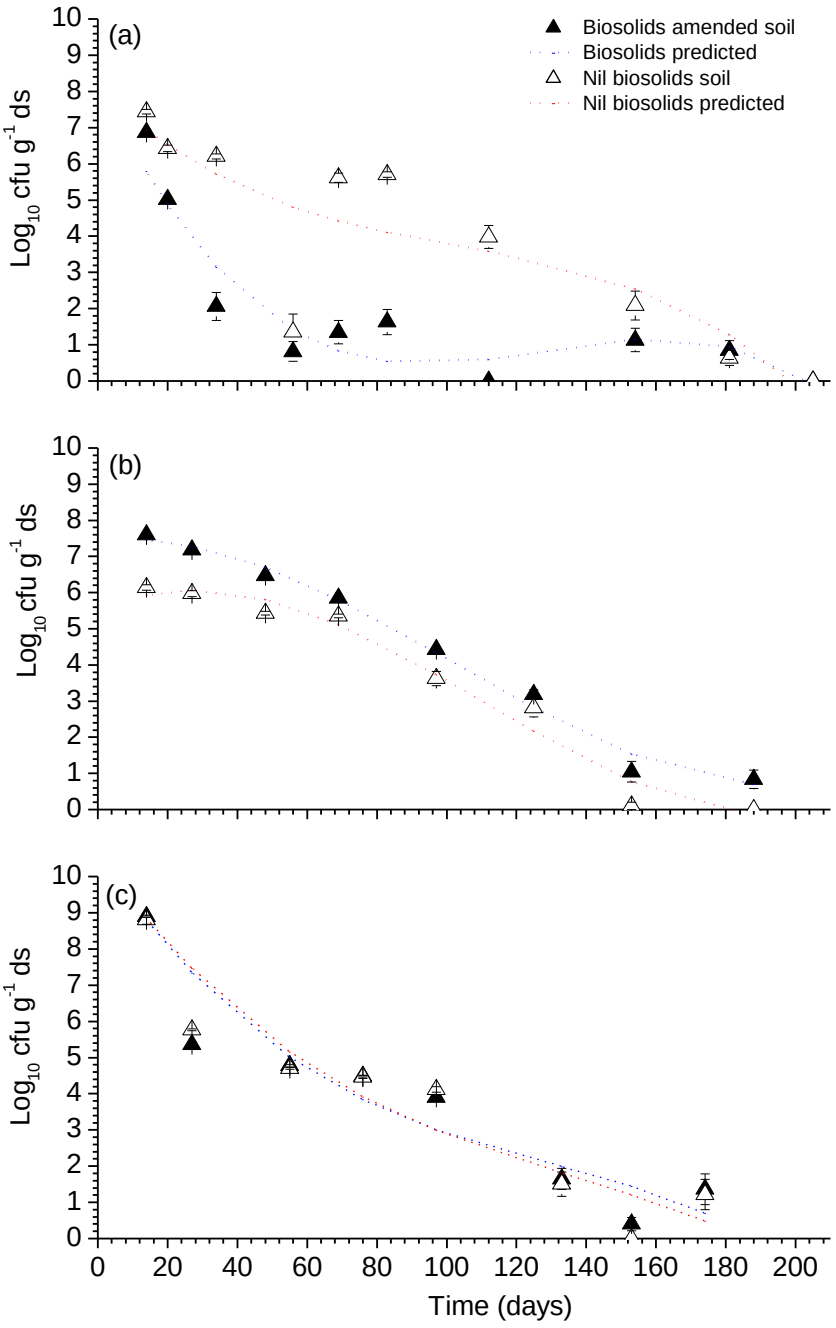


Figure 6-16: *E. coli* decay in biosolids-amended and nil-biosolids soil with standard error bars where (a) is Moora 2006 Site A; (b) is Moora 2008 Site B; and (c) is Mt Compass 2008 Site C.

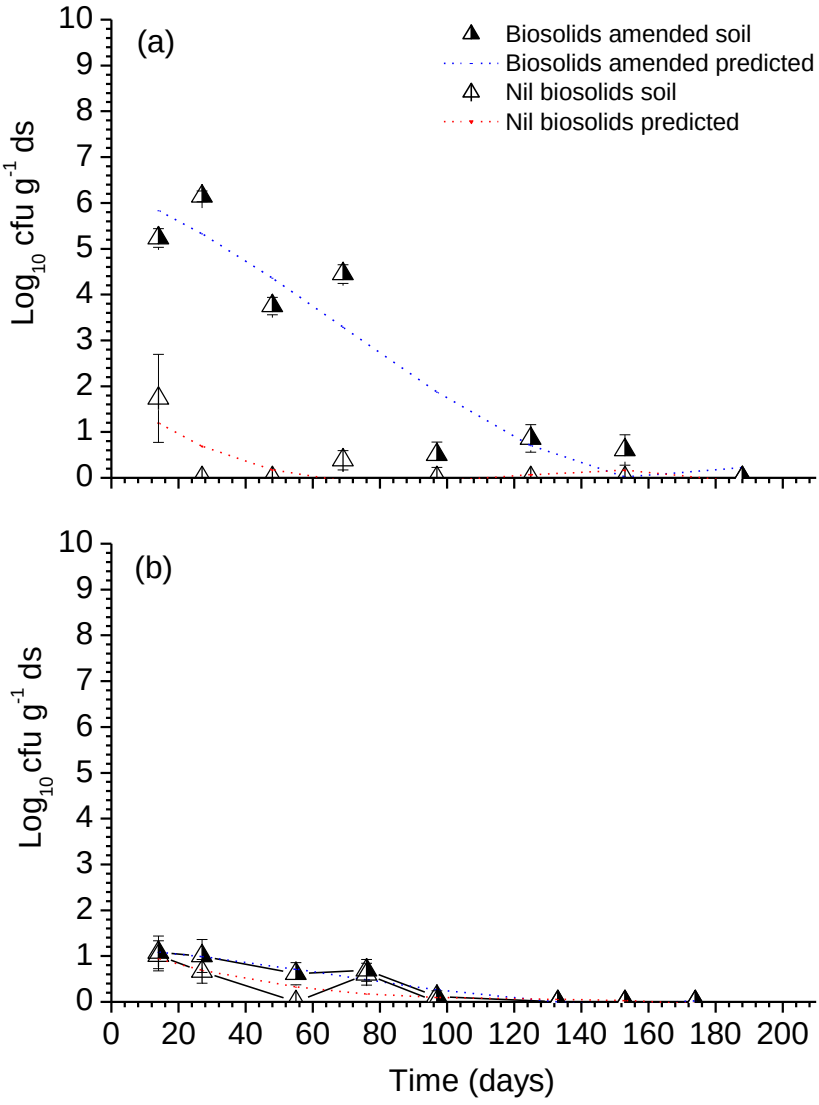


Figure 6-17: *E. coli* numbers in biosolids-amended and nil-biosolids soil (outside chambers) with standard error bars where (a) is Moora 2008 Site B; and (b) is Mt Compass 2008 Site C.

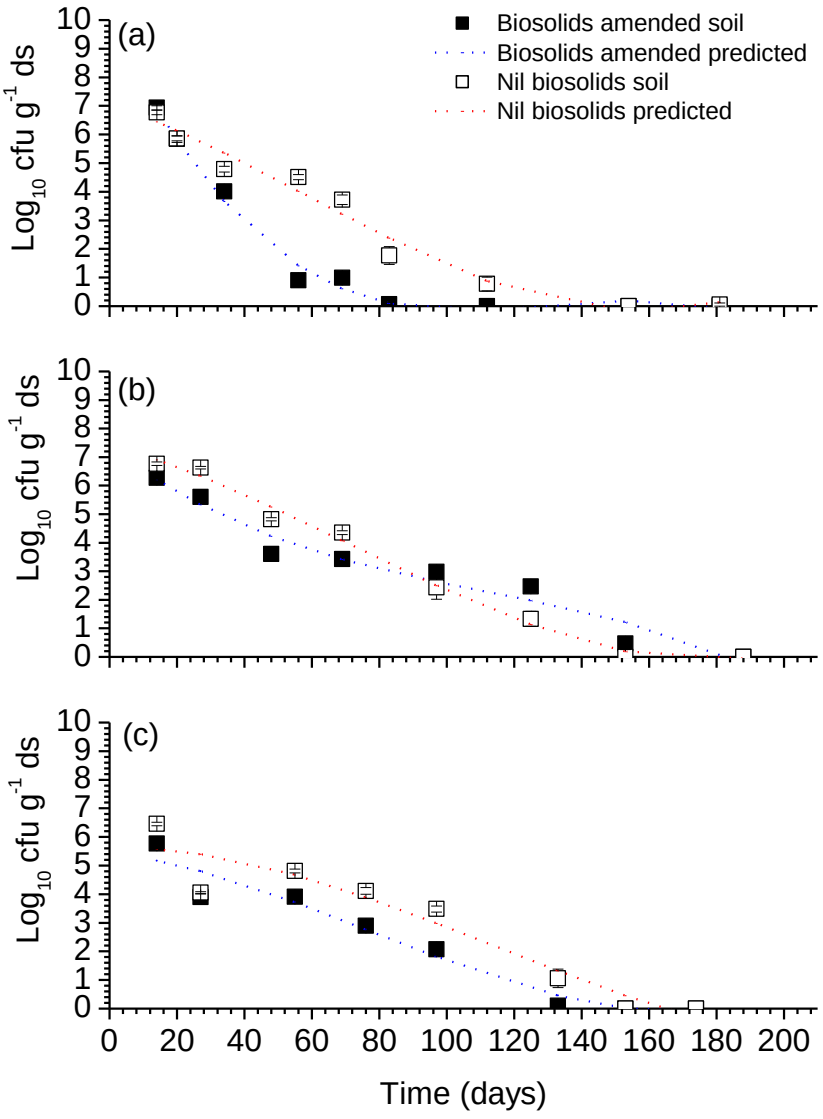


Figure 6-18: *S. enterica* decay in biosolids-amended and nil-biosolids soil (with SE bars) where (a) is Moora 2006 Site A; (b) is Moora 2008 Site B; and (c) is Mt Compass 2008 Site C.

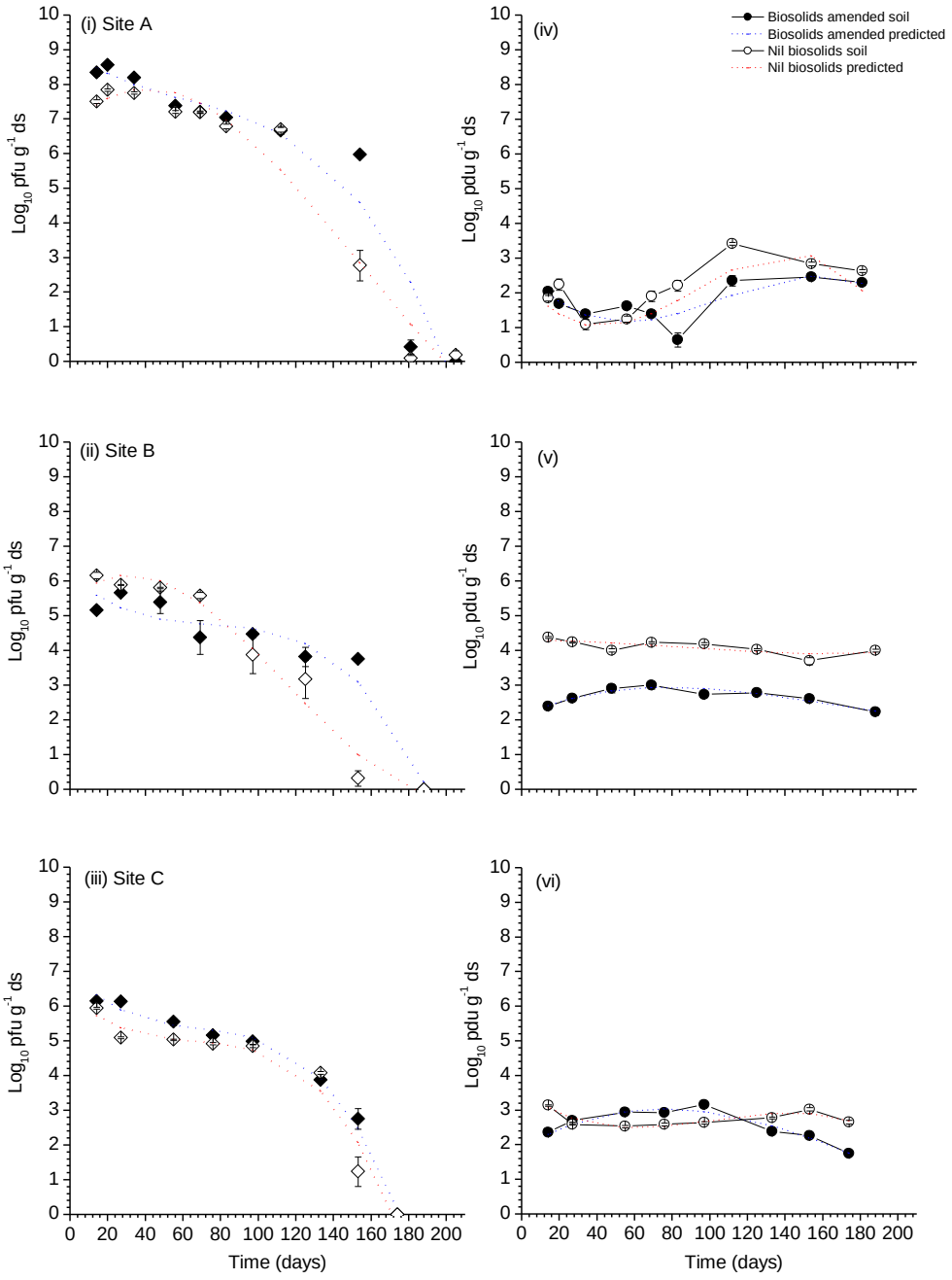


Figure 6-19: The decay patterns of MS2 and adenovirus in biosolids-amended and nil-biosolids soil (with SE bars) where (I and iv) are at Moora 2006 Site A; (ii and v) are at Moora 2008 Site B; and (iii and vi) are at Mt Compass 2008 Site C, respectively.

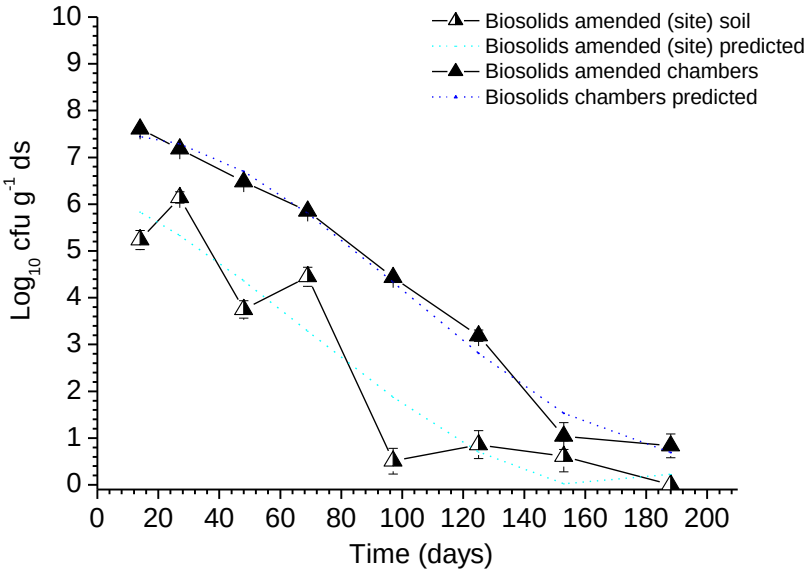


Figure 6-20: Comparison of *E. coli* (inoculated) into chambers with *E. coli* (environmental strain) in topsoil at biosolids-amended plots, Site B Moora 2008.

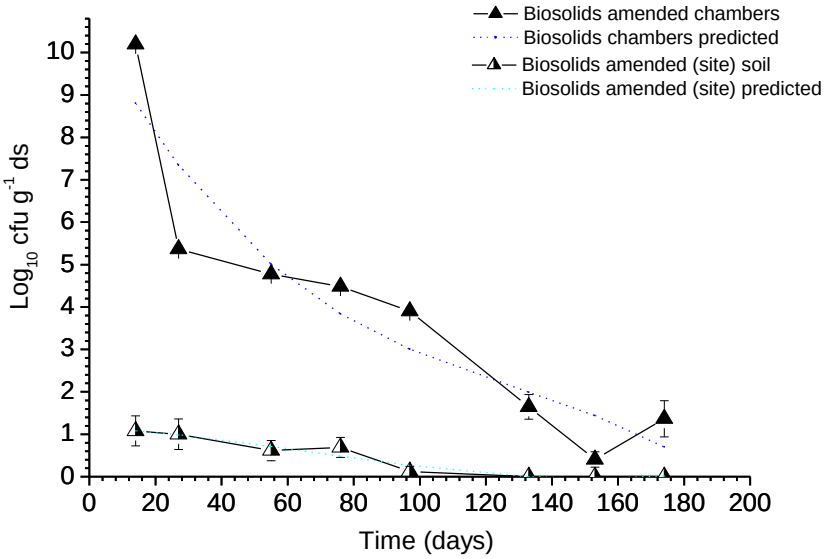


Figure 6-21: Comparison of *E. coli* (inoculated) into chambers with *E. coli* (environmental strain) in topsoil at biosolids-amended plots, Site C Mt Compass 2008.

Table 6-7: Time for a one log_{10} reduction (T_{90}) to occur for enteric microorganisms in soil at three field sites.

	Estimated T_{90} times (d)					
	Site A – Moora 2006		**Site B – Moora 2008**		**Site C – Mt Compass 2008**	
Microorganism	Biosolids	Nil-biosolids	Biosolids	Nil-biosolids	Biosolids	Nil-biosolids
E. coli	5	12	56	83	7	8
E. coli site	#	#	29	21	109, 189	59
S. enterica	4	21	12	25	37	57
MS2	36	108	29	90	22	29
Adenovirus	20, 102, 198	18, 71, 189	>200	>200	>200	44, 80, 18

 # Not tested

NB: The standard deviation values on individual sampling events are provided in the figures.

6.4.3. The effects of climate variables on microbial numbers

The changes in *E. coli, S. enterica,* MS2 and adenovirus (inside the chambers) at Moora in 2008 (Site B) were significantly influenced ($P<0.05$) by the changes in soil moisture (taken from inside moisture chambers) over the duration of the experiment in the biosolids-amended soil (Figure 6-22) and the unamended soil (Figure 6-23). The only exception was adenovirus in the biosolids-amended soil (Figure 6-22), which was not significantly correlated ($P=0.28$) to soil moisture patterns.

At Mount Compass in 2008 (Site C), the decay patterns for *E. coli, S. enterica* and MS2 in the unamended soil were significantly correlated ($P<0.01$) to changes in soil moisture (Figure 6-25). In the biosolids-amended soil, the changes in MS2 and adenovirus were significantly correlated ($P<0.05$) to soil moisture changes at the same site (Figure 6-24).

Soil temperature changes over the duration of the experiment were significantly correlated ($P<0.05$) with changes in *E. coli* and MS2 in both soils at Site B (Figures 6-22 and 6-23). At Site C, decay patterns for MS2 were significantly correlated ($P<0.01$) to soil temperature changes in the biosolids-amended soil and unamended soil (Figures 6-24 and 6-25). In addition, the decay patterns of adenovirus in the biosolids-amended soil and *S. enterica* in the unamended soil were significantly correlated ($P<0.02$) with soil temperature changes over time at the same site.

The changes in *E. coli,* taken from the biosolids-amended soil outside the chambers, were significantly correlated ($P=0.05$) to changes in soil moisture at Site B at Moora in 2008 (Figure 6-26). There was no correlation between changes in *E. coli* (outside the chambers) and soil temperature at Site B, and no correlation between soil temperature and soil moisture at Site C ($P>0.10$).

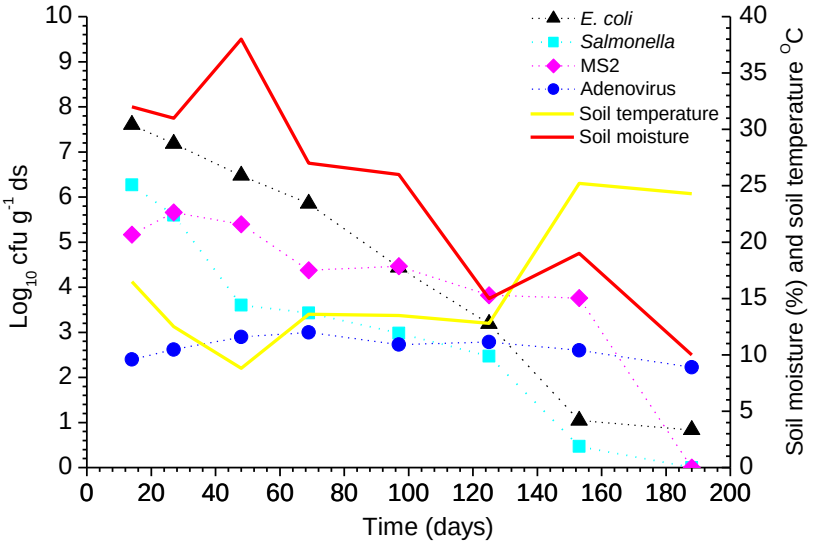


Figure 6-22: Decay patterns of *E. coli, S. enterica,* MS2 and adenovirus in biosolids-amended soil chambers with soil moisture levels (%) from biosolids-amended moisture chambers along with soil temperature (from auto-probes) at Site B, Moora 2008.

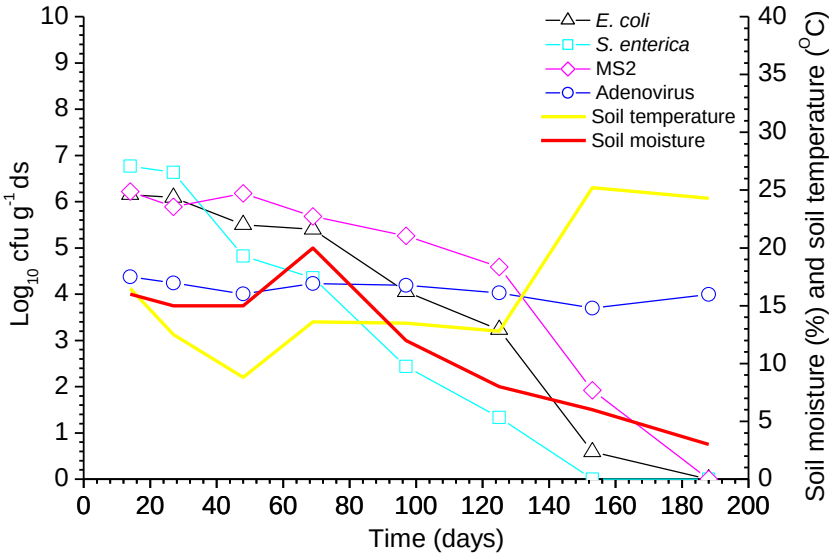


Figure 6-23: Decay patterns of *E. coli, S. enterica,* MS2 and adenovirus in unamended soil chambers with soil moisture levels (%) from unamended moisture chambers alongside soil temperature changes at Site B, Moora 2008.

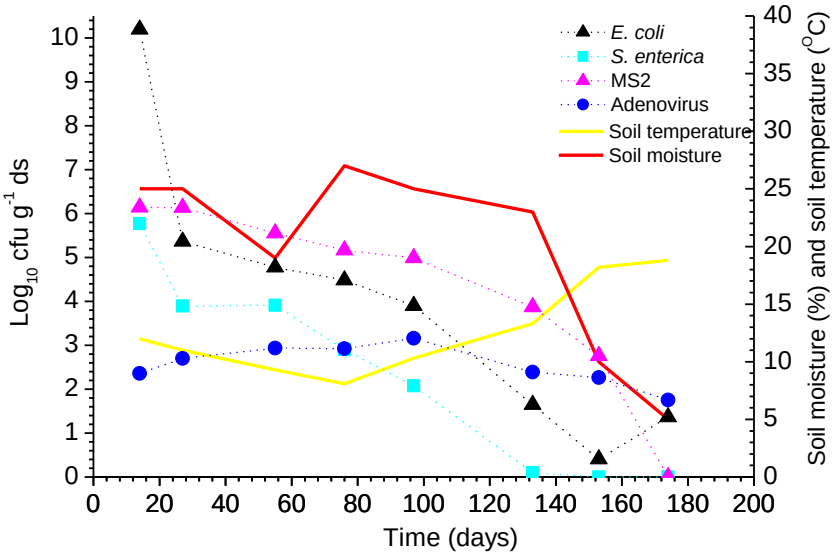


Figure 6-24: Decay patterns of *E. coli, S. enterica,* MS2 and adenovirus in biosolids-amended soil chambers with soil moisture levels (%) in biosolids-amended moisture chambers along with soil temperature changes (probes) at Site C, Mt Compass 2008.

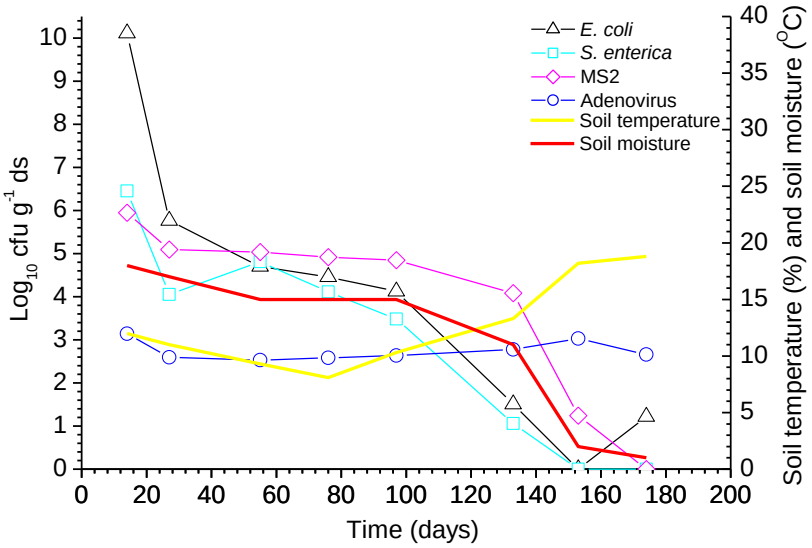


Figure 6-25: Decay patterns of *E. coli, S. enterica,* MS2 and adenovirus in unamended soil chambers with soil moisture (%) from unamended moisture chambers along with soil temperature changes at Site C, Mt Compass 2008.

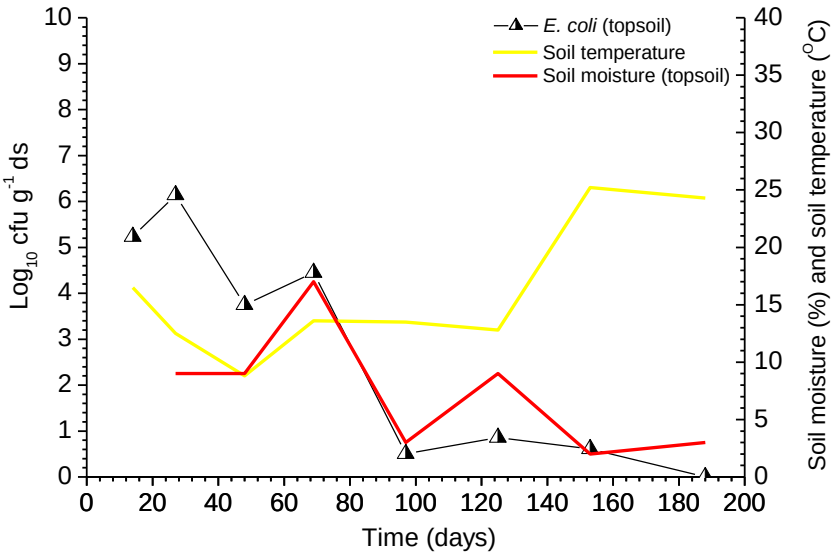


Figure 6-26: Decay pattern of *E. coli* (environmental strain) in biosolids-amended topsoil alongside soil moisture (outside chambers) and soil temperature at Site B, Moora 2008.

Table 6-8: Levels of signficance (*P*<0.05) of correlations between the decay patterns of enteric microorganisms with soil moisture and soil temperature.

	Soil moisture		**Soil temperature**	
Microorganism	Biosolids-amended	Unamended	Biosolids-amended	Unamended
SITE B				
E. coli	***P*=0.00**	***P*=0.00**	***P*=0.03**	***P*=0.01**
S. enterica	***P*=0.02**	***P*=0.01**	*P*=0.08	*P*=0.09
MS2	***P*=0.01**	***P*=0.00**	***P*=0.05**	***P*=0.01**
adenovirus	*P*=0.28	***P*=0.05**	*P*=0.06	*P*=0.18
E. coli (site)	***P*=0.05**	***P*=0.03**	*P*=0.18	*ND*
SITE C				
E. coli	*P*=0.10	***P*=0.01**	*P*=0.14	*P*=0.12
S. enterica	*P*=0.09	***P*=0.00**	*P*=0.06	***P*=0.02**
MS2	***P*=0.00**	***P*=0.00**	***P*=0.01**	***P*=0.00**
adenovirus	***P*=0.03**	*P*=0.69	***P*=0.00**	*P*=0.27
E. coli (site)	*P*=0.76	*ND*	*P*=0.14	*ND*

ND = Not determined

6.5. Discussion

This is the most comprehensive study in Australia where the survival of enteric microorganisms in land-applied biosolids, particularly on broadacre grain farms in Australia, has been undertaken. Results from this study demonstrate that enteric bacteria (after inoculating at high levels) can be expected to remain above detection limits for 6 to 7 months in agricultural soil amended with biosolids, particularly in dry temperate cropping regions (when spring temperatures increase and soil moisture levels decrease). Enteric viruses can be expected to survive for longer periods of time than bacteria with slower patterns of decay over time.

6.5.1. Decay times of enteric bacteria in the soil

The inactivation times (T_{90}) of seeded enteric bacteria were 4 to 12 d for *S. enterica* at Moora and 5 to 7 d for *E. coli* at Moora in 2006 and Mt Compass in 2008 (Table 6-6). In a similar study, Crute (2004) reported decay times of 4 d for *E. coli* and 12 d for enterococci sampled directly into biosolids-amended soil at Toodyay, WA. Similar decay times were reported in soils irrigated with farm effluent in Victoria as 15 d for *E. coli* and 10 d for *Salmonella* (Chandler and Craven 1980), and 8 to 15 d for *S. enterica* in sewage sludge in New Zealand (Horswell *et al.* 2010).

The Australian soils used in this study had fairly low levels of organic carbon (~ 2 to 3%) (Table 6-1). Higher inactivation rates of *E. coli* have been associated with low organic carbon content (<1.65%) (Vidovic *et al.* 2007). The decay times of *E. coli* (Table 6-6) were similar at Moora (2006) to Mt Compass (2008) despite being different seasons, different locations and different biosolids (with different treatment processes), but much longer decay times were found for Moora in 2008 (Table 6-6). Some possible explanations for this are that the biosolids application rate was almost three times higher in 2008 compared with 2006, although this does not explain the longer decay time in the unamended soil of 83 d (Site B). The clay (7.5%) and silt (5.5%) content at the Moora site (Site B) was higher than at Mt Compass (Site C) where the clay content was 1.5% and the silt content was 2.5%. Clay soils not only have a better moisture holding capacity than sandy soils, the moisture is not as easily lost which also improves chances of bacterial survival (Platz 1980) which are more

sensitive to loss of soil moisture. The soil components at both Moora sites should have been similar, being close in location, and therefore this does not explain the longer decay times at Moora in 2008 (Site B). Soil moisture content at Site B (Figure 6-9) underwent a dry period from August to September which would normally cause bacterial deaths but despite this, there was no pattern of sudden decay during this period. Cools *et al.* (2001) reported that moisture content did not affect the survival of *E. coli* or *Enterococcus.*

Based on the observed data (Figure 6-16), the *E. coli* at all three sites decreased approximately seven-$\log_{10}$ over 150 to 200 d with *E. coli* numbers below one-$\log_{10}$ by approximately 5 to 6 months. These results indicate that estimated decay times (T_{90} values) should be considered in conjunction with the observed data. In previous work, Crute (2004) reported that *E. coli* and enterococci were able to be detected in biosolids-amended soil for up to 6 months at Toodyay, WA. Eamens *et al.* (2006) reported longer detection times of 10 to 17 months for *E. coli*, *C. perfringens* and *Salmonella* spp. in soils amended with anaerobically-digested biosolids at Goulburn, New South Wales. These survival times may have been longer due to samples collected directly from biosolids 'clumps' as opposed to the biosolids being diluted with the soil.

Actual survival times have been reported as up to 3 months in New Zealand and the United Kingdom (Horswell *et al.* 2007; Lang *et al.* 2007) and 7 months up to 11-12 months in Australia (Eamens *et al.* 2006; Eamens and Waldron 2008). These survival (detection) times are related to various locations, soil characteristics at the site, the type of waste applied (Williams *et al.* 2007), starting numbers at the beginning of the experiment, detection limits, methods of enumeration, methods for determining decay times, and the microorganism type (Cools *et al.* 2001) used across the various studies.

The difference between the survival times reported in other studies such as Horswell *et al.* (2007), Lang *et al.* (2007), Eamens *et al.* (2006) and Eamens and Waldron 2008 and the present study, could be that these studies commonly report the 'losses' or removal of microorganisms from the soil rather than the actual decay times calculated from the decay slope. This means that the reductions, or the time before

the microorganisms fell below detection, as reported in these studies, may have been as a result of factors such as leaching, random distribution or run-off. Some of the bacteria may have been inside of biosolids 'clumps' and others not. As the biosolids would have broken down over time, there may have been greater variation and more difficulty associated with the ability to detect them. In the present study, the unique aspect of the research was that actual decay times were examined. Since the microorganisms were inoculated and confined to the sentinel chambers, the reductions reflected in the observed data were not a result of microorganisms being leached or washed away. Rather, the viable microorganisms present inside the chambers were cultured at each sampling event and therefore provided a more accurate representation of the decay patterns for each microorganism studied.

The decay times (T_{90}) of *S. enterica* were shorter at Moora in the biosolids-amended soil (4 to 12 d) compared with Mt Compass (37 d). The same pattern occurred in the unamended soils, where decay times were shorter at Moora (21 and 25 d) compared with Mt Compass (57 d), although this difference was not as great. This may have been due to higher moisture and lower temperatures that occur on the South Australian peninsula compared with the drier, warmer climate that is common inland from the coast of Western Australia. Soil temperatures and humidity were lower at Mt Compass than Moora (Table 6-3) with higher annual rainfall recorded at Mt Compass (328 mm pa). Similar results were found by Lang *et al.* (2007) where temperature, particularly soil temperature, and soil moisture were identified as the most influential environmental parameters affecting inactivation.

Several factors need to be taken into account when reviewing the reduction times found in the present study. Firstly, biosolids were added to soil at far higher rates than would be allowable (for release onto land) under the current land application guidelines. Biosolids applications at Moora in 2008 (Site B) and Mt Compass (Site C) were 1.5 times the nitrogen limited biosolids application rate (NLBAR) or normal district practice (DEP, WRC and DOH 2002) so that the treatment effect of biosolids (on pathogen survival) could be examined. In the sample chambers, the biosolids to soil rates were 25% biosolids to 75% soil. In the field (based on the NLBAR) the application rate would be approximately 7 to 10 dry t ha^{-1} which would equate to 1% biosolids to 99% soil in the chambers. Based on previous findings (Crute 2004), the

rate of 1% biosolids was not high enough to show up any treatment effect (i.e. the effect that biosolids have on the decay rates of the pathogens). The biosolids from Western Australia were fresh, were despatched directly from the wastewater treatment plant and were applied to land immediately after delivery to the site. Biosolids from South Australia had been stockpiled for approximately 2 years before application. This could have resulted in any a higher level of predation occurring in fresh biosolids thus the increased inactivation times from the Western Australian plots. Alternatively, the bacteria remaining in the stockpiled biosolids may have been more robust and may have resulted in longer decay times at the South Australian site.

Secondly, the laboratory-cultured pathogens tested in this study were inoculated into the soil and biosolids-amended soil at very high levels; at levels which, if present in biosolids, would not normally be permitted for land application. The higher starting numbers at the beginning of each experiment (from the use of inoculants), coupled with higher biosolids application rates, served to provide a worse-case scenario or overestimation of the levels of risk to be expected where biosolids are used in the field. Therefore, where biosolids are applied under normal conditions (according to NLBAR), the risks to public health from enteric pathogens would be expected to be lower.

Inoculation was used to provide a longer time-frame to examine decay patterns before die-off occurred, and for statistical purposes. Given these factors, *E. coli* was also tested from the topsoil of the paddock (outside the chambers), where biosolids had been applied at a normal district rate of approximately 8 dry t ha^{-1} for comparison with the *E. coli* inside the chambers. The decay pattern for both were significantly correlated ($P<0.05$). The starting numbers of *E. coli* inoculated into the biosolids-amended soil at Moora in 2008 (Site B) were very similar, being 7 log cfu g^{-1} in the chambers compared with 6 log cfu g^{-1} in the topsoil at the biosolids-amended site and as a result, the decay times (T_{90}) were similar (i.e. 56 d in the chambers and 29 d in the topsoil). However, at Mt Compass (Site C) the starting numbers were 10 log cfu g^{-1} inside the chambers compared with 3 log cfu g^{-1} outside the chambers at the biosolids-amended site, as expected, and the decay times were vastly different (7 d in the chambers and 109 d in the topsoil) and yet still significantly correlated ($P<0.05$). The presence of *E. coli* in the unamended soil at

Mt Compass may be attributed to the grazing of cattle and kangaroos at the site prior to the experiment since faecal pathogens have been known to be introduced into the soil in this manner (Wu *et al.* 2009).

The soil moisture content inside the chambers was higher in the biosolids-amended soil than the unamended soil (from data collected at Sites B and C – Table 6-5). As a possible link, the decay times were shorter for *E. coli*, *S. enterica* and MS2 in the biosolids-amended chambers than in the unamended soil chambers (Table 6-6). In addition, despite the same mean content (of 7% in Table 6-5), the moisture content in the topsoil at Site B was higher across the experiment in the unamended soil (Figure 6-11) than the biosolids-amended soil (Figure 6-10), and as a possible result, the decay time of *E. coli* was shorter in the unamended soil where the soil moisture patterns were higher (Table 6-6). This suggests that decay may be increased (or decay times shorter) for the study microorganisms (*E. coli, S. enterica* and MS2) in soils with higher moisture content. This pattern, however, was not evident in the topsoil at Site C (with a lower T^{90} in the unamended soil of 59 days), where there was less variation in moisture levels between treatments (17 and 14% in Table 6-5 and Figures 6-14 and 6-15).

6.5.2. Decay times of *E. coli* inside chambers compared with outside chambers

At Moora in 2008, the estimated decay time (T_{90}) of *E. coli* inoculated into the biosolids-amended soil inside the sentinel chambers (56 d) was similar to that of the *E. coli* (not inoculated) into the biosolids-amended soil (topsoil) outside the chambers (29 d). The decay patterns of *E. coli* outside the chambers showed greater variability in the observed data than the *E. coli* inside the chambers (Figures 6-16b and 6-17a); however, these decay patterns were significantly correlated ($P<0.001$) across the duration of the experiment. This significance ($P<0.05$) also occurred for *E. coli* outside the chambers at Site C. Bacteria outside the chambers (in the topsoil) may have been more exposed to the elements, resulting in shorter decay times. At this site (Site B), soil moisture content was lower in the topsoil (where decay time was also shorter at 29 d) than in the chambers (where decay time was longer at 56 d) demonstrating that the chamber may provide more stabilised conditions from rapid drying, thus the effect of moisture in increasing decay times as previously suggested may not always be the case.

The variability in the observed data may have also been because the biosolids incorporated with the soil contained more clumps. To avoid soil dilution factors, the clumps were sampled in an effort to obtain a direct measurement of the actual survival of bacteria within the biosolids (Crute 2004; Eamens *et al.* 2006). This method of sampling into the soil does not truly reflect the inactivation of microorganisms when incorporated with the soil (i.e. the dilution factor for any given application rate). In addition, the method may introduce more random error between samples, particularly as the clumps begin to dry and become difficult to penetrate with a sample corer. For this reason, the chambers were useful for reducing random error and for providing uniformity without compromising the general survival decay patterns in the soil.

6.5.3. Decay times of enteric viruses in the soil

Bacteriophage decay times (T_{90}) in the biosolids-amended soil were less than 36 d at all three sites (Table 6-6); however, decay times were much longer at Moora than Mt Compass. This may have been influenced by higher clay content in the soil at Moora (7.5%) compared with Mt Compass (1.5%), as was suggested for the bacterial decay patterns. Assadian *et al.* (2005) found that the extended persistence of bacteriophage MS2 in soil was reported to be due to higher clay content. Goyal and Gerba (1979) found that increased persistence of virus through soil adsorption was highly strain dependent and was influenced by the type of soil. In addition, better adsorption of viruses occurs in soil with a saturated pH of less then 5.0. In the present study, bacteriophage in the unamended soils had longer decay times than the biosolids-amended soils (Table 6-6).

The decay rate of enteric microorganisms is known to be influenced by the microorganism type (Sidhu *et al.* 2008). In the present study, the enteric bacteria were inactivated more rapidly than the surrogate virus (MS2). This was also found by Lasobras *et al.* (1999). Moce-Llivina *et al.* (2003) found that phages were significantly more resistant (to higher temperatures) than bacterial indicators. For this reason, it does not seem appropriate to rely on indicator microorganisms to act as a true representation of other enteric pathogens in soil/biosolids-type samples, even though they may provide accurate representation in water or other substances.

However, the author does recommend that indicator microorganisms such as *E. coli* be used for other (*E. coli*) pathogenic strains, for example.

From the observed data (Figure 6-19) a general trend occurred at all three sites where MS2 numbers decayed slowly up to approximately 150 d before rapidly decreasing to below one-$\log_{10}$ at 180 d or 6 months. It was during this time that soil temperatures also increased, there were fewer rainfall events and soil moisture levels rapidly declined (Figure 6-6 to 6-11). F-specific bacteriophages have been observed to be sensitive to temperatures over 25ºC, resulting in a reduction of more than 2 $\log_{10}$ units following exposure (Lasobras *et al.* 1999; Moce-Llivina *et al.* 2003; Guzman *et al.* 2007). At Moora, soil temperatures rose above 20ºC from 130 to 140 d with increasing periods where temperatures were above 25ºC from approximately 160 d onwards. Soil temperatures at Mt Compass reached 20ºC from 150 d onwards. At both sites, the changes in MS2 was significantly correlated (P<0.05) with changes in soil temperature and soil moisture over time (Table 6-7).

No notable inactivation of adenovirus occurred in both the biosolids-amended and unamended soils over the growing season of the wheat crop and, as a result, the decay times (T_{90}) were more than 180 d. This is in agreement with previously reported findings of Charles *et al.* (2009) and Schlindwein *et al.* (2010) where there was no change in adenovirus numbers across the duration of their experiments. In the present study, this may have either been due to the strong adsorption of the viruses to the soil minerals (Hurst *et al.* 1980; Horswell *et al.* 2010) or, more likely, the use of quantitative PCR to detect viral DNA rather than the detection of infective viruses by culture. Wei *et al.* (2009) examined the 'detection' of human Adenovirus 41 (Ad41) in manure and biosolids and found no significant loss of viral DNA after 60 d. When they tested 'infectivity', the adenovirus decay times (T_{90}) were much shorter at 4 and 8 d (in dairy manure), 12 and 28 d (in biosolids) and 19 and 51 d (in swine manure). Horswell *et al.* (2010) also observed that adenovirus could be detected by PCR in the soil where no culturable viruses were detected and attributed this to greater sensitivity of PCR to detect viable and nonviable viruses alike. This agrees with others who stated that PCR methods do not quantify the human heath risk associated with enteric pathogens since they do not distinguish between infectious (living) and noninfectious (dead) pathogens (Lasobras *et al.* 1999; Guzman *et al.* 2007; Charles *et*

al. 2009; Schlindwein *et al.* 2010). Further experimentation is required to quantify virus decay (i.e. using an indicator such as bacteriophage) to compare cultural methods with molecular techniques and thus 'infective' decay times with 'detectable' decay times. For these reasons, it is difficult to draw conclusions from the results because the PCR detects live and dead virus.

6.5.4. The effect of adding biosolids to soil

In the present study, the decay times were often shorter in the biosolids-amended soil compared with the unamended soils. This indicates that, in some cases, the addition of biosolids to the soil may actually increase the inactivation times of these enteric microorganisms. Ingham *et al.* (2004) also reported that *E. coli* decreased more rapidly in manure-fertilised soils. Jiang *et al.* (2002) found that *E. coli* O157:H7 was inactivated more rapidly in non-autoclaved soil compared with autoclaved soil since the antimicrobial activities of microorganisms in manures and indigenous soil microorganisms are thought to contribute to the more rapid inactivation of *E. coli* in soils amended with biosolids or manures. Along with this, the organic substrate in sludge may contribute towards the reduction of indigenous populations by stimulating the activity of predatory and competing soil flora (Lang *et al.* 2007). In Hurst *et al.* (1980), the presence of sewage did not influence virus survival.

Increasing manure content can result in decreased attachment, in particular for bacteria. Guber *et al.* (2005; 2007) found that maximum *E. coli* attachment occurred to soils in the absence of manure colloids. The opposite trend was found by Cools *et al.* (2001), Platz (1980) and Holley *et al.* (2006) where the addition and incorporation of manure to the soil enhanced bacterial survival. In Holley *et al.* (2006) this was thought to be due to possible nutrient availability. In other studies, the presence of manure enhanced the survival of *E. coli* in no-till soil which was thought to be due to enhanced microsite habitat and the addition of nitrogen (Gagliardi and Karns 2000). Horswell *et al.* (2007) found that bacterial die-off was significantly correlated with per cent solids of sludge. Eamens *et al.* (2006) also found no significant difference between survival in biosolids, with or without incorporation with the soil, however the raw data indicated a trend towards slightly greater survival of bacteria where biosolids had been incorporated, suggesting a possible protective effect under the

soil. In the present study, the addition of organic matter was not attributed to prolonged survival of bacteria.

6.5.5. The effect of climate and location

Bacteria respond to seasonal patterns in the environment by declining with increasing soil temperature and decreasing soil moisture (Holley *et al.* 2006; Lang *et al.* 2007). In the present study, the decay of bacteria was significantly correlated ($P<0.05$) with declining moisture levels, particularly at Site B and in the unamended soil at Site C; however, the same significant relationship with soil temperature did not occur at Site C ($P>0.10$) but was evident at Site B ($P<0.03$). Lang *et al.* (2007) found that *E. coli* populations declined in warm, drier soil in the summer and increased in cool, moist soils in the winter. Horswell (2007) also reported that *E. coli* die-off was faster when temperatures increased in conjunction with reduced rainfall events resulting in increased moisture loss. Cools *et al.* (2001) reported that increasing temperature caused a decrease in survival of *E. coli* at levels from 15 to 25ºC. Unc and Goss (2006) also found that *E. coli* populations decreased faster at a soil temperature of 20ºC compared with 12 and 4ºC. In the present study, soil temperatures rose above 15ºC at Site A from September to November, and at Sites B and C from October to November, however there was no obvious rapid decline of *E. coli* relating to soil temperature changes within these periods. Horswell *et al.* (2007) also found that *E. coli* numbers reduced to background levels following a week of increased temperatures and low rainfall (15 mm). Chandler and Craven (1980) reported that moisture-availability was a dominant factor in the survive-ability of *E. coli*. Hurst *et al.* (1980) also found that temperature and soil moisture content had a large influence on virus survival along with the degree of virus adsorption to the soil (adsorption increased as soil pH decreased) along with the presence of aerobic microorganisms. In the present study, the decay of the surrogate virus (MS2) was significantly correlated ($P<0.05$) with soil temperature and soil moisture at Sites B and C (Table 6-7), indicating that the inactivation of this virus was influenced by increasing temperatures and decreasing moisture levels in the surrounding soil environment. This agrees with the information presented in the Literature Review, p. 23.

Overall, when introduced to agricultural soil, enteric pathogens do decay and this decay may be influenced by climatic conditions, in particular, soil moisture and soil

temperature. Decay times and the influence of climatic variables may vary between sites, thus more research is required to refine the understanding of the drivers influencing decay at different sites under different conditions.

6.6. Conclusions

The key findings from this chapter were as follows:

- Enteric microorganisms in the biosolids-amended soil had shorter decay times than those in the nil-biosolids soil;
- The enteric bacteria and bacteriophage (inoculated and in sentinel chambers) in the field experiments, were detected in the soil (chambers) for 6 to 7 months;
- The changes in *E. coli* numbers inside the chambers were significantly correlated ($P<0.05$) with the changes in *E. coli* numbers outside the chambers (topsoil) at Sites B and C, thus indicating that the chambers were suitable microcosms to represent the external environment;
- Decreasing soil moisture over the duration of the experiment significantly influenced ($P<0.05$) most enteric microorganisms at Sites B and C, particularly in the unamended soils (Table 6-7);
- Increasing soil temperature significantly influenced ($P<0.05$) virus (MS2) decay patterns at Sites B and C, as well as *E. coli* decay patterns at Site B in the biosolids-amended and unamended soil (Table 6-7);
- The changes in moisture content inside the chambers was significantly correlated ($P<0.001$) with the moisture content outside the chambers (topsoil) at Site B, thus demonstrating that gaseous and moisture exchange had occurred between the surrounding soil and the chamber membranes;

Further conclusions were made from this chapter:

- It was observed that the enteric bacteria decayed faster than the enteric viruses in the soil (chambers), therefore decay times were dependant on microorganism type;
- The decay of *E. coli* at Moora in 2006 (Site A) was similar to that of Site C at Mt Compass in 2008 with reduction times (T_{90}) of less than 12 d, but Site B (Moora 2008) was vastly different with decay times of up to 83 d;
- The decay times for *S. enterica* in the nil-biosolids soils at Moora were higher than the biosolids-amended soils (21 to 25 d), and both treatments at Mt Compass were between 37 and 57 d;
- Based on the results, the author recommends that *E. coli* should not be used as an indicator to represent the patterns of other pathogen types, in particular from soil or biosolids medium (water samples may provide different results);
- Due to the use of inoculation and higher biosolids application rates, decay times reported were overestimations of what would normally be expected to occur in the field, and this must be taken into consideration when reviewing the results;
- A general trend occurred at three sites where MS2 experienced a rapid decrease between 150 to 180 d. This may have been related to increases in temperature (above 20ºC) that occurred over this period;
- Adenovirus showed no significant decay across the duration of the experiment. This may have been a result of the use of a molecular technique (PCR) which is able to detect the adenovirus regardless of the level of infectivity. The use of 'detection' provided a more conservative estimation, which was preferable over underestimating decay in relation to the protection of public health;
- The enteric bacteria and viruses, inoculated into the soil at high numbers, were below one-$\log_{10}$ by the end of the growing season of wheat (6 to 7 months);

The results of this chapter suggest that the period of time where the public would be at greatest health risk is when biosolids are first applied to the cropping site. Increased risk occurs where temperatures are low and rainfall is high, usually from the autumn to spring months in regions where wheat is produced (below the Tropic of Capricorn) in Australia.

CHAPTER 7 THE DECAY TIMES OF *E. COLI, S. ENTERICA* AND MS2 FROM THE PHYLLOSPHERE AND ON GRAINS OF WHEAT

7.1. Introduction

The concern that foodborne illnesses may occur from food products contaminated with microbial pathogens has mostly been associated with the fresh fruit and vegetable industry, in particular from contaminated animal manures and irrigation water (Beuchat 1996; Doyle 2000a, 2000b; Buck *et al.* 2003; Johannessen *et al.* 2005). In broadacre cereal crop production, the risk to consumers from contaminated plant and grains due to the use of biosolids is not fully understood since very little research has been conducted on the quantification of human enteric pathogens in the phyllosphere of plants (Ibekwe *et al.* 2004). In particular, no studies could be found on the decay times of enteric pathogens from the plant components of wheat or other cereal plants.

There is a potential risk or concern that enteric pathogens, present in biosolids-amended soil, could transfer onto the grain heads of food crops and thus be transmitted to humans. Contaminated soil, manure compost and irrigation water have been responsible for the contamination of salad and vegetables (Solomon *et al.* 2002; Ibekwe *et al.* 2004; Islam, Morgan *et al.* 2004; Ibenyassine *et al.* 2006; Ibekwe *et al.* 2009). This has resulted in diseases being transmitted from wastewater sources via the faecal-to-oral route (Abdulraheem 1989). The survival of enteric pathogens in soil, and subsequent recontamination onto plant leaves during rainfall events, may be a primary source of transmission from sludges (Brown *et al.* 1980). Pathogens such as faecal bacteria and viruses may be dispersed from the soil onto plant leaves by factors such as rain splash and may persist through natural physical barriers (Boyer 2008). If this was to occur, contaminated grains and fodder may transmit diseases to humans and livestock at consumption. Primarily, the responsibility of consumer safety has moved from final cook or consumer across the food supply-chain back to the producer. Thus, from a marketing point of view it is particularly important to

assess the bio-safety of cereal crops grown on biosolids-amended land (Chaney *et al.* 1996; Tauxe 2002).

Most studies on pathogen survival in the plant phyllosphere have been conducted on fruits and vegetables which are consumed raw or have minimum preparation time prior to consumption. Prolonged survival of *E. coli* O157:H7 on the plant phyllosphere has been previously reported, for example on lettuce (Solomon *et al.* 2002; Solomon *et al.* 2003; Islam, Doyle *et al.* 2004; Ibekwe *et al.* 2009), ryegrass (Sjogren 1995), grassland (Bolton *et al.* 1999) and onions or carrots (Islam *et al.* 2005).

The comparison of pathogen survival times is difficult across these studies due to the different methodology used (Franz *et al.* 2005). Results from these studies may not directly relate to cereal crops due to different plant types, growing seasons and climatic conditions. No studies could be found on the presence of, or inactivation times, of enteric pathogens on cereal crops contaminated from biosolids.

The purpose of the research reported in this chapter was to examine the decay times of *E. coli, S. enterica* and bacteriophage MS2 from the phyllosphere of wheat and the decay times of the same microorganisms on wheat grains. The specific objective was to compare the decay times of individual microorganisms on two locations of the wheat plant – 1) the leaves and 2) the spikelets at flowering time. In addition, the decay times of individual microorganisms on the grains of two wheat varieties was also investigated.

7.2. Materials and methods

7.2.1. Experimental site

Two glasshouse experiments were carried out at the CSIRO Land and Water, Floreat, WA. The first experiment, a plant experiment, was conducted during the spring of September 2006 and the second experiment, a grain experiment, was conducted during the winter from May to July 2007.

7.2.2. Establishment of wheat plants

Three pots were prepared (TerraBoxes™, Planterra, 450 mm length x 150 mm width x 100 mm depth) with sieved soil (<2 mm) from Moora Site A (as described in Chapters 3 and 6). Soil was amended with non-sterile biosolids sourced from Beenyup wastewater treatment plant, Perth, WA at a rate equivalent to 10 t DS ha^{-1}. Biosolids were incorporated with the topsoil (<10 cm) to resemble a similar environment (i.e. soil moisture and temperature) where biosolids are applied in the field. Noodle wheat (*Triticum aestivum cv.* Calingiri) was sown into the soil (10 grains pot^{-1}) at a depth of 2.5 cm and 4.5 cm wide row spacing at a rate equivalent to the field seeding rate of 60 kg ha^{-1}. Fertilisers applied were diammonium phosphate (77 mg kg^{-1}) and urea (53 mg kg^{-1}) at rates equivalent to what would be applied in the field. Pots were maintained at gravimetric soil water holding capacity without leaching during the plant development stage (for approximately 4 months). At the time of the experiment, each pot contained approximately 10 flowering plants with well-formed heads (stage Z65-71 Zadoks scale (Zadoks *et al.* 1974) as indicated in Figure 7-1).

The microorganisms tested were *E. coli, S. enterica* and bacteriophage (MS2), cultured and prepared as described in Section 3.3, p.54. The final bacteria suspensions had a final cell count of more than 1 x 10^8 cfu mL^{-1} and the bacteriophage had a final suspension of more than 1 x 10^8 pfu mL^{-1}. Inoculant cultures of *E. coli, S. enterica* and MS2 were applied to the leaves and the spikelets in two to three applications using a sterile brush for even distribution. A 10 min drying time was allowed between applications.

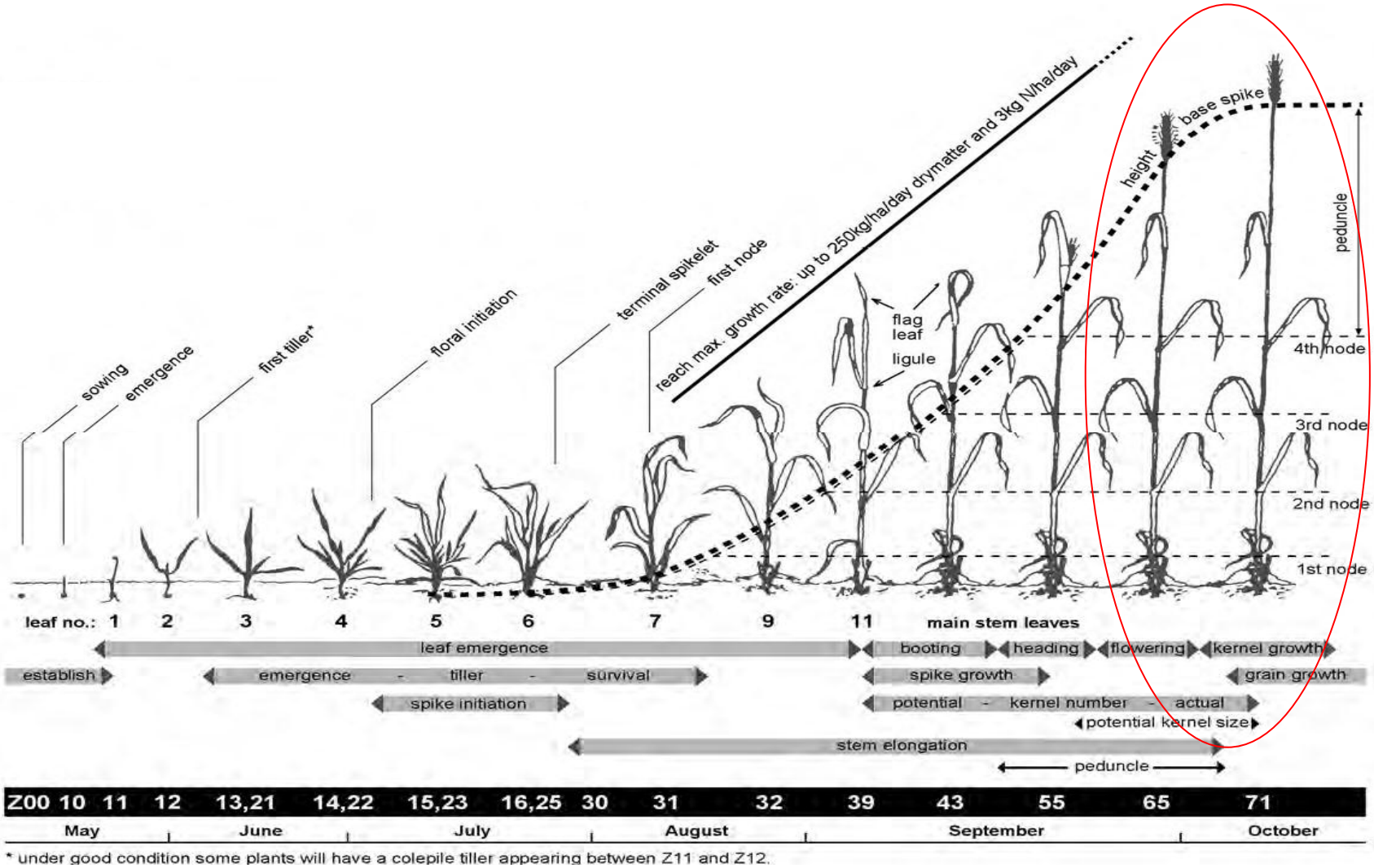


Figure 7-1: Zadoks growth stages for cereal plants (Zadoks *et al.* 1974) showing the stage used for experimental work.

Plant samples were collected at hours 0, 1, 2, 4, 6 and 8. Sampling frequency was then reduced to 26, 28, 53, 64, 206 h to a maximum of 9 d. At each sampling event, three plant leaves and three spikelets (grain heads) from three pots were randomly selected (n=3 samples per treatment). All samples were placed directly into sterile Bag Filter® (Interscience) bags and transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA for processing within 5 h.

7.2.3. Establishment of harvested grains

Wheat (*Triticum aestivum*) was collected from Co-operative Bulk Handling Ltd (CBH, Forrestfield, WA) where grain produced across WA is pooled together and stored according to segregation. The two varieties tested were noodles (NN), typically used for pasta, and Australian Soft White (ASW), used to produce flour for biscuits or cakes. Feed wheat (FF), typically used for livestock, was also examined but not reported in this chapter due to similarities in results with the ASW wheat.

One portion (1.5 kg) of wheat grains was inoculated with *E. coli*, *S. enterica* and bacteriophage MS2 inoculums, prepared as described in Section 3.3. Inoculant cultures were applied by evenly distributing the grains across a tray and spraying the inoculums onto the grains with a fine-mist atomizer. The other control portion (1.5 kg) was sprayed with sterile water to match the moisture content of the amended grains. Grains were tossed several times and allowed to air-dry for 10 min between applications for even distribution. Seven tins (500 g) with lids were established to represent stored grains - three containing inoculated grains, three containing non-inoculated grains (control), and one test tin for monitoring moisture and temperature changes inside the tin. The grain moisture and temperature was tested using an Infratec at CBH, Forrestfield, WA. Tins were stored in the glasshouse, under direct sunlight (non artificial), with the lids on to represent grain storage silos.

Grain samples were collected at days 0, 1, 2, 7, 14, 21, 30 and 35. Sampling frequency was then reduced to fortnightly intervals (days 50 and 63) to a maximum of 63 d. At each sampling event, three representative samples of grain from three tins were selected for each treatment (n=9 samples per treatment). All samples were placed directly into sterile Bag Filter® (Interscience) bags and transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA for processing within 8 h.

7.2.4. Enumeration of microorganisms

The net weights of sample contents in each stomacher bag (Bag Filter® Interscience) were obtained (i.e. ~3 g plants and ~15-25 g for grains). P-buffer (pH 7.2) was aseptically added (20 mL to plant samples and 50 mL to grain samples). All sample bags were placed in the stomacher (Bag Mixer®, Interscience) and mixed for 2 min (speed no. 7). The supernatant was collected, transferred into sterile polypropylene tubes (Sarstedt) and serial 10-fold dilutions were made in P-buffer. The quantification of pathogens was performed as described in Section 3.4, p. 54.

7.2.5. Glasshouse conditions

Air temperature in the glasshouse was maintained at 17˚C (± 0.25) by an air-conditioning unit and relative humidity maintained at 72% (± 0.97). Temperature and solar radiation levels were monitored inside the glasshouse during the grain experiment. Global solar radiation was recorded from inside the glasshouse using an automated Global Radiation Instrument (Unidata model 6501-F/G) connected to data logger (Unidata Starlogger 6004-2). Daily air temperature and relative humidity during the winter (May to July 2007) were automatically recorded from inside the glasshouse every 20 min using a Tinytag Plus 2 (Gemini Data Loggers Ltd, UK).

7.3. Data analysis

7.3.1. Data preparation

Prior to statistical analysis, pathogen counts were normalised from the raw data as described in Section 3.5, p. 62. This was done to account for different dilutions, plating volumes, phosphate buffer levels and leaf, spikelet or grain volumes used. The log values of each microorganism were plotted over time and the decay times determined.

7.3.2. Statistical analysis

All statistical analyses were performed with the generalised linear model (GLM) using SAS version 9.1 (SAS Institute Inc. 2005).

7.3.2.1 Analysis of variation sources for decay of individual microorganisms

The generalised linear model of ANOVA was used for the plant experiment to identify significant variation sources affecting final pathogen counts ($\log_{10}$ *Count*) in individual experiments. The variation sources included the fixed effects (treatment, linear terms of a covariate - sampling date, their interactions, pot and sample effect). The statistical model can be written as described in Section 5.3.1 (Equation 4), p. 85:

$$Log_{10}Y_{ijl} = \mu + T_i + S_j + (TS)_{ij} + C_l\,(T)_i$$

a) in the plant experiment Ti =1, 2 corresponds to spikelet or leaf, respectively; and $C_l\,(T)_i$ is the effect of the lth sample collected (l=1, 2, 3) nested within the ith treatment;
b) in the grains experiment, T is replaced by B which corresponds to each block. B_k is the effect of the block (k =1, 2 corresponding to grain segregations ASW and NN); $(BS)_{jk}$ is the interaction between the kth block by the jth sampling date; $C_l\,(B)_i$ is the effect of the lth sample collected (l=1, 2, 3) nested within the kth block.

The least-square effects of the fixed factor comparisons were then produced for both experiments. The regression coefficients of sampling date (linear terms) within each treatment were used as the indication of pathogen decay times.

Based on the regression coefficients of corresponding terms in the simple linear model ("sdate" and "sdate*treatment"), where sample date was involved for either experiment, one-$\log_{10}$ decay time (T_{90}) for each microorganism in the plants experiment (h) and the grains experiment (d) were determined from each pot using the formula described in Section 5.3.1 (Equation 5).

7.3.2.2 Comparison of decay rates across all microorganisms

A linear mixed model ANOVA was formulated for the plant experiment to compare significant effects within trial across microorganisms as described in Section 4.3.1.2 (Equation 3), p. 70.

$$Log_{10}Y_{ijmn} = \mu + T_i + O_m + S_j + (OS)_{jm} + (OT)_{im} + (OTS)_{ijm} + C_l\,(OT)_{im} + e_{ijmn}$$

with the addition of the following modifications where, i =1, 2 corresponds to the spikelet or leaf; m = *E. coli*, *S. enterica*, MS2; and the block effect (B) was not fitted in the model due to confounding effects between block and treatment.

A similar model was also used for the grains experiment to compare significant effects within trial across microorganisms. The modification to the above equation was that the fixed effect of treatment (T_j) was replaced with (B_k), the fixed effect of the $_k$th (k =1, 2 corresponding to the different grain types ASW or NN) in each block.

7.4. Results

7.4.1. Environmental conditions

Temperatures inside the glasshouse during the plant experiment averaged 16.5ºC (Table 7-1). Mean solar radiation readings (manually recorded) were 124.9 W m^{-2} with a maximum of 656.3 W m^{-2}. Manual light intensity readings taken at each sampling event are presented in Figure 7-2.

The temperature and relative humidity recorded (automatically) inside the glasshouse for the grains experiment is presented in Figure 7-3. Temperatures inside the glasshouse during the experiment averaged 17.4ºC and relative humidity averaged 72.2% (Table 7-1).

Table 7-1: Climate conditions in the glasshouse during the plant component and grain variety experiments.

Glasshouse condition	Mean	Maximum	Minimum
Plant experiment – Spring 2006			
Temperature (ºC)	16.5 (±1.3)	23.4	14.3
Solar radiation (W m^{-2})	124.9 (±13.9)	656.3	1.37
[a]External temperature (ºC)	16.6 (±0.26)	18.9	14.1
Grain experiment – Winter 2007			
Temperature (ºC)	17.4 (±0.25)	22.9	13.9
Relative humidity (%)	72.2 (±0.97)	87.8	43.9

[a]Recorded by Swanbourne weather station (www.bom.gov.au)

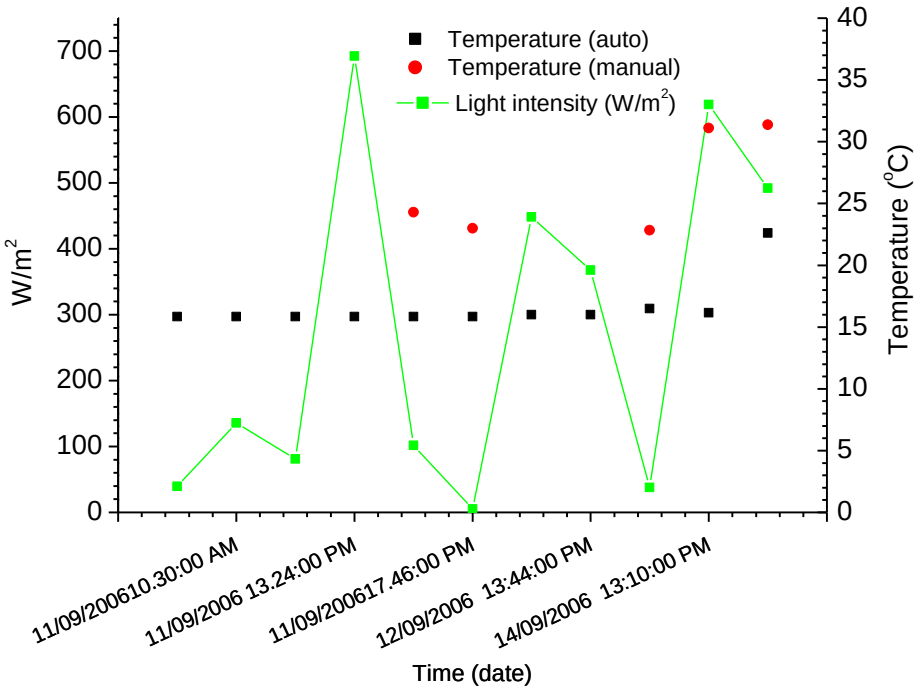


Figure 7-2: Daily temperatures (°C), temperature during sample events and solar radiation levels (W m^{-2}) recorded for the plant experiment.

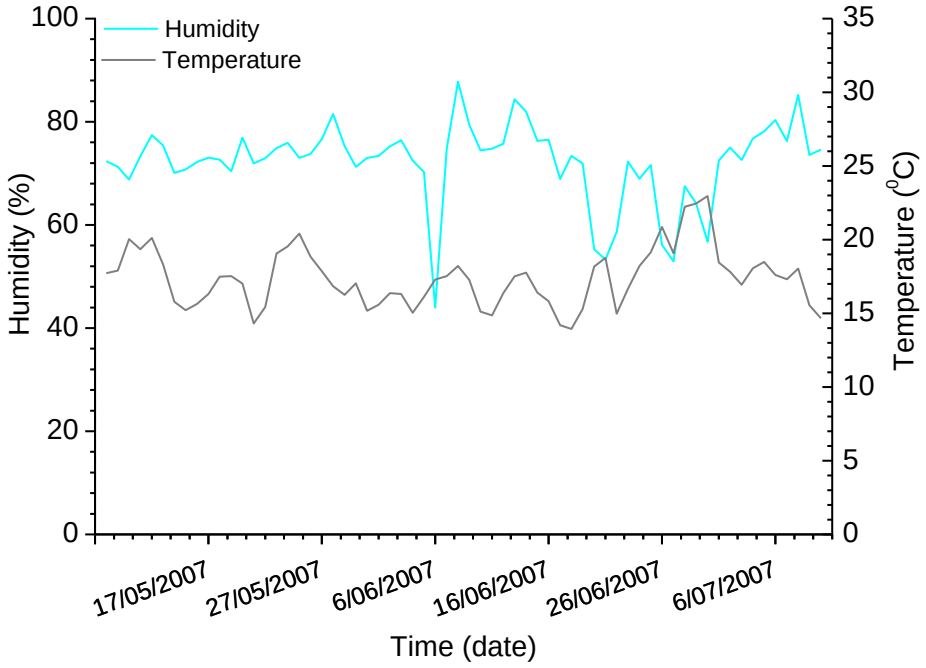


Figure 7-3: Temperature and humidity recording during the grains experiment.

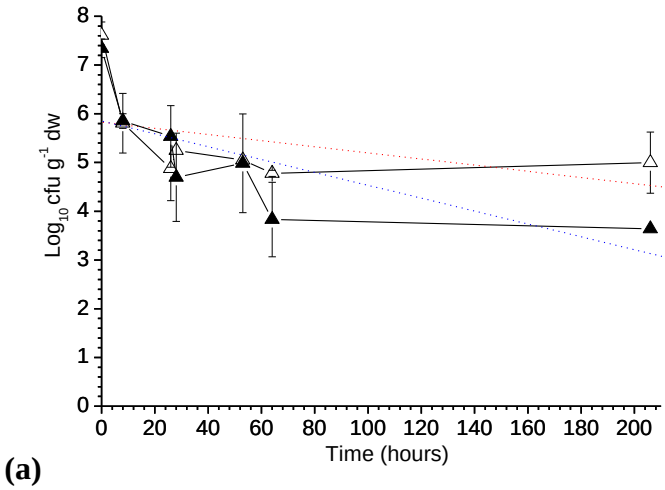
7.4.2. Survival patterns of microorganisms on the phyllosphere

The inactivation rate of *E. coli, S. enterica* and MS2 was examined from the leaves and spikelets of wheat to determine decay times. The experiments were conducted in relation to the potential transfer of pathogens from the soil up onto the cereal plants from the use of biosolids.

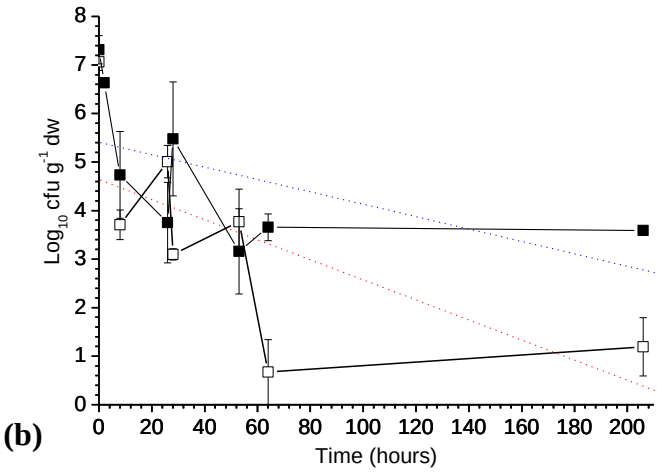
The observed changes in *E. coli, S. enterica* and MS2 numbers are presented in Figure 7-4. The error bars in the following figures represent the standard deviation between the means of the replicates. *E. coli* and *S. enterica* on the wheat leaves decreased approximately four to six-$\log_{10}$ cfu g^{-1} over the duration of the experiment (200 h or 8 d). Inactivation of MS2 was faster with approximately three-$\log_{10}$ cfu g^{-1} loss over the same period. *E. coli* and *S. enterica* from the spikelets decreased approximately three to four-$\log_{10}$ cfu g^{-1} over the duration of the experiment. Inactivation from the spikelets was slower with decreases of approximately four to six-$\log_{10}$ for *E. coli, S. enterica* and MS2. Overall, MS2 decayed the fastest from the spikelets and the leaves (Figure 7-4c).

The decay times for *E. coli, S. enterica* and MS2 are presented in Table 7-2. The decay times (T_{90}) of the enteric microorganisms on the leaves tended to be longer (35 to 72 h) compared with the spikelets (23 to 51 h), however this was not significant for *E. coli* (P=0.23) nor *S. enterica* (P=0.08).

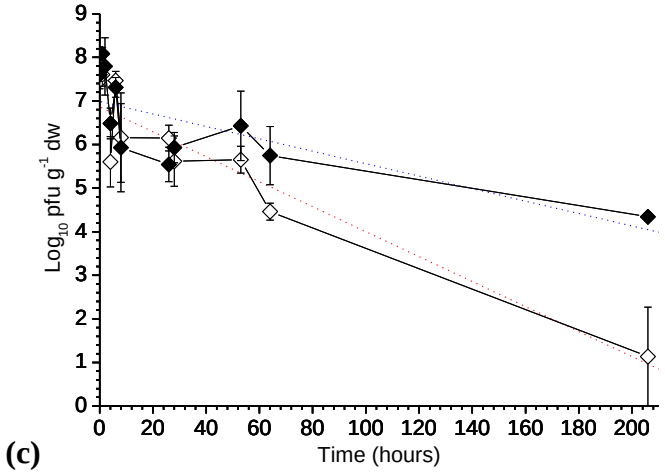
The three microorganisms behaved significantly different (P<0.001). On the leaves, *E. coli* decayed significantly slower than *S. enterica* (P<0.001) and MS2 (P=0.001). The difference in *S. enterica* and MS2 decay times was also highly significant (P<0.001). The decay time of MS2 on the leaves was the shortest (35 h). *S. enterica* on the spikelets decayed significantly faster than *E. coli* (P<0.001) on the spikelets. The difference in decay times between *E. coli* and MS2 on the spikelets was also significant (P=0.001). Again, the decay time for MS2 on the spikelets was the shortest (23 h).



(a)



(b)



(c)

Figure 7-4: Decay patterns on wheat leaves (■) and spikelets (□) with linear regression lines for the leaves (—) and spikelets (—) where (a) is *E. coli;* where (b) is for *S. enterica*; and (c) is for MS2. Standard error bars are shown.

Table 7-2: Time for a one log_{10} reduction (T_{90}) to occur for enteric microorganisms on the leaves and spikelets of wheat.

	T_{90} times (h)	
Microorganism	**Wheat leaves**	**Spikelets**
E. coli	72	51
S. enterica	57	34
Bacteriophage MS2	35	23

NB: The standard deviation values on individual sampling events are provided in the figures.

Table 7-3: Time for a one log_{10} reduction (T_{90}) to occur for enteric microorganisms on wheat grains.

	(T_{90}) times (d)	
Microorganism	**Noodle grains (NN)**	**ASW grains**
E. coli	9	10
S. enterica	10	12
Bacteriophage MS2	60	71

NB: The standard deviation values on individual sampling events are provided in the figures.

7.4.3. Survival patterns of microorganisms on grains

The decay times of *E. coli, S. enterica* and MS2 were examined on stored wheat grains so that the potential contamination risk to humans and livestock, where biosolids have been used, could be evaluated. Figure 7-5 presents the decay patterns of the three enteric microorganisms from the stored wheat grains. The error bars in the following figures represent the standard deviation between the means of the replicates.

It can be seen that *E. coli* decreased approximately four-$\log_{10}$ cfu g^{-1} and *S. enterica* decreased approximately three-$\log_{10}$ cfu g^{-1} over the duration of the experiment (~60 d). The decay of MS2 was much slower with a loss of less than one-$\log_{10}$ pfu g^{-1} over the experimental period.

The decay times for *E. coli, S. enterica* and MS2 are presented in Table 7-3. The decay times (T_{90}) of enteric bacteria from both grain varieties were less than 12 d. MS2 decay times were longer at 60 to 71 d. There was a significant decay of *E. coli* and *S. enterica* (P=0.001 and P=0.003, respectively) from the noodle grains compared with the ASW grains; however, this difference was not significant (P=0.81) for MS2.

The decay patterns across all three microorganisms on the noodle grains was significantly different (P<0.001). The decay times of *E. coli* and *S. enterica* were shorter than MS2 and there was a significant difference (P=0.009) between the changes in all three microorganisms across the grain varieties tested.

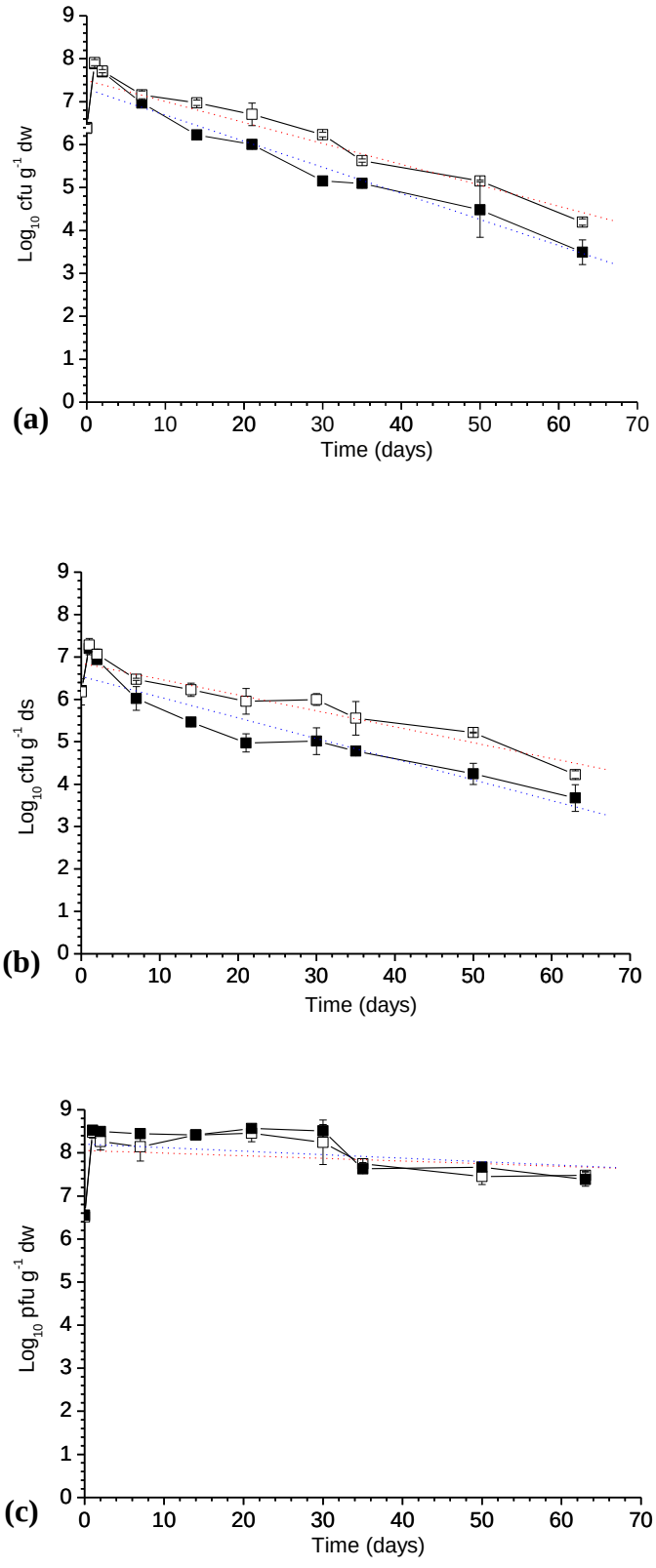


Figure 7-5: Decay patterns of (a) *E. coli,* (b) *S. enterica,* and (c) MS2 on wheat grains where NN is (■) and ASW is (□), and linear regression lines are NN (—) and ASW (—). Standard error bars are shown.

7.5. Discussion

7.5.1. The decay times from the phyllosphere and grains

The estimated inactivation times (T_{90}) of *S. enterica* and *E. coli* on the phyllosphere were 2 to 3 d (57 to 72 h, respectively) on the leaves and 1 to 2 d (34 to 51 h, respectively) on the spikelets. In comparison, on lettuce much longer decay times of 15 to 77 d have been reported for *E. coli* O157:H7 (Beuchat 1999; Solomon *et al.* 2002; Solomon *et al.* 2003; Islam, Doyle *et al.* 2004; Ibekwe *et al.* 2009) and 63 d for *Salmonella typhimurium* (Islam, Morgan *et al.* 2004). *E. coli* O157:H7 has also been reported to persist for 41 d on ryegrass (Sjogren 1995), 99 d on grassland (Bolton *et al.* 1999) and 74 to 168 d on onions and carrots, respectively (Islam *et al.* 2005). Patel *et al.* (2010) reported *E. coli* O157:H7 survival times from 7 to 14 d on spinach leaves when co-inoculated with non-pathogenic *E. coli.* Islam, Morgan *et al.* (2004) found that *S. enterica* persisted for 161 d on parsley. Kroupitski *et al.* (2009) found no decline in *Salmonella* on lettuce over 9 d.

The longer survival times of bacteria on plant surfaces such as parsley and lettuce (Islam, Doyle *et al.* 2004) may have been attributed to crop density. Crops such as alfalfa have shown increased survival times of enteric microorganisms due to the reduction of desiccation and sunlight effects (El Hamouri *et al.* 1996). Such complex leaf structures may provide protection for enteric pathogens on the phyllosphere whereas enteric pathogens on plants with flat, wider leaves (similar to wheat) may be more exposed to drying and sunlight inactivation. The longer survival times for *E. coli* O157:H7 on onions and carrots (Islam *et al.* 2005) may be attributed to the protective effect under the soil, in moist conditions and in the absence of direct sunlight.

Estimated decay times (T_{90}) for MS2 were 35 h on wheat leaves 23 h on spikelets (i.e. approximately 1 d). These results were similar to Choi *et al.* (2004) where one log reduction times of 1 to 2 d were reported for bacteriophage (MS2 and PRD1) on the surface of lettuce. The short decay times were thought to be due to high temperatures over the duration of the experiment. In other studies, poliovirus survival was reported as actual survival times of more than 76 d on celery and 55 d on spinach maintained at 4ºC in a humid atmosphere in the dark (Ward and Irving

1987). Brown *et al.* (1980) observed that coliphage on grass could not be detected after 24 h followed by simulated rainfall events. Croci *et al.* (2002) observed that only a slight decrease in hepatitis A virus occurred (over 9 d) and saw complete inactivation within 7 d for fennel.

Despite the different growing conditions, irrigation sources and methods used in the above studies, the persistence of viruses remained within the growing season and consumption times of the harvested produce. The growing season for cereal crops is much longer than that of vegetable and salad crops, and the consumable parts for wheat are not grown close to the soil. Along with this, the time available for enteric pathogens to be inactivated is longer for cereal crops grown in dryland conditions than for vegetable and salad crops, therefore the risk of disease transmission is considered to be lower. This is supported by Wilkinson *et al.* (2003) who found that *E. coli* numbers were higher in the soil during the crop growing period but had dropped markedly by harvest time. Wilkinson *et al.* (2003) chose food crops that are consumed raw because of the higher risk of food-borne illness that is evident in the absence of cooking.

Due to the rapid reduction times (<3 d) reported in the present study from the phyllosphere of wheat, the risks to the consumer are considered much lower than salad and vegetable crops. For wheat crops, this means that the use of biosolids is considered a safe option and that minimal risk of disease transmission is imposed. This is particularly evident when comparing the decay times of perishables such as lettuce, alfalfa, parsley and carrots which have actual longer survival times (<168 d) and are consumed immediately following harvest.

7.5.2. The effect of microorganism type on survival times

The survival times of enteric bacteria on the phyllosphere and grains depended on the type of microorganism. *S. enterica* had significantly faster inactivation times than *E. coli* when seeded onto the leaves, and bacteriophage was significantly faster than enteric bacteria. In general though, the survival patterns were similar for both microorganisms. This may be related to poor natural attachment capacity of *Salmonella* to the plant surface. It has been observed that plants have the ability to deter the attachment of *Salmonella* (Barak *et al.* 2008). In the plant experiment, the

opposite occurred for the spikelets where the inactivation time of *E. coli* was significantly faster than *S. enterica*. Different enteric bacteria have different attachment properties, indicating species variability with regard to attachment (Critzer and Doyle 2010). In the present study, the different microorganisms tested had different inactivation rates from the different parts of the wheat plant. This demonstrates the inability of an indicator microorganism (such as *E. coli*) to truly represent an enteric pathogenic patterns (such as *Salmonella*). In particular, *E. coli* as an indicator should not be expected to represent a whole suite of pathogens that may be present in biosolids.

7.5.3. The effect of climatic conditions on survival

Virus inactivation can increase with increasing temperatures (Abdulraheem 1989; Aruscavage *et al.* 2006). On the leaves, the decay time (T_{90}) of bacteriophage was significantly shorter than enteric bacteria (i.e. 35 h). The same trend occurred on the spikelets where the decay time for bacteriophage was significantly shorter than the bacteria (i.e. 23 h). This may be due to bacteriophage irreversibly binding onto the plant surfaces or just dying off more rapidly. This also occurred in Sidhu *et al.* (2008) where inactivation times for bacteriophage on grass in the shade during winter were faster compared with enteric bacteria. However, this trend was not seen across any of the other microorganisms tested in their study.

Since viruses are commonly more resistant to adverse climatic conditions than bacteria, it was expected that bacteriophage should have survived longer than the bacteria cells on the wheat leaves. The average temperature in the glasshouse during the spring was 16.5ºC with a maximum of 23.4ºC and thus, the faster reduction of bacteriophage numbers in this case, may have been influenced by temperature as was also found by Choi *et al.* (2004).

7.5.4. The effect of location on plant on microorganism inactivation

The specific survival times of enteric pathogens on wheat plants may be influenced by the location of the microorganisms on the plant. The inactivation times of *E. coli, S. enterica* and bacteriophage tended to be shorter (P>0.05) from the spikelets than from the leaves. This was expected since the spikelets are located in the upper region of the phyllosphere where they are exposed to factors such as higher UV light intensity, desiccation and higher temperatures. This means that the region of the plant where grains are produced (and later consumed) is also the region of lower risk where the survival of enteric pathogens is significantly reduced.

The desiccation of enteric microorganisms exposed to light and temperature was also found by Sidhu *et al.* (2008). The leaves are more subject to shading, increased moisture levels and other microbial communities (Lindow and Brandl 2003). Since enteric pathogen populations may reside and survive between the leaves and stems of the plants (Brown *et al.* 1980; Ibekwe *et al.* 2004), it would be expected that longer detection times of pathogen numbers should occur. However, the leaves are also subject to rapid and large fluctuations in temperature, humidity, and osmotic pressures (Wilson *et al.* 1999) which may be detrimental to the survival of enteric pathogens. Other factors such as waxes (Aruscavage *et al.* 2006) may restrict bacterial attachment to leaf surfaces, and the competition for nutrients and moisture makes enteric pathogen survival on leaf surfaces more difficult (Mercier and Lindow 2000).

7.5.5. The effect of grain variety on microorganism inactivation

The survival times of enteric pathogens on grains appears to be influenced by grain variety. The inactivation of enteric bacteria was faster on the noodle grains (NN) than the soft wheat (ASW) grains, with all T_{90}s being less than 10 d. Likewise, the inactivation of MS2 was also faster on the noodle grains compared with the ASW grains, with T_{90} times of 60 to 71 d. Grain varieties contain different properties which are suited to different end uses i.e. noodle wheat is used for the production of pasta, ASW for the production of flour, doughs and biscuits, and feed wheat is used for consumption by livestock. As a result of the different properties, the grains vary in strength i. e. noodle grains are softer and ASW grains are more brittle. In any case,

grain varieties such as noodles and ASW are subjected to milling (for flour production) and cooking prior to consumption and such processes result in a further reduction of any enteric pathogens that may be present. This means that the risks to the consumer (from pathogens) are also reduced.

Bacteriophage is more persistent in humid conditions than in dry conditions (Choi *et al.* 2004). The prolonged persistence of bacteriophage on the grains in this study may have been due to the higher humidity levels that occurred inside the tins. At seeding, the grains were spray-inoculated with cultures and then stored in sealed tins. Prior to inoculation, grain moisture levels were approximately 10% and with the added moisture from the inoculant cultures, the grains would have started the process of 'sweating' inside the tins. Moisture levels in wheat grains are known to increase approximately 1 to 2% (i.e. from 11.5% to 13.5%) when sealed in enclosed areas and stored in the sun such as in trucks covered with tarpaulins or grain silos. Therefore, the longer inactivation times of bacteriophage on the grains in stored tins may have been influenced by increased humidity levels along with the absence of direct exposure to UV rays and thus avoided desiccation from the sun.

Based on the results of the present study, the risks to the consumer from wheat products grown from biosolids-amended land are considered to be very low. In the experiments reported in this chapter, the enteric microorganisms were inoculated onto the wheat leaves, spikelets and grains at higher numbers than would be expected to occur where biosolids have been land-applied in the field. In addition, the results from the field soil studies showed that the same enteric microorganisms, when inoculated at high starting numbers into the soil in late autumn-winter, were mostly reduced to low levels (<4 $\log_{10}$ cfu g^{-1}) in the soil by the spring and summer time. It would be expected that only a small percentage of pathogens would be transferred from the soil onto the phyllosphere through transmittable means such as rain-splash or by becoming wind-borne. The results of the present study demonstrate that , if present, microorganism numbers are rapidly reduced. In addition, climatic conditions in the spring and summer months such as increasing temperatures, decreased moisture levels, decreased humidity and increased UV intensity do not favour the persistence of pathogens on wheat plants or grains.

7.6. Conclusions

The following conclusions were made from this chapter:

- The type of microorganism affected the decay times. The enteric bacteria seeded onto the wheat plant had longer decay times (T_{90}) of 34 to 72 h than the virus (23 to 35 h);
- The enteric bacteria seeded onto the stored grains had decay times of less than 12 d. The surrogate virus persisted for longer with decay times of 60 to 71 d;
- The location of the microorganisms on the plant made a difference to the decay times. The enteric microorganisms tested in the phyllosphere experiment generally persisted longer on the leaves of the wheat plant than the spikelets even though this was not statistically significant;
- The grain variety made a significant difference to the decay times of the enteric microorganisms tested. The enteric microorganisms on the biscuit variety of grains (i.e. ASW) tended to persist significantly longer than those on the pasta variety (NN);
- The risk that enteric pathogens may persist on wheat plants and grains (until consumed) is considered to be low. In addition, the climatic conditions that occur in the spring and summer are not favourable to the persistence of enteric pathogens on the phyllosphere or grains of wheat.
- Since most foods produced from wheat involve some form of processing such as grinding, milling, rolling, steaming and baking, the risks of human enteric pathogens originating from land-applied biosolids and transmitting to humans at consumption is considered to be very low.

CHAPTER 8 THE PRESENCE OF BACTERIA IN BIOAEROSOLS WHERE BIOSOLIDS ARE USED; AND EFFECT OF THRESHING ON PATHOGEN NUMBERS

8.1. Introduction

Bioaerosols, or aerosolised biological particles such as enteric pathogens, can travel over significant distances (Pillai *et al.* 1996; Pillai and Ricke 2002). The aerosols containing enteric pathogens may result in a potential health hazard if inhaled (Pillai and Ricke 2002; Pepper *et al.* 2006).

There is a concern regarding the occupational and public health safety associated with the exposure of bioaerosols from harvesting crops where biosolids have been previously applied. Farmers may be exposed to high levels of microorganisms when working with grain dust during threshing and grain storage work (Halstensen *et al.* 2007). The immediate risk of transfer of bioaerosolised pathogens from soil and wheat plants during harvesting is unknown. To date, the only information available is related to aerosols from wastewater treatment plants, the land application of wastewater, the health effects at composting plants and the risks at application of animal manures (Pepper *et al.* 2006).

There has been a series of studies carried out on airborne microorganisms such as fungal spores, mycotoxins (Ayalew *et al.* 2006), nephrotoxins, endotoxins and hyphae in grain crops (Halstensen *et al.* 2004; Halstensen *et al.* 2007) but only a few studies have been conducted on the generation of bioaerosols from land applied biosolids (Brooks *et al.* 2005b). Of these studies, most have linked waste application practices, biosolids handling, wind patterns and micrometeorological fluctuation to the aerosolisation of microbial pathogens (Pillai *et al.* 1996; Dowd *et al.* 1997; Pillai and Ricke 2002).

Since enteric pathogens can survive for several months in the soil and may transfer onto standing wheat plants, it is possible that any pathogens present in cereal crops at the time of harvesting may become airborne during threshing and potentially be

transmitted to humans through inhalation of associated dust or ingestion of contaminated grains and processed products. No studies have examined the fate and transport of enteric pathogens from cereal crop into wheat dust (bioaerosols) that is generated during harvesting and threshing. The purpose of the research presented in this chapter was to examine the potential presence of bacteria in bioaerosols generated during the harvesting operations of a wheat crop where biosolids have been applied. The fate of seeded microorganisms on mature wheat plants during threshing was also examined. The specific objectives were: to compare indigenous bacterial levels at biosolids application sites with unamended sites to examine whether biosolids influence bacteria numbers at the site; and to test the enteric microorganism numbers (on the spikelets, chaff, grains, thresher drum and dust) following threshing to determine whether threshing reduces microorganism numbers.

8.2. Material and methods

8.2.1. Experimental sites

The potential dispersal of pathogens via bioaerosols during harvesting and threshing of grains was examined in two separate studies. The experiment to examine bioaerosol generation during the threshing of wheat grains was carried out in an undercover area at Muresk Institute (Curtin University), Northam, WA. The experiment to examine the dispersal of aerosolised pathogens during wheat crop harvesting was carried out on a broadacre cropping farm at Moora, WA. Both studies were conducted over the summer harvesting period (December) over two years, 2008 and 2009.

Four field sites were selected for the harvester study: Site NA – a nil-biosolids site (30º48’19.53”S, 116º04’43.44”E), Site BB – a biosolids application site (30º49’10.14”S, 116º01’45.87”E), Site NC – a nil-biosolids site (30º50’18.4”S, 116º06’06.8”E) and Site BD – a biosolids application site (30º51’27.1”S, 116º05’22.1”E). The four sites were within 5 km radius of each other on the same property and consequently had the same climatic conditions (temperature and rainfall) and soil type (i.e. gravely sandy-loam). The sites in this study were different to those described in Chapter 6 although were carried out on the same property. Anaerobically-digested dewatered biosolids (Beenyup Wastewater Treatment Plant,

Perth WA) had been previously applied to Site BB in May 2006 (i.e. 3 years before harvest) and to Site BD in May 2009 (i.e. the same season). No biosolids had previously been applied to Sites NA and NC. All sites contained matured standing wheat crops, ready for harvest at the time of the experiments.

8.2.2. Bioaerosol samplers

The bioaerosol samplers used to collect the dust samples for both studies were SKC BioSamplers® (SKC West Inc., Fullerton, California) operating at an air intake rate of 12.5 L min^{-1} (Figure 8-1). All glassware was autoclaved at 121˚C for 15 min prior to use. In preparation for this study, an experiment was conducted to test three collection mediums (i.e. mineral oil, P-buffer and sterile distilled water) for the optimal recovery of the study microorganisms. Collection mediums were placed in the collection basin of the BioSamplers®. Results found that sterile distilled water provided the best recovery efficiency with a 1 to 2 log_{10} higher recovery rate than the P-buffer or mineral oil. For this reason, sterile distilled water was selected for use in the thresher and harvester experiments.

For the thresher experiment, samplers were clamped to a custom-built mount and placed approximately 5 to 10 cm from the dust outlet (Figure 8-2). The samplers were sealed with a large plastic bag to recover all the dust generated by the thresher.

For the harvester study, the samplers were mounted to a Toyota Landcruiser tray back utility parked approximately 20 to 30 m away downwind from the operating combine harvester (Figure 8-3). Air intake was provided through the use of three SKC Vac-U-Go pumps (SKC West Inc.) that were run for 2 min during sample collection.

NB: The following photos are for illustrative purposes only and were not taken while the thresher was in operation or where any inoculated pathogens were present. Full protective gear such as gloves, masks, suits and safety glasses is required to be worn during sample collection.

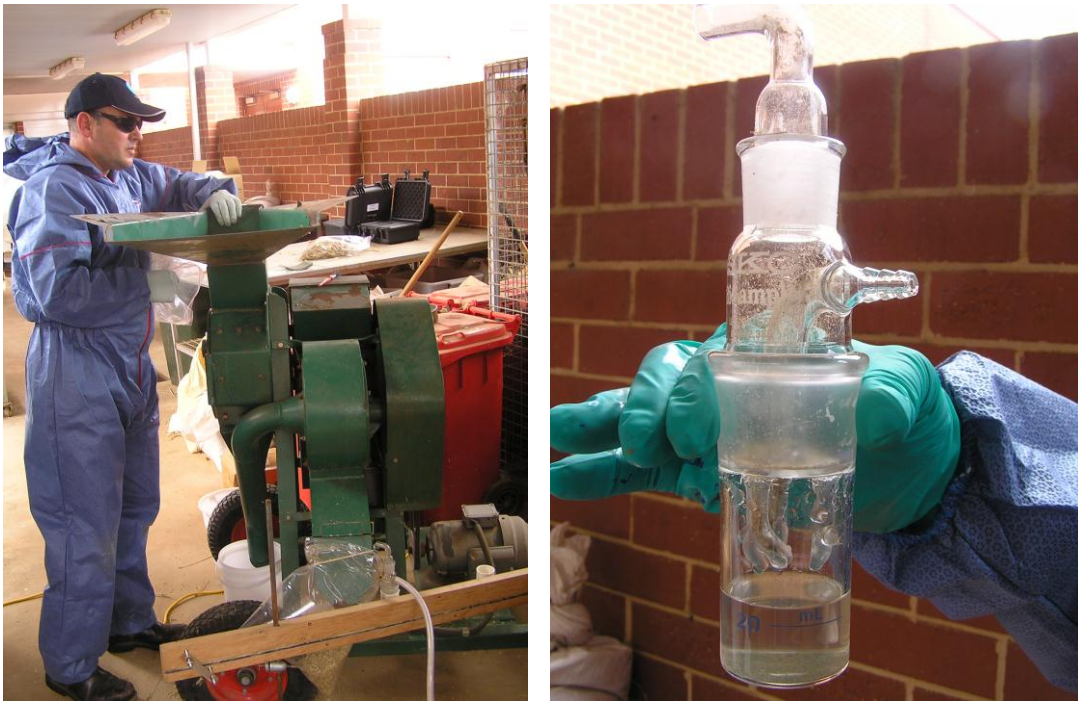


Figure 8-1: Left: Venables small seed thresher; Right: BioSampler collection vials.

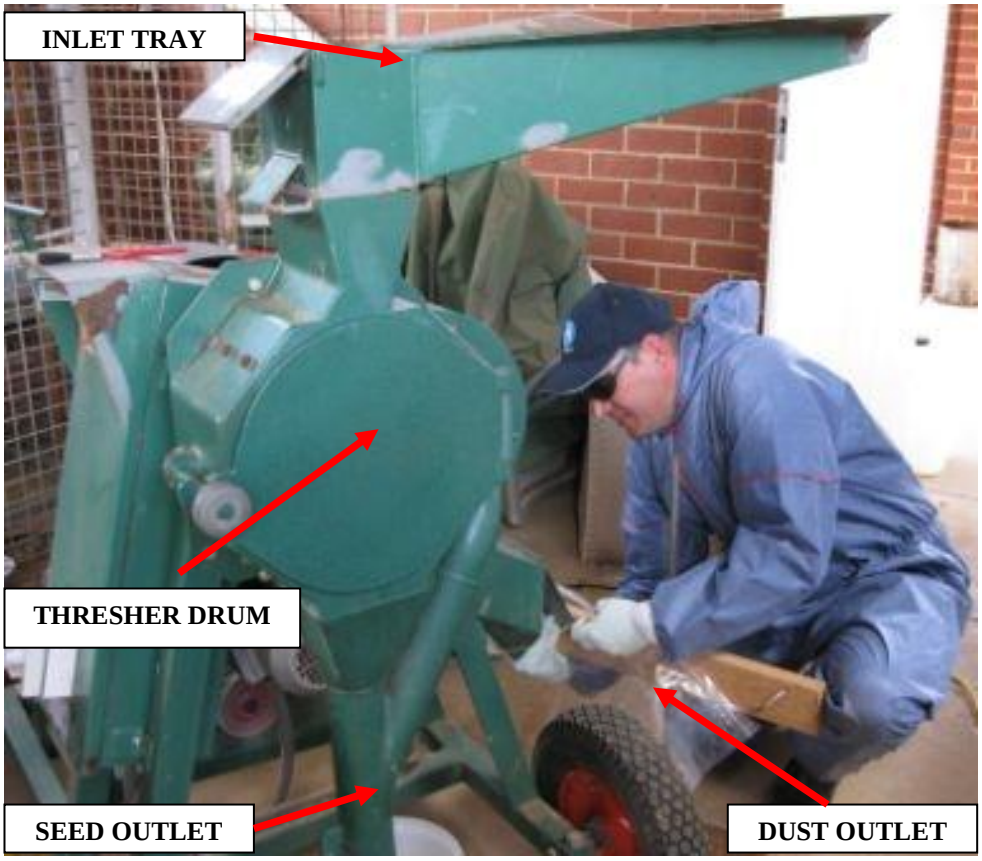


Figure 8-2: The sampling points on the Venables small seed thresher.

Figure 8-3: Collecting dust samples during wheat harvesting using three BioSamplers® mounted onto the frame of the Landcruiser utility.

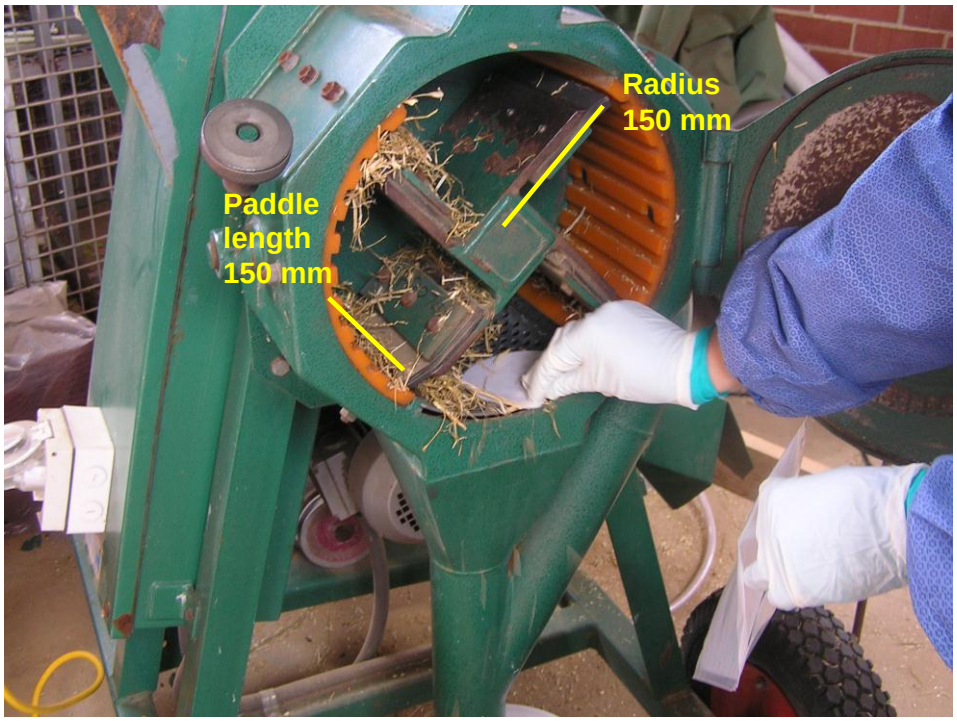


Figure 8-4: Taking swab tests from inside the thresher drum – 150 mm paddle length and 150 mm radius is shown.

8.2.3. Thresher experiment

Three individual microorganisms (*E. coli, S. enterica* and bacteriophage MS2) were tested across the stages of threshing to examine where the microorganisms were being directed during the process of threshing. The thresher experiment comprised three treatments: wheat spikelets (thresher control), wheat spikelets (control) and inoculated wheat spikelets (treatment). Wheat spikelets were weighed into 200 g portions. Wheat spikelets for the thresher control were established in triplicate (n=3). Seven replicates of the control wheat spikelets were established (n=7) and seven replicates of the inoculated wheat spikelets were prepared (n=7).

A Venables small seed thresher (L & T Venables, Wembley WA) was used for the thresher study (Figure 8-2). The swab samples were taken from the threshing drum (dimensions 300 mm diameter, paddle length 150 mm, slotted screen 150 x 150 mm = 225,000 mm^2). Dry, matured wheat plants (*Triticum aestivum cv.* Calingiri) were collected from the farming property at Moora where biosolids had been previously applied. Wheat stalks were cut approximately 25 cm below the seed head or spikelet (Figure 8-5) to match the approximate harvesting height used in the field and to reduce trash handling.

The microorganisms tested in the thresher experiment were *E. coli, S. enterica* and bacteriophage (MS2), cultured and prepared as described in Section 3.3, p. 54. The final suspensions had final cell counts of 1 x 10^6 cfu mL^{-1} of *E. coli,* 1 x 10^5 cfu mL^{-1} of *S. enterica* and 1 x 10^6 pfu mL^{-1} of MS2. Inoculants were seeded onto the spikelets with a fine-mist atomiser using three applications with 10-15 min drying time allowed between applications.

The pathways undertaken by microorganisms throughout the process of threshing and harvest is important to understand the potential exposure pathways (of pathogens) to humans or livestock from wheat crops cultivated where biosolids have been applied. The target microorganisms (*E. coli, S. enterica* and MS2) were seeded onto wheat spikelets and their dispersal following threshing was examined. An initial experiment was conducted in 2007 (unpublished data) where *E. coli, S.*

enterica and MS2 were inoculated onto spikelets and their numbers were tested in the dust samples. Following threshing (in the 2007 test), a 5-$\log_{10}$ cfu g^{-1} loss across all microorganisms had occurred. This initial (unpublished) experiment highlighted the need to investigate whether microorganisms were being deposited onto other threshing products such as the grains and chaff during threshing or whether they were being inactivated. Following this, the samples for the present study were then collected from the threshing drum, chaff and grains.

Samples were collected during operation of the thresher across a time period of approximately 2 h. At each sampling event, samples from each treatment were collected (i.e. spikelets, drum swabs, grains, chaff and dust) to a total of seven samples per treatment.

Swab tests were collected on 90 mm filter paper (Qualitative Advantec) from inside the threshing drum (Figure 8-4) and placed immediately into sterile, pre-weighed Bag Filter® bags (Interscience). Grain samples were collected from the seed outlet of the thresher (Figure 8-2) and placed into sterile plastic bags. A large sterile plastic bag was attached to the chaff outlet (to the rear of the thresher, not shown) to capture the chaff samples. To capture dust samples, each BioSampler® was loaded with 25 mL of sterile distilled water and clamped onto the dust outlet of the thresher (Figure 8-2). Samples were collected for 2 min during each operation, while spikelets were being processed through the thresher. BioSamplers® were sterilised and flushed with sterile water between runs. Following collection, samples were aseptically removed from the sample basin and placed into sterile 50 mL polypropylene centrifuge tubes (Sarstedt). All samples were transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA for processing within 24 h.

Figure 8-5: An example of wheat spikelets with encasing around the grains that later becomes chaff following threshing.

Figure 8-6: The Case IH 8010 Axial-Flow harvester used to generate dust.

8.2.4. Harvest (dust) experiment

The harvester study was designed to compare the numbers of individual bacteria at two sites to determine any effect of biosolids application to bacteria levels. The two treatments tested were a wheat crop previously applied with biosolids (i.e. the first trial in 2008 where biosolids had been applied 3 years prior, and the second trial in 2009 where biosolids had been applied in the same year) and a wheat crop where no biosolids had been applied (unamended). Dust samples were collected while the wheat crop was being harvested. A Case IH 8010 Axial-Flow harvester was used to harvest the wheat crop and to generate the wheat dust (Figure 8-6). In each year, the nil-biosolids site was harvested first followed by the biosolids-application site to avoid any cross-contamination.

Dust samples were examined for the presence of indigenous *E. coli*, enterococci and heterotrophic bacteria. *E. coli* and enterococci are known to be present in biosolids in Western Australia and they have been detected in the soil where biosolids have been applied in previous work (Crute 2004). Heterotrophic bacteria was selected as a relative indicator of the overall presence of aerosolised microorganisms, as was also studied in Brooks *et al.* (2004). Collectively, this selection of microorganisms enabled comparisons to be made across the biosolids application sites and the unamended sites.

At each harvesting site, site samples of the clean air, soil and spikelets were collected prior to the harvester entering the site. Soil samples were collected by randomly selecting three to six core samples from the topsoil (at 0-10 cm depth) and were placed directly into sterile plastic bags. Spikelet samples were collected by randomly selecting approximately six standing wheat plants (6 plants = 1 sample) in triplicate (n = 6 samples in 2008 and 3 samples in 2009) for each treatment, cutting them approximately 25 cm below the seed head and placing the samples directly into sterile paper bags. Clean air samples were collected in triplicate using three SKC BioSamplers® with Vac-U-Go pumps as described in Section 8.2.2. BioSamplers® were sterilised with ethanol and flushed between runs with deionised distilled water.

Dust samples were collected downwind (20-30 m) from the operating harvester over a period of approximately 5 h. At each sample event in 2008, four samples were collected in triplicate (n = 12 samples) for each treatment. In 2009, two samples were collected in triplicate (n = 6 samples) for each treatment.

Dust samples were collected using three SKC BioSamplers® with Vac-U-Go pumps as described in the thresher study (Figure 8-1). BioSamplers® were clamped approximately 30 cm apart and mounted onto the frame of a Toyota Landcruiser utility above approximate human breathing height at 2 m (Figure 8-7). Samplers were filled with 25 mL of sterile distilled water as collection media and the collection basins were covered with alfoil to eliminate the effect of UV light on microorganism survival. Samplers were run for 2 min and samples were aseptically removed from the sample basin and placed into sterile polypropylene 50 mL centrifuge tubes (Sarstedt). BioSamplers® were sterilised with ethanol and flushed between runs.

At each site, chaff and grain samples were collected following harvesting operations. Chaff samples were collected by randomly selecting three grab-samples of plant material deposited by the harvester at each site. Samples were placed directly into sterile paper bags. Grain samples were collected from the field storage bin following dispatch from the harvester using collection buckets. All samples were placed on ice (~4˚C) and transported to the CSIRO Microbiology Laboratory, Floreat, WA for processing within 24 h.

8.2.5. Climatic conditions

At each site, wind speed (km/h), temperature (˚C), relative humidity (%), wind chill (˚C), heat index (%), dew point (˚C), wet bulb (˚C) and barometric pressure (hPa) were automatically recorded using a Kestral® Communicator 4000 Pocket Weather Station Version 1.4 (Nielsen-Kellerman Co., Boothwyn, PA.). Data was recorded at 2 h intervals in 2008 (by error in the instrument setup) and every 2 min in 2009.

Figure 8-7: Attaching BioSamplers® to the mounting frame.

8.2.6. Enumeration of microorganisms from thresher and harvester samples

All sample contents for the spikelets (~10 g), chaff (~5 g), grains (~40 to 60 g) and soil (~30 g) were transferred into pre-weighed sterile polypropylene tubes (Sarstedt) and net weights were obtained. After addition of P-buffer (30 mL) samples were placed in a stomacher (Bag Mixer®, Interscience). Samples were stomached for 2 min (speed no. 7) and then the supernatant was aseptically collected. Net weights for the thresher swab samples (in pre-weighed stomacher bags) and field soil samples were obtained (~3 g) and P-buffer (30 mL) was added. Swab samples were placed into the stomacher and the supernatant collected. Soil samples were vortexed for 2 min, left to settle, then vortexed again for 1 min. One mL samples of the resulting supernatant from the soil samples were collected without disturbing the pellet. Thresher dust samples (in 25 mL sterile distilled water) were vortexed and then serial 10-fold dilutions were made in P-buffer for the spikelet, chaff, grains, soil, dust and swab samples. A total of 5 mL of the sample buffer (in triplicate) was screened for the quantification of *E. coli* and enterococci in air and dust samples collected from the harvesting sites by passing through a vacuum manifold filtration system (PALL) using 0.45 µm membrane filters (Millipore). The quantification of pathogens was performed as described in Section 3.4, p. 55.

8.3. Data analysis

8.3.1. Data preparation

Prior to statistical analysis, pathogen counts were normalised from the raw data as described in Section 3.5, p. 62. This was carried out to adjust for the variations due to different sample volumes used, different dilutions, plating volumes and phosphate buffer levels. Box plots were performed in Origin® 6.1 (OriginLab Corporation 1991-2000). Bacterial number in bioaerosol samples were determined as cfu per cubic meter (m^3) of air based on the following conversion formula (Equation 11):

Equation 11

Pump flow rate x operating time (min) ÷ 1000 L (or 1 m^3) **(11)**

The microorganism numbers in the swab samples were determined as cfu or pfu per cm^2 of area based on the following conversion formula (Equation 12):

Equation 12

Average cfu ÷ area of the swab (i.e. 225,000 mm^2) **(12)**

8.3.2. Statistical analysis

A generalised linear ANOVA model was used to identify significant variation sources affecting final pathogen counts ($\log_{10}$ *Count*) in individual microorganisms or sites as well as across microorganisms or sites. All analyses were performed using a GLM model in SAS package version 9.1 (SAS Institute, 2005). The least-square effects of all the fixed factors were estimated and examined for their significance.

8.3.2.1 Analysis of variation sources for individual microorganisms

The variation sources in the thresher and harvester experiments included the effects of treatment and individual sample within a treatment. The model can be written as described in Section 5.3.1 (Equation 4), p. 85 with the following modifications:

$$Log_{10}Y_{iln} = \mu + T_i + C_l\,(T)_i + e_{iln}$$

where, T_i is the treatment effect and i =1, 2 corresponds to biosolid and nil-biosolid treatment. C_l is the sample effect within each treatment and l =1, 2, ..5 represents chaff, dust, grain, spikelet or swab in the thresher study and l =1, 2, 3…..12 corresponds to air, chaff, dust, grain, soil and spikelet at the biosolids-amended harvesting site or the nil-biosolids harvesting site.

8.3.2.2 Comparison of microorganisms numbers

The generalised linear ANOVA model as described in Section 4.3.1.2 (Equation 3), p. 70 was used to compare significant effects within the individual trial across microorganisms in the thresher study with the following modifications where, Ti =1, 2, 3…..5 corresponds to the treatments chaff, dust, grain, spikelet or swab samples.

The same equation was used for the harvester study with the modification that Cl =1, 2, 3…..12 corresponds to air, chaff, dust, grain, soil and spikelets at the biosolids-amended harvesting site or at the nil-biosolids harvesting site); and O_m is replaced by S_m to provide the 'site' effect of the $_m$th organism (*m=E. coli, S. enterica* or MS2).

8.4. Results

8.4.1. Environmental conditions during harvest

The climatic parameters during harvest in 2008 and 2009 are presented in Table 8-1. Mean wind speeds were 6 and 7 km h^{-1} with a range of maximum speeds of 15 to 27 km h^{-1}. Average air temperatures were 24 to 25ºC across the sampling days during both seasons. Maximum daily temperature ranged from 24 to 35ºC with minimum daily temperature ranging from 18 to 20ºC. Average relative humidity was 28 to 32% and average altitude was 270 to 271 m. Average heat index on the sampling days ranged from 22 to 23ºC with a maximum of 32ºC at Sites C and D. No rainfall events occurred during either of the sampling events.

Table 8-1: Climatic parameters from experimental sites during harvesting in 2008 and 2009.

	Sites NA and BB (2008)			Sites NC and BD (2009)		
Measurement	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**
Wind speed (km h^{-1})	6	15	2	7	27	0
Temperature (ºC)	24	24	20	25	35	18
Relative humidity (%)	32	44	22	28	49	15
Barometric press. (hPa)	981	984	979	981	986	978
Altitude (m)	270	289	239	271	297	226
Dew point (ºC)	5	8	1	4	13	0
Heat index (ºC)	22	22	18	23	32	16
Wet bulb (ºC)	14	14	12	14	20	12
Wind chill (ºC)	24	24	20	25	35	18
Density altitude (m)	681	698	540	718	1074	459

8.4.2. Survival patterns of enteric microorganisms at threshing

The observed changes in *E. coli, S. enterica* and MS2 numbers throughout the process of threshing are presented in Figure 8-8. It can be seen that the microorganism numbers on the spikelets were highest prior to threshing, as expected following inoculation. Following threshing, *E. coli, S. enterica* and MS2 numbers on the grains had reduced the most, with less reduction seen on the chaff. The means of the study microorganisms is presented in Table 8-2. The seeded spikelets had the highest number ($P<0.001$) of *E. coli* and *S. enterica* prior to threshing with mean values of 6.56 and 4.86 $\log_{10}$ cfu g^{-1}, respectively; however, the highest number of MS2 was found on the chaff samples following threshing (i.e. 7.01 $\log_{10}$ cfu g^{-1}) (Table 8-2 and Figure 8-8).

Following threshing, the *E. coli, S. enterica* and MS2 on the chaff were significantly higher than those on the grains ($P<0.001$). The study microorganisms on the grains were the lowest in number compared with those detected on the chaff or the spikelets (Table 8-2 and Figure 8-8). The higher number of MS2 on the chaff compared with the spikelets was not statistically significant ($P=0.10$).

The numbers of *E. coli, S. enterica* and MS2 from the thresher drum (swab) and in the wheat dust samples following threshing are presented in Table 8-3. The drum swab samples contained 7.99 x 10^2 cfu per cm^2 of *E. coli*, 4.67 x 10^0 cfu per cm^2 of *S. enterica* and 1.20 x 10^3 pfu per cm^2 of MS2 after threshing. Dust generated during threshing contained 1.19 x 10^5 cfu per m^3 of *E. coli,* nil *S. enterica*, and 4.18 x 10^6 pfu per m^3 of MS2.

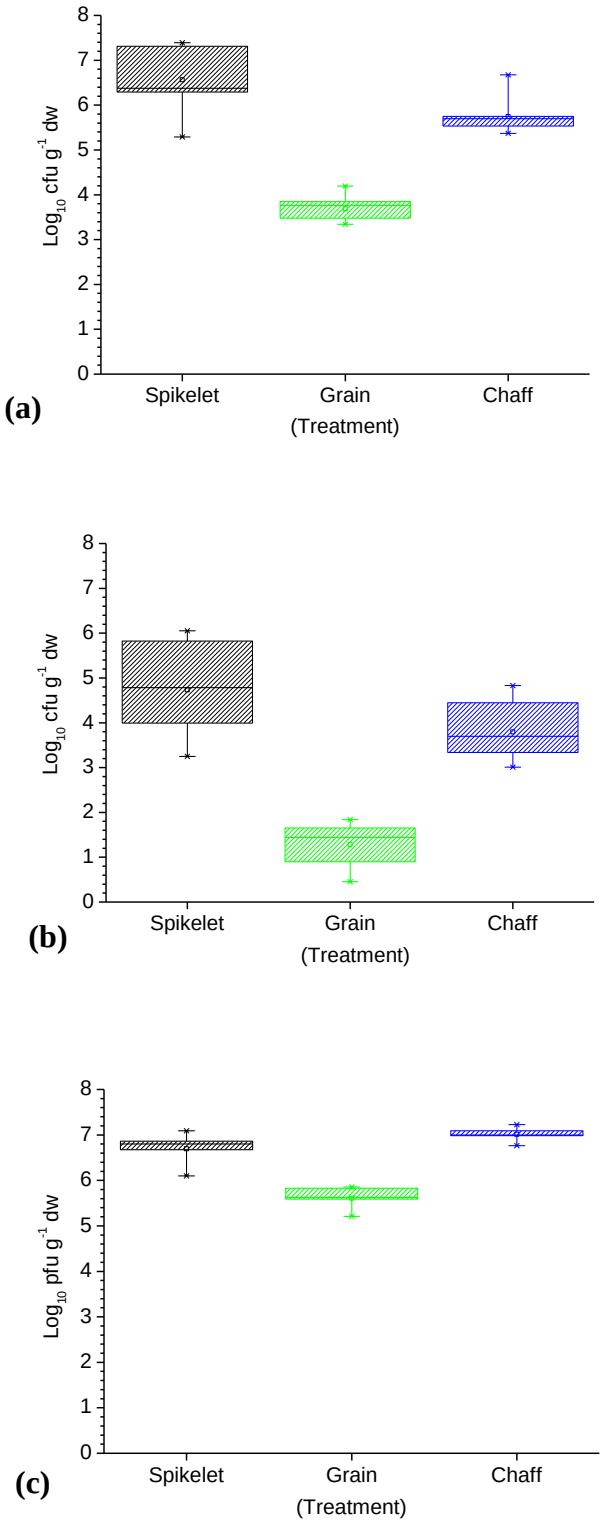


Figure 8-8: Microorganism numbers from thresher study on inoculated spikelets and on grain and chaff following threshing where (a) is *E. coli,* (b) is *S. enterica,* and (c) is MS2. Standard error bars are shown.

Table 8-2: Estimated least square means of microorganisms across treatments (Log count).

	Least square means (Log_{10} cfu g^{-1} dw)		
Sample type	***E. coli***	***S. enterica***	**MS2**
Chaff	5.74 (± 0.01)	3.59 (± 0.13)	7.01 (± 0.03)
Grain	3.69 (± 0.01)	1.21 (± 0.13)	5.60 (± 0.03)
Spikelets (seeded)	6.56 (± 0.01)	4.86 (± 0.13)	6.67 (± 0.03)

Table 8-3: Summary of *E. coli, S. enterica* and bacteriophage numbers present in the threshing drum and dust samples following threshing.

	Cfu or pfu per area sampled		
Sample type	***E. coli***	***S. enterica***	**MS2**
Drum swab (per cm^2)*	7.99×10^2	4.67×10^0	1.20×10^3
Dust (per m^3)*	1.19×10^5	Nil	4.18×10^6

**Note: different volumes for swabs as for dust samples*

8.4.3. The presence of bacterial microorganisms at harvest

This study was conducted to compare bacterial numbers in aerosols through the cereal harvesting process at biosolids application sites with unamended sites. Clean air, spikelet and soil samples were taken prior to harvest, the dust samples were collected during harvesting, and the chaff and grain samples were collected following harvest.

No *E. coli* were detected in the clean air, soil, spikelet, wheat dust, chaff or grain samples at either the biosolids-amended site or the unamended site (Sites NA and BB) at harvest time. For this reason, the nil results were not presented. Sites NC and BD were not tested for *E. coli* in 2009.

Clean air vs. dust

The observed numbers of heterotrophic bacteria (and enterococci) in the clean air and dust samples are presented in Table 8-4. Heterotrophic bacteria numbers in the clean air samples, taken prior to harvesting operations, were significantly lower ($P<0.001$) than those in the dust samples at all sites.

Heterotrophic numbers in the clean air samples at the unamended sites (NA and NC) were lower than those at the biosolids sites (BB and BD) in 2008 (Table 8-4). In 2008, this difference was significant ($P<0.001$) but in 2009, this difference was not significant ($P=0.97$). In 2009, the heterotrophic bacteria in the dust at the unamended site (NC) was significantly lower ($P<0.001$) than that at the biosolids site (BD). The results of the heterotrophic bacteria in the dust samples at the biosolids-application site in 2008 were not available due to a laboratory processing error.

Total heterotrophic bacteria

The numbers of heterotrophic bacteria on the spikelets, chaff, grains and soil at the biosolids site and the nil-biosolids site are presented in Figures 8-9 and 8-10. Across both sites, the chaff contained the highest number of heterotrophic bacteria (Table 8-5, Figures 8-9 and 8-10). In 2008, heterotrophic bacteria numbers (Figure 8-9) were significantly ($P=0.05$) higher in the chaff at the unamended site compared with the biosolids site (Table 8-5). There was no significant difference in 2008 between the

heterotrophic bacteria numbers found in the soil (P=0.15), on the spikelets (P=0.46) or on the grains (P=0.18) at the nil-biosolids site compared with the biosolids site. Apart from the chaff, the application of biosolids did not significantly affect heterotrophic bacteria numbers.

In 2009, the heterotrophic bacteria numbers (Figure 8-10) on the grains and in the soil were significantly higher at the biosolids application site compared with the nil-biosolids site (P<0.001). There was no significant difference between the heterotrophic bacteria numbers on the chaff (P=0.21) or the spikelets (P=0.08) between sites.

Table 8-4: Summary of heterotrophic bacteria and enterococci numbers in air and dust samples at the Moora field sites in 2008 and 2009.

	Heterotrophic bacteria (cfu m^3)		Enterococci (cfu m^3)	
Harvesting site	**Clean air**	**Dust**	**Clean air**	**Dust**
2008 Site NA Nil-biosolids	1.00×10^4	1.93×10^5	#	#
2008 Site BB Biosolids-amended	1.89×10^4	Not available	#	#
2009 Site NC Nil-biosolids	2.24×10^4	3.10×10^5	Nil	7.27×10^2
2009 Site BD Biosolids-amended	5.37×10^4	4.57×10^5	Nil	2.71×10^3

 # Enterococci not tested in 2008

Table 8-5: Least square means for heterotrophic bacteria (on Log numbers) on plant and soil samples at both sites.

	Least square means for heterotrophic bacteria (Log numbers)	
Sample type	**2008**	**2009**
Chaff	5.09 (± 0.17)	6.51(± 0.13)
Grain	4.25 (± 0.17)	5.28 (± 0.13)
Spikelet	4.24 (± 0.12)	5.74 (± 0.13)
Soil	4.19 (± 0.12)	5.04 (± 0.13)

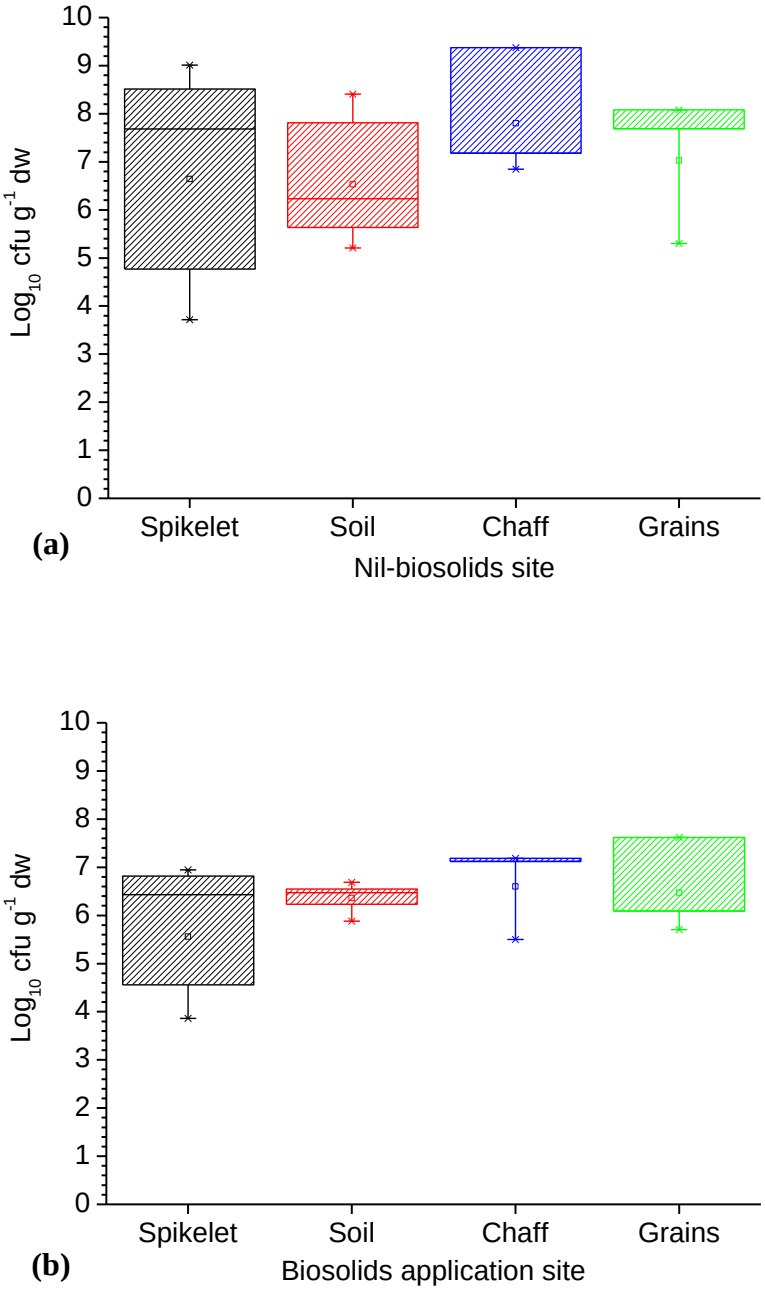


Figure 8-9: The mean total heterotrophic numbers on spikelets, soil, chaff and grains at harvest time in 2008 where (a) is Site NA with nil-biosolids applied; and (b) is Site BB with biosolids applied in May 2006. Standard error bars are shown.

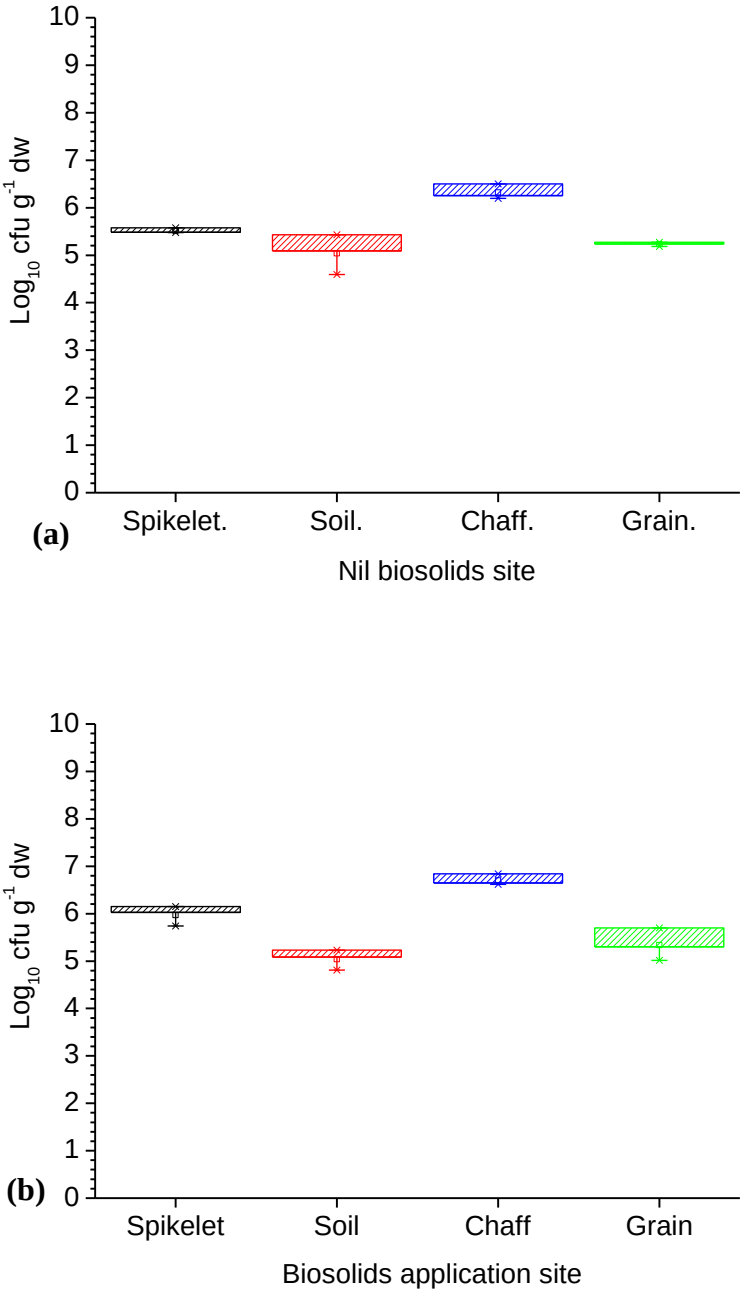


Figure 8-10: The mean total heterotrophic numbers on spikelets, soil, chaff and grains samples at harvest 2009 where (a) is Site NC with nil-biosolids applied; and (b) is Site BD with biosolids applied in May 2009. Standard error bars are shown.

Enterococci

The dust samples in 2009 contained enterococci (Table 8-4) and although the numbers tested were lower at the nil-biosolids site (7.27 x 10^2 cfu per m^3) compared with the biosolids site (2.71 x 10^3 cfu per m^3) this difference was not significant (P=0.22). There were no enterococci detected in the clean air samples at either site prior to harvesting. Sites NA and BB were not tested for enterococci in 2008.

The observed numbers of enterococci on the spikelets, soil, grain and chaff samples for both sites is presented in Figure 8-11. A similar trend occurred for the heterotrophic bacteria, where enterococci numbers were significantly the highest (P<0.001) in the chaff samples compared with the spikelets, soil and grains (Table 8-6). Also, the enterococci numbers on the chaff were significantly higher (P=0.04) at the nil-biosolids site compared with the biosolids site. In the soil, enterococci numbers at the biosolids site were significantly (P<0.001) higher than those at the nil-biosolids site (since there were no enterococci detected at the nil site). A general trend occurred where the enterococci numbers on the grains and spikelets were higher at the biosolids site compared with the nil site, but this was not significant (P=0.96 and P=1.00).

Table 8-6: Least square means for treatment effect for enterococci (on Log numbers) on plant and soil samples at Moora in 2009.

	Least square means for enterococci (Log numbers)	
Sample	**Biosolids site (NC)**	**Nil-biosolids site (BD)**
Chaff	1.69 (± 0.21)	1.96 (± 0.21)
Grain	1.21 (± 0.22)	0.36 (± 0.22)
Spikelet	0.70 (± 0.11)	0.00 (± 0.11)
Soil	1.58 (± 0.04)	0.00 (± 0.04)

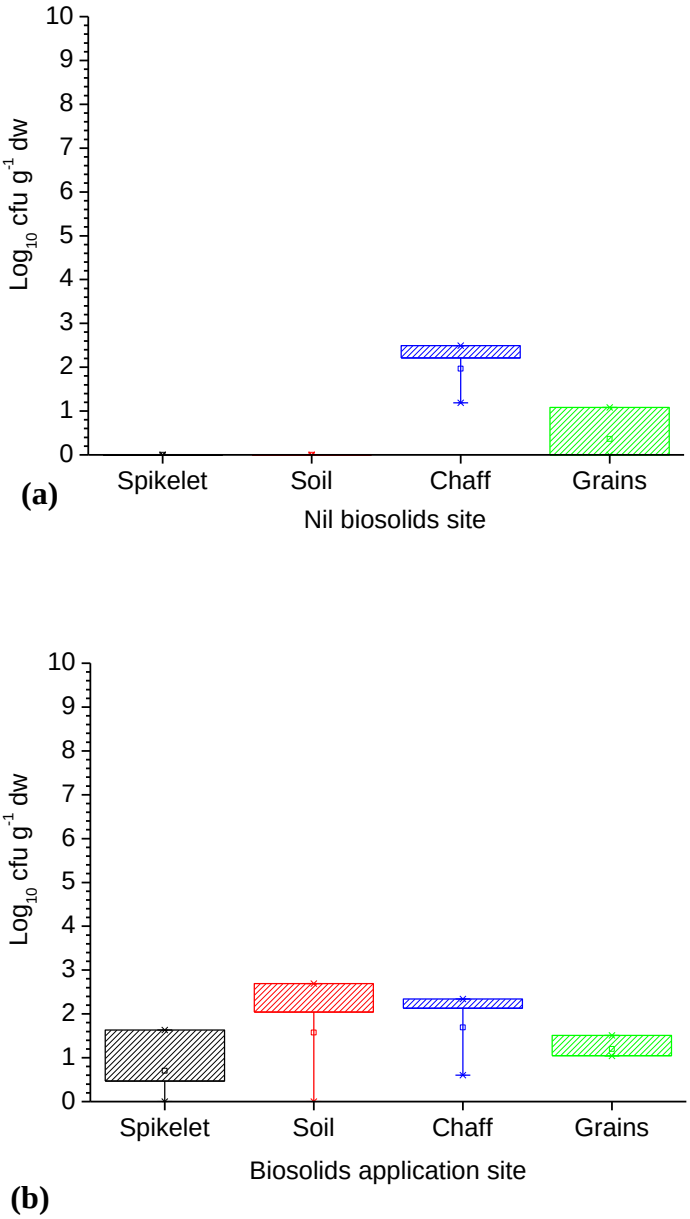


Figure 8-11: Enterococci numbers in the harvester study on the spikelets, soil, chaff and grains in 2009 where (a) is the nil-biosolids application site; and (b) is the biosolids application site. Standard error bars are shown.

8.5. Discussion

8.5.1. The effect of threshing on microorganisms

The results from the thresher study indicated that most microorganisms were distributed from the wheat plant to areas such as the threshing drum, chaff and grains and that only a low level of microorganisms passed out of the thresher as bioaerosols in the wheat dust (Tables 8-2 and 8-3). The observed low numbers of microorganisms in the dust (reduced rapidly over a short period of time) suggest that decrease in numbers is most likely associated with gravitational settling (that is, the drag or frictional force exerted on that particle) rather than biological inactivation alone (Pillai and Ricke 2002).

Forcier (2002) suggested that the shorter the transportation time, the less effect the inactivation processes would have on microbial numbers. In the present study, the microorganisms appeared to have attached to other sources during threshing before they became airborne, rather than undergoing the processes of inactivation since the threshing time was less than one minute. The microorganisms on the grain samples were higher than those in the dust samples which suggest that microorganisms could have bound onto the grains. If so, they may pose more risk to humans and livestock at consumption of the grains rather than be of risk as airborne contaminants present in the dust.

8.5.2. Survival patterns of bacteria and bacteriophage MS2

In the present study, the surrogate virus (MS2) was more stable on the wheat grains and chaff throughout the process of threshing than the bacteria. High numbers of MS2 were detected in the dust samples following threshing. In aerosols, viruses may be more stable than bacteria due to their ability to remain airborne for prolonged periods because of their low settling velocity (Tellier 2006).

In the present study, no *S. enterica* were detected in the dust samples. Brooks *et al.* (2005a) found that bacteria, particularly Gram negative, were inactivated much more quickly than the virus (coliphage). Also, *E. coli* did not survive aerosolisation but coliphages could be routinely detected. In Carducci *et al.* (2000), the rate of viral

isolation was higher than bacterial and coliform counts. And, in Fannin *et al.* (1977), coliforms were less stable than coliphages in the airborne state. Tanner *et al.* (2005) also found that more coliphages than coliforms were able to be detected in samples collected downwind of a biosolids application site. This suggests that viruses could be of greater risk to humans and therefore further studies should be conducted (Carducci *et al.* 2000).

8.5.3. The microorganism levels in chaff

In the thresher study, a general trend occurred where *E. coli, S. enterica* and MS2 numbers were higher in the chaff samples following threshing than the grains. The same trend occurred in the harvesting experiment where heterotrophic bacteria were significantly higher on the chaff samples, compared with the other samples. One possible explanation for this in the thresher study was that the microorganisms were sprayed directly onto the spikelets (grain heads) at the beginning of the experiment and the chaff, being the outer casing of the grains, absorbed the majority of the inoculant. In the field, it is possible that bacteria may be present on the spikelets from aerosols or rain splash (and thus the chaff), and consequently, higher bacterial numbers may be found on the chaff. Therefore, it could be expected that any natural contamination that may occur in the field should also be present primarily on the chaff. The spikelets may provide a 'protective effect' for any enteric microorganisms lodged inside the grain and chaff areas; however, results from Chapter 7 demonstrated that the decay times of enteric pathogens were shorter on the spikelets (where chaff is located) compared with the plant leaves.

8.5.4. The effect of the biosolids application site on bacteria numbers

One of the main reasons for conducting the experiments reported in this chapter was to examine the effect that biosolids application had on the levels of bacteria in bioaerosols at harvest time. To achieve this, the bacteria numbers in dust samples from the biosolids application site were compared with an unamended site. The hypothesis under examination was that the numbers of indigenous bacteria in aerosols at the biosolids application site would be higher than those at the unamended site.

In the present study, enterococci were present in the soil and the spikelets at the biosolids application site but not at the unamended site. Despite this, enterococci were still present in the dust, chaff and grain samples at both sites. The enterococci numbers were significantly higher in the spikelet, soil, dust and grain samples at the biosolids site when compared with the unamended sites. Therefore, the presence of biosolids in the soil resulted in higher numbers of enterococci at these sites.

Heterotrophic bacteria, already present in the environment, were selected to compare the overall potential transfer of bacteria from soil and spikelet to dust, grains and chaff at a biosolids site compared with an unamended site. In the first season (2008), heterotrophic bacteria numbers in the chaff were significantly higher at the nil-biosolids site than at the biosolids site. An opposite trend occurred in the second season (2009) where heterotrophic bacteria were significantly higher at the biosolids-application site in the grains and soil samples compared with the nil-biosolids site. This suggests that the presence of biosolids in the soil increased the heterotrophic bacterial levels at the site, as it did with enterococci; however, this suggestion is non-conclusive. Since in 2009 the biosolids were applied to the soil in the same year as the harvesting experiment (May 2009), it may be possible that the enterococci and heterotrophic bacterial numbers may only be higher at biosolids application sites where biosolids have been applied in the same year. This was suggested since the same trend did not occur in the spikelet, soil, chaff and grains samples where biosolids had been applied to the site more than two years before to the harvesting experiment in 2008 (i.e. where heterotrophic numbers were higher at the nil-biosolids site) and thus any microorganisms present at the site would have had a longer period of time to decay.

In a study by Brooks *et al.* (2004), significant numbers of heterotrophic bacteria were found in air samples during biosolids application and therefore it was suggested that the bacteria may have arisen from soil particles being aerosolised at application. It was also inferred that aerosolised soil might contribute to the number of aerosolised microorganisms since soil particles are small in particle size and low in mass and may be able to aerosolise more readily. However, in their work the aerosolised heterotrophic bacteria at the biosolids application site were similar to those found at

the non-biosolids site, which suggests that the bioaerosols tested were present at both sites in similar number and that biosolids do not contribute any bacteria into aerosols. No *E. coli* were detected in the samples collected from the first season. One possible explanation was that the biosolids were applied to the site (Site BB) in May 2006, which was more than two and a half years before the experiment occurred in December 2008. It is possible that any *E. coli* present in the field, from biosolids applications, were not able to persist across three summers. Indeed, this has been found in other studies, particularly with environmental strains of *Salmonella* spp. and *E. coli* such as Eamens *et al.* 2006, Eamens and Waldron 2008 and Horswell *et al.* 2007.

In the present study, harvest was selected for the aerosol study since it is the main time of the cropping year, apart from seeding, where farmers or workers may be exposed to bioaerosols from land-applied biosolids. Brooks *et al.* (2007) stated that the levels of aerosolised culturable microorganisms were shown to be greatly reduced where soils were moist. Aerosol samples were not collected during biosolids application since the soils are normally moist for seeding (autumn). In addition, the biosolids contained approximately 20% solid material (unlike sprayed wastewater) and, as a result, little or no dust spray was expected during spreading. Between seeding time and harvest in WA, wheat crops are usually only accessed for herbicide, fertiliser and insecticide spray applications. During this time the soil is not disturbed, therefore this period of time (i.e. June to October) was not tested.

The use of large-scale spray application of inoculants for the field study was not considered necessary for this project since extensive research has already been carried out by Moore *et al.* (1979), the Water Environment Research Foundation (Peccia and Paez-Rubio 2007), Brooks *et al.* (2004; 2006), Brooks *et al.* (2005a), Brooks *et al.* (2005b) and Tanner *et al.* (2005). For the present study, the research plan was designed to represent similar conditions to the operations that normally occur where biosolids are used in agriculture. In this way, the research reported in this chapter is unique and specific to the climatic conditions, harvesting methods, biosolids-type and agricultural land for the Australian wheatbelt.

8.5.5. The risk of bacteria in aerosols

It was observed that only a low level of bacteria could be detected in the dust and air samples during both experiments, indicating that only a portion of the microorganisms present on surfaces such as spikelets and soil were able to become airborne. The incidence of low levels or no levels of aerosolized biological agents present in air samples was raised by Brooks *et al.* (2005a) who suggested that this may be because no biological contaminants were present, since microbial numbers in aerosols in their study were consistently below the detection limits. In the thresher and harvest experiments conducted in the present study, the low levels of bacteria in the dust and air samples demonstrated that the process of threshing may significantly reduce pathogen numbers and therefore decrease the risk to the farmer or field worker.

At this point, it must also be noted that the dust generated from a harvester does not originate from the soil, but is wheat dust generated from the threshing of chaff. Therefore, any enteric pathogens present in the (biosolids-amended) soil at the time of harvest are not likely to be transferred onto the chaff and grains from the process of harvesting. In contrast, any enteric pathogens already present on the chaff are threshed and are able to become airborne, therefore are at risk of becoming bioaerosols. To ascertain the levels of risk, it is recommended that the numbers of enteric pathogens on the chaff in the field should first be tested.

8.5.6. The effect of climatic conditions

In the present study, the average wind speed was low (6 to 7 km h^{-1}) and relative humidity levels were low (28 to 32%) at all field sites. The average temperature on the sampling days was approximately 25ºC with maximum heat indexes of 22 to 32ºC. Biological parameters may not appear to have any evident correlation with meteorological factors such as temperature, relative humidity or wind characteristics (Carducci *et al.* 2000), and yet, several studies have found that relative humidity may affect bacterial and virus survival in aerosols (Fannin *et al.* 1977; Pillai and Ricke 2002; Brooks *et al.* 2004; Tellier 2006; Karra and Katsivela 2007). Microbial populations may be inactivated by environmental stresses such as UV radiation, temperature and desiccation (Lighthart and Frisch 1976; Brooks *et al.* 2005a; Paez-

Rubio and Peccia 2005; Karra and Katsivela 2007). Bacteria such as *E. coli* and enterococci are known to rapidly decline (in soils) at temperatures above 22 to 25ºC (Wang *et al.* 1996; Holley *et al.* 2006; Vidovic *et al.* 2007) and simarly, temperatures at harvest time do not favour the survival of bacteria.

The chance of transferring pathogens to humans may be more likely through the handling of biosolids, particularly during loading operations (Brooks *et al.* 2004), than from the pathogens becoming airborne (Forcier 2002; Pillai and Ricke 2002). This risk of transmission (of airborne pathogens) has been reported to be low where biosolids and municipal sludges have been applied to land (Sorber *et al.* 1984; Pillai *et al.* 1996; Brooks *et al.* 2004; Brooks *et al.* 2005b; Tanner *et al.* 2005; Rusin *et al.* 2003). Nevertheless, while earlier studies have failed to show significant levels of enteric microorganisms present during spray applications, there could still be a potential for low-level transmission of pathogens during application (Dowd *et al.* 2000; Gerba and Smith 2005; Nikaeen *et al.* 2009).

8.6. Conclusions

The following conclusions were made from this chapter:

- No *E. coli* were detected at the biosolids application site or the unamended site at harvest in 2008;
- Low levels of enterococci were detected in the dust samples in 2009. These levels were slightly higher at the biosolids application site than the unamended site, but this was not significant;
- The number of heterotrophic bacteria and enterococci were highest in the chaff and a general trend occurred where these bacteria were higher in the chaff at the nil-biosolids site;
- The bacteria numbers were higher in the dust samples compared with the clean air samples;
- The number of heterotrophic bacteria and enterococci was higher in dust and air samples at the biosolids application site compared with the unamended site;
- Apart from the spikelets (which were inoculated), the enteric microorganism numbers in the thresher study were higher on the chaff samples than on the grains samples following threshing;
- In the field, the same trend occurred as in the thresher study where bacterial levels were highest on the chaff samples;
- In the thresher study, low levels of seeded *E. coli* and MS2 passed through the stationary thresher into the dust samples. *S. enterica* was not able to be detected in the dust samples following threshing;
- Bacteriophage numbers were more stable than bacteria throughout the process of threshing and resulted in higher numbers on the grains and chaff following threshing;
- The highest numbers of microbial contaminants were found on the chaff in the field and, as a result, this region of the plant should be tested first for any potential contaminants that could become airborne in wheat dust;
- Overall, since the process of threshing was found to reduce enteric microorganism numbers, the risks of unsafe levels of bioaerosols in the dust at harvest was considered to be low.

CHAPTER 9 GENERAL DISCUSSION

9.1. Research significance

The die-off rate of enteric pathogens introduced into the soil after biosolids application provides the final barrier to transmission in a multi-barrier risk management approach, thus affecting the permissibility for biosolids to be applied to agricultural land around the world. The reduction of pathogen numbers during sludge treatment is also critical in this approach, along with the crop-types to which biosolids are applied. Efficient processes to remove high levels of pathogenic contaminants at treatment plants, along with fast rates of inactivation once introduced into the soil, decrease the risk of dissemination of disease-causing microorganisms and optimise the opportunities for biosolids reuse. However, the survival patterns of enteric pathogens once introduced into field conditions, particularly in Australia, is not fully understood.

The research in this thesis originated because there is very little scientific data available on the fate of enteric pathogens in agricultural soil following the land application of biosolids in Australia. Since a large proportion of biosolids throughout Australia are already being applied to land, there was a significant gap in the knowledge as to the survival patterns of pathogens once introduced to the soil.

The scientific knowledge gained on the risks of using biosolids where food crops are grown is important. There is a potential that the producers of food crops are at risk during application of biosolids and when accessing the sites, including at harvest. The consumers of the produce may also be considered to be at risk if pathogens are ingested. Similarly, the global markets for cereal grains produced from biosolids-amended soil may be at risk. The implications of this lack of knowledge may lead to negative perceptions influencing grain markets (i.e. international customers discontinuing to import grain and hay products produced off biosolids-amended land, or domestic customers rejecting grain products normally used to supply local flour mills and feedlots). Such types of outcomes could result in the practice of biosolids land-application being discontinued. Therefore, the survivability of pathogenic

contaminants across the growing season of the grains (and further supply chain if necessary) needed to be examined.

9.2. Current land release practices

Currently, the level of pathogenic contaminants in biosolids is graded according to microbiological criteria. Pathogen grading may restrict the possible end uses for the product. For example in Western Australia, the release of sewage sludge for use as biosolids is determined by the levels of just one or two bacteria (i.e. usually thermo-tolerant coliform (TTC) and *Salmonella*) in the sludge. In addition, TTC are more sensitive to wastewater treatment processes and environmental changes than other more resistant bacteria, viruses, protozoan cysts and Helminth eggs (Toze 1997) and therefore may not be a true reflection of other pathogenic contaminants present.

While these levels are only used as a guide, they do not represent the whole suite of pathogens that may be present in any batch of biosolids. Along with this, no data is available on the individual survival times or the types of the pathogens present in the sludge. This is important for land release since some viruses, helminths and protozoa can persist in the soil for longer than bacteria (Sorber and Moore 1987; Sidhu and Toze 2009).

Once the acceptable bacterial (and other contaminant) levels are reached and the biosolids are dispatched and land-applied, there is no current monitoring system to ensure the further reduction of pathogens. Some consider this unnecessary as the cropping and harvesting restrictions are designed to be very conservative to allow the natural loss of viability of pathogens to take place. Instead, withholding periods are applied to prevent public access to the biosolids application sites for a periods such as 30 to 45 d for grazing animals, 30 d before crops are permitted to be harvested, 1 y for turf farms and 12 months for forestry sites (DEP *et al.* 2002). The withholding periods are not established for all types of pathogens and they are not specific to the conditions that may occur in each region. In addition, most of the guidelines used around the world are based on the United States Environmental Protection Agency (USEPA) guidelines where the conditions differ from other countries such as Australia.

These guidelines (i.e. USEPA) were established more than a decade ago based on what we know about the survival times of pathogens in soil and on plants. The main requirements from the standards are that sludge be treated to two class types. Class A ensures that sludge is treated to eliminate pathogens and Class B sludges are treated to a lesser degree and may contain residual numbers. Land use restrictions apply to Class B biosolids but are unnecessary for Class A biosolids. In this way, sludge is treated to reduce pathogenic microorganisms to below acceptable limits before being applied to land. Alternatively, the treatment processes may be reduced provided that adequate time is allowed for natural attenuation of pathogens to occur from the soil before public are allowed to access the area or food crops are grown at the site (Gerba and Smith 2005). The guidelines are not exact and specific to individual locations, conditions, sludge type or soil types, nor can they be expected to be, therefore a method suitable for field monitoring of several pathogen types is desirable in order to reduce any potential environmental health risks.

9.3. Field monitoring method

Because there is currently no standard method for monitoring the decay times of enteric pathogens in the field, there is very little comparable scientific data available on the persistence of various pathogens such as viruses, protozoa or helminths in biosolids-amended soils given different locations, soil types and climates. To obtain quantitative estimates of the effect that biosolids may have on the persistence of individual enteric pathogens in agricultural soil, adequate methodology for field sampling and processing the samples in the laboratory was needed. This method was required to enable individual regions to test their own pathogen decay times over time so that safe withholding periods could be more accurately estimated and biosolids managed more specifically. In addition, a more accurate method for determining decay times from the data collected in the field (where biosolids are normally applied) was also required (i.e. the use of the quadratic model).

The sentinel chamber (Jenkins *et al.* 1999) was developed from commercially-available products. It was selected to provide a more controlled environment for the biosolids and microorganisms in the field where conditions are often unable to be controlled. In particular, in the present study the aim of the chambers was to reduce variability and heterogeneity.

The methodology used in the research reported in this thesis was based on:

(1) The development of a soil microcosm (sentinel chamber) to eliminate the possibility of loss of pathogens via leaching;
(2) The ratio of biosolids to soil (or application rate);
(3) The number of enteric pathogens present in the soils (i.e. inoculums) so that patterns of decay could be examined over a sufficient time period;
(4) The ability to enumerate the enteric microorganisms in the laboratory to enable quantification and the plotting of the data over time;
(5) The statistical analysis method to estimate decay times (T_{90}'s) more accurately;
(6) The choice of field sites (location) and the relationship to soil type and climatic conditions at these sites;
(7) Suitable methodology so that the experimental plots could be managed from remote distances.

For this research, extraction methods were developed from wastewater protocol for the processing of soil and biosolids samples in the laboratory. This allowed for the microorganism numbers to be quantified at each sampling event and the data plotted. The resulting data enabled the decay times for enteric bacteria and viruses to be estimated. It was not possible, however to estimate the decay times of a whole suite of enteric pathogens or determine the absolute reasons for the difference in decay times across different conditions. Theoretically, these decay times could be estimated through a modelling system using inputs such as climatic data from each site; however, this research project has shown that this would be very difficult to do accurately due to varying conditions, seasons, soil types, biosolids-types etc.

The development of a quadratic statistical model in this research provided more accurate estimates of decay times from the non-linear data. These decay times, along with the observed data, are now available to provide essential information to the key stakeholders such as the facility administrators, operators, regulators, politicians, scientific community, wastewater generators, users of biosolids and the general public, wherever it is required. The survival patterns for each enteric pathogen still needs to be adequately replicated at various sites to determine any reoccurring patterns and to further understand the factors that contribute to die-off in the field.

9.4. Comparison of decay times

The research reported in this thesis collected scientific data from three field sites in Australia. From the studies reported in this thesis, decay rates (T_{90} values) of enteric pathogens were estimated. Quantitative estimates of enteric pathogen survival are necessary to enable accurate withholding periods to be applied in the interest of protecting public health. Comparison of pathogens and indicators (in biosolids) across other studies is difficult because there are no standardised analytical techniques, and sludge characteristics are diverse and subject to seasonal variation (Sidhu and Toze 2009).

The estimated decay times from the present study were difficult to compare with other published studies conducted around the world. Most of the research on enteric pathogens in soils has been conducted in animal manures (Wang *et al.* 1996; Lau and Ingham 2001; Jiang *et al.* 2002; Hutchison *et al.* 2004; Holley *et al.* 2006), soil alone (Chandler and Craven 1980; Hurst *et al.* 1980; Gagliardi and Karns 2002; Vidovic *et al.* 2007) and different sludge types such as effluent (Sidhu *et al.* 2008), irrigation water or composted biosolids (Sidhu *et al.* 2001). They have been carried out in laboratories or glasshouses, using different sampling methods (as described in Chapter 4) and different methods of recovery and enumeration. Different methods for calculating decay times have also been used. Currently, the main research apart from this study on land-applied biosolids has been conducted in the United States of America (Pepper *et al.* 2006; Zerzghi *et al.* 2010), the United Kingdom (Lang *et al.* 2007; Lang and Smith 2007), New Zealand (Horswell *et al.* 2007; Horswell *et al.* 2010) and Australia (Crute 2004; Eamens and Waldron 2008; Schwarz *et al.* 2010). Very little scientific data is therefore available, specifically for Australia, adding importance to the outcomes of this current research.

Despite this, the comparison of results from different regions (where different methodology has been used) is not as relevant as each region being able to collect their own scientific data specific to soil types, biosolids types and climatic conditions. The methods developed in this thesis mean that the decay times of enteric pathogens can be tested and repeated anywhere in the world. The experimental site in South Australia proved that the chambers can be constructed in a remote laboratory

(such as Western Australia), sent out to the site, inserted into the soil, collected periodically and shipped back to the analysis laboratory for processing.

In addition, the use of the observed data is an important consideration for use in conjunction with the T_{90} decay times since it was found that the estimated decay times alone may not always reflect the survival patterns (to below detection) of the individual microorganisms at any given site.

9.5. Major research findings and their implications

The major findings and their implications discussed below, directly refer to the land application of biosolids and related findings, collated from the experiments conducted in the research reported in this thesis. Such findings have been derived across the growing season of wheat - from the field (soil) studies, the phyllosphere experiments, the grains experiments and the dust study – this being the unique aspect of this research.

9.5.1. The effect of adding biosolids to soil

One of the key areas of concern for biosolids stakeholders is that the application of biosolids to agricultural soil could result in prolonged persistence of human enteric pathogens. In the field experiments conducted in the present study, biosolids were applied at higher rates than are normally allowable under the guidelines. Despite this, no apparent trend was found where the inoculated microorganisms survived for longer in the soils where biosolids had been applied (compared with unamended soils). By contrast, it was often seen that the decay times for the study microorganisms were more rapid in the amended soils compared with the unamended soils, indicating that the application of biosolids to the soil may have actually increased the inactivation processes of the enteric microorganisms in the soil.

For industry, this means that while the application of biosolids may introduce harmful enteric pathogens to the field, the pathogens (in biosolids-amended soils) are reduced over time. In addition, the climatic conditions that are typical for southern Australia are generally not favourable to the survival of enteric pathogens. When biosolids are applied in the summer or autumn months, and the sites are sown to grain crops, the enteric pathogens are rapidly reduced with the onset of spring and

summer climatic conditions. In the present study, it was demonstrated that the all of the study microorganisms (except for adenovirus) decreased rapidly with increasing soil temperatures and decreasing soil moisture (through reduced rainfall events).

9.5.2. The decay times (soil)

Another issue of concern for biosolids stakeholders is the accuracy of withholding periods. The established time periods, preventing public access to biosolids application sites, is important under the current legislation (i.e. with regard to duty-of-care).

The estimated decay times in this research, calculated from data collected in the field, are a result of overestimation rather than underestimation. The use of laboratory-cultured inoculants, seeded at high numbers, along with higher than normal biosolids application rates, provided for a worst-case scenario to be examined. Along with this the microorganisms were confined to sentinel chambers. It is possible that under normal field conditions they may have leached down through the soil profile, or have been re-distributed to other areas of the field through run-off during rainfall events, and therefore not be of such high risk at the soil's surface. As a result, the decay times (at normal field application rates with normally-occurring pathogenic levels) may actually be shorter. Therefore, it could be assumed that the risk of enteric pathogens at biosolids-application sites may actually be lower than what is reflected in the results of the present study.

To best determine this, the decay patterns of *E. coli* from outside the chambers should be viewed (Figure 6-17, p. 124). *E. coli* numbers at Moora in 2008 decreased approximately five-$\log_{10}$ cfu g^{-1} to below one-$\log_{10}$ cfu g^{-1} over approximately 100 days. At Mt Compass, *E. coli* starting numbers were one-$\log_{10}$ cfu g^{-1} and decreased to below one-$\log_{10}$ cfu g^{-1} also over approximately 100 days. This indicates that *E. coli* numbers (normally found in biosolids) were decreased to safe levels within approximately 3 months in the field, bearing in mind that some of these *E. coli* may have been leached away, been re-distributed, or were not able to be cultured.

The current guidelines for Western Australia prevent public access (or grazing animals) at the biosolids application site for 30 to 45 d. Based on this, and the findings from this research, the following recommendations can be made:

- That public access to the site be restricted to at least 6 to 7 months, based on the fact that the risk from *E. coli* may be present for 3 to 4 months but the risk from viruses may be longer (e.g. more than 6 or 7 months);
- The risk of transfer of human enteric pathogens to livestock, and the presence of pathogens in biosolids that may affect livestock health (such as *Clostridium perfringens*), should be examined. Thereon, the current withholding periods for each region for grazing animals should also be reviewed;
- That decay times be derived using normal district application rates with normal expected microorganism starting numbers;
- That research be conducted in different regions with different climatic conditions such as Queensland. Tropical climatic conditions with summer rainfall, warmer temperatures and higher humidity levels, along with soils that contain higher clay content, may favour the survival (and potential regrowth) of enteric pathogens. This may result in different decay times, and thus different withholding periods, and therefore different guidelines; and
- That the survival times of microorganisms across different biosolids types from different wastewater treatment plants across Australia be conducted.

9.5.3. Survival of enteric pathogens on the phyllosphere

Where fodder crops are grown for livestock feed, from biosoloids-amended paddocks, there is concern that pathogenic contaminants will transfer from the soil to the plant and be of risk at consumption. The key areas of risk are evident where enteric pathogens may be transferred to livestock via hay and silage and then to humans across the supply chain. In the present study, the study microorganisms were detectable for longer in the soil (6 to 7 months) than on the plant leaves (less than 1 month). It was also stated by Epstein (1998) that pathogens commonly survive for longer periods of time in the soil than on plant leaves.

The phyllosphere can present a harsh environment for enteric pathogen survival (Aruscavage *et al.* 2008). The plant microenvironment above the soil is considered

to be unfavourable for pathogen survival because of environmental factors such as humidity and temperature (Lindow and Brandl 2003). Choi *et al.* (2004) observed greater inactivation rates of bacteriophage (MS2 and PRD1) from the leaves of lettuce compared with the soil, due to the protective effect (of the soil) from environmental conditions such as solar radiation and desiccation. If cereal crops are used to make hay or silage, then correct airing and drying times would potentially further reduce pathogen numbers.

The main consumers of plant leaves at flowering (for spring crops) are livestock. Cereal crops may be cut, raked and baled for hay or silage and contamination from any pathogens such as pathogenic enterobacteria *Salmonella* and toxin-producing *E. coli* may affect the safety and quality of forage crops and silage (Weinberg *et al.* 2004). The distance between the ground (where biosolids are present) and the edible portion of the crop is also important when considering potential risk of transmission (Abdulraheem 1989).

In the present study, the bacteria persisted for longer than the virus on the leaves. Enteric pathogens on plant leaves would mostly be of risk to livestock if grazed as a fodder crop, but pathogen ingestion is usually considered low where withholding periods are followed (Eamens and Waldron 2008). Still, very little is known about the risk to domestic grazing animals (Toze 2004) particularly from bacterial pathogens that may infect livestock.

The results of the soil field studies (Chapter 6) showed that inoculated bacteria were generally below four-$\log_{10}$ cfu g^{-1} by the spring time (at approximately 110 d). For hay, fodder or silage crops this would be the time when crops would be cut, raked and baled. In the phyllosphere experiment, the bacteria survived the longest on the plant leaves (compared with the virus and the bacteria and virus on the spikelets). Based on the estimated decay times (T_{90}) for *E. coli* and *S. enterica* on wheat leaves (Table 7-2, p. 157) of 72 and 57 h (respectively) to a one-$\log_{10}$ reduction, these bacteria should theoretically fall below detection (from four-$\log_{10}$ cfu g^{-1}) within 9 to 12 d. Given favourable weather conditions for hay and silage production, the time from cutting to baling is approximately 1 week and therefore the risks to livestock from enteric pathogens is considered low. Having said this, further research should

be conducted on pathogen survival for hay and silage products across the stages of production.

For pastures where animals are grazed, current withholding periods (under the WA guidelines) are approximately 30 to 45 d. Given that pastures would normally be grazed within only months following sowing, these withholding periods may be considered too liberal. Ruminants, particularly sheep, graze closely to the ground and if biosolids have been applied within the same year, would be at risk of direct ingestion of biosolids and any pathogenic contaminants present. Further research is required specific to the types of pathogens (found in Australia) that may be of direct risk to sheep and cattle, and specific to lactating or pregnant livestock. This is important to ensure that the biosolids-to-pathogen-to-livestock/humans-to-biosolids cycle of disease transmission does not occur (refer to pathways of transfer, Table 2-6, p. 18).

9.5.4. Decay of enteric pathogens from stored grains

There is a concern that any enteric pathogens present in biosolids may survive the growing season in the soil and be present on the end-product, the grains, at harvest time. This is important scientific data to have available in the event that grain buyers are concerned about the safety of the grains produced for human consumption.

In the present study, the decay times (T_{90}) for bacteria on stored grains was 9 to 12 d (Table 7-3, p. 157). Based on starting numbers of seven to eight-$\log_{10}$ cfu g^{-1}, the total estimated time required for *E. coli* and *S. enterica* to fall below detection would be approximately 80 to 90 d. The virus (MS2) persisted for longer with decay times (T_{90}) of 60 to 71 d. Based on high starting numbers of more than eight-$\log_{10}$ cfu g^{-1}, the total estimated time for MS2 to fall below detection would be approximately 604 d or 20 months. Since viruses are often highly contagious and result in a high rate of transmission (Koopmans and Duizer 2004) they may be of greatest risk to humans at consumption (of the grains). However, the risk of disease transmission should be considered in relation to the initial concentrations present in the grains, the storage period and the processing methods of the grains prior to consumption.

The risk of pathogenic contaminants in stored grains needs to be placed into context. Based on the results of the field studies, the enteric microorganisms in the soil were reduced to low levels by harvest time. At harvest time (approximately 170 d), *E. coli* and MS2 numbers were below two-$\log_{10}$ cfu g^{-1}, *S. enterica* were below detection and adenovirus was below four-$\log_{10}$ pdu g^{-1}. Based on a worse-case scenario, using the decay times of MS2 inoculated onto stored grains (two-$\log_{10}$ pdu g^{-1}) and given that all of the viruses could transfer from the soil onto the grains, the total estimated time to below detection would be approximately 142 d or almost 5 months. Since grains consumed by humans and livestock are usually stored for several months, transported by truck or rail, warehoused and then shipped, and then subjected to further processing methods, such as milling to produce flours or flakes (Zhang *et al.* 1997; Toze 2004; Fastnaught *et al.* 2006), this level of risk is considered to be low. In addition, the time period from harvesting (of the grains) to consumption is normally several months. During this period, it would be expected that the numbers of any enteric pathogens present, would be reduced to safe levels or have completely died off; however, some cross-contamination could still be a cause for concern.

9.5.5. The risk of bioaerosols during harvest

Another concern relating to the land application of biosolids to agricultural soil is the risk of transfer of airborne (pathogenic) contaminants from the soil to field workers during harvesting. Results from the dust study (in Chapter 8) showed that indigenous heterotrophic bacteria and enterococci numbers at the site where biosolids had been applied in the same year were higher than where no biosolids had been applied. It was also found that the highest numbers of bacteria (and inoculated microorganisms) were found on the chaff region of the plant. Despite this, it was determined that the process of threshing also significantly reduced microorganism numbers on wheat.

As discussed above (in Section 9.5.4), the study microorganisms in the soil (after inoculation at seeding time) were at low levels (<two-$\log_{10}$ cfu g^{-1}) by harvest time. Since it is difficult to test aerosol samples for every type of enteric pathogen (due to the logistics and inconvenience subjected on the farmer), it is suggested from the harvest results, that chaff be tested instead. It was found that any pathogens that may be present in aerosols would first be found in highest numbers on the chaff.

Provided that most field workers remain inside vehicles at harvest time (in sealed cabs of harvesters, trucks and utes) or use dust protection, the risks from bioaerosols in wheat dust is considered to be low. In addition, the climatic conditions in the field at harvest time do not favour the prolonged survival of bioaerosols due to high summer temperatures, low humidity and dry conditions.

9.6. Research limitations

The main research limitation associated with the research reported in this thesis was the ability to extract and enumerate pathogens from soil and biosolids samples. For example, experimentation was carried out on protozoa in this study but difficulties were encountered with visibility of the oocysts under the microscope because of the viscous and muddy nature of the biosolids. Other difficulties have been encountered with virus extraction using molecular methods because of PCR inhibitors. Bacterial colonies can become overgrown on agar plates with indigenous populations thus affecting the ability for quantification. Despite the many challenges, many improvements were also made to the method over the course of this research period by adapting wastewater protocols to the processing of biosolids and soil samples. However, more work is required to further develop laboratory methods suitable for processing more enteric pathogen types from soil and biosolids. This work is intensive and requires much experimentation and therefore is also expensive.

The use of indicator microorganisms to represent enteric pathogens has long been an issue of debate. In the present study, indicator bacteria (*E. coli*) and a virus (bacteriophage MS2) were selected for testing alongside pathogenic bacteria (*S. enterica*) and a virus (adenovirus). It was demonstrated that each microorganism behaved differently. While it is not possible to extract and enumerate any enteric pathogen of choice from biosolids (for reasons discussed above), it is obvious that each type of microorganism (indicator or pathogenic) had its own decay time. In addition, when placed into different soil types, seasons and locations these decay patterns varied. Therefore indicator microorganisms, particularly *E. coli*, are not an accurate representation for enteric pathogens within the same pathogen group. This agrees with Sidhu *et al.* 2009 that survival characteristics vary in the environment therefore no single microorganism can indicate the patterns or presence of all pathogens.

The benefits of using *E. coli* as a bacterial indicator in soil-amended biosolids (in this research) were the following: 1) they were easy to detect and quantify; 2) *E. coli* are consistently present in faecal matter; 3) they are unable to multiply outside the host, apart from possible regrowth; 4) survival times were not too short (compared with *Salmonella*); and 5) they don't originate from sources other than of faecal origin (Sidhu *et al.* 2009).

Another issue of debate is the use of laboratory-cultured strains versus strains originating from the environment. In the present study, it was demonstrated that similar patterns of decay occurred for the laboratory-cultured strain of *E. coli* used inside the chambers compared with the *E. coli* found outside the chambers (Figures 6-20 and 6-21, p. 127. This is a matter requiring further research and multiple sets of scientific data if suitable indicators (or pathogens) are to be selected for field monitoring.

9.7. Further research

From the research conducted in this thesis, key areas for further research have been identified. Further studies on the disease transmission from biosolids to food crops would augment the present knowledge of pathways from pre-harvest sources, aerosols and post-harvest sources. Such pathways may include:

- Grazing animals on pastures fertilised with biosolids. Even though this practice may not be common, the pathogens of harm to sheep and cattle particularly pregnant or lactating animals, needs to be examined. The pathogens specific to the location from which they are tested (for example *Cryptosporidium, Salmonella* spp., *Campylobacter* and toxigenic *E. coli*) need to be selected with the intention of preventing the cycle of infection occurring from biosolids use;
- The potential for, and levels of, enteric pathogens that may transfer onto food plants via rain splash where biosolids have been applied needs to be studied; and
- The fate of protozoa is an important area where more information is needed due to their recognised presence in biosolids (Pepper *et al.* 2006; Sidhu and Toze 2009) and their ability to infect both humans and animals (DuPont *et al.* 1995; Bradford and Schijven 2002).

Further work is required in the laboratory to develop methods for extraction and quantification of enteric pathogens from biosolids-amended soil samples. This would enable more scientific data to be collated on the decay patterns of such microorganisms, where introduced into agricultural soil from use of biosolids. In particular, enteric pathogens that may persist for prolonged periods of time in the soil such as viruses, helminths and protozoa needs to be examined. There are a number of factors in the laboratory that affect the ability for such microorganisms to be enumerated from biosolids/soil samples. These include factors such as inhibitors, visibility, background flora, recovery efficiencies, ability to culture and source the pathogens and the pathogens binding onto soil or biosolids particles. In addition, the decay patterns of laboratory-cultured strains compared with environmental strains needs to be examined for selection of suitable indicators for monitoring. The use of

PCR is an area requiring further development in terms of the ability to determine infective cells as opposed to the detection of non-viable cells.

The effect of different biosolids types on enteric pathogen survival times needs to be understood. Biosolids produced from different wastewater treatment plants possess different characteristics such as solids to moisture ratios, nutrient levels, pH levels and carbon content, and this may influence the decay times of enteric pathogens across different locations. In addition, the decay patterns of enteric pathogens in a variety of soil types, climatic conditions and locations needs to be tested to find any reoccurring patterns or conclusive results.

Further scientific data on the effect that biosolids have on decay times in soil needs to be collected. This includes the testing of different application rates to determine the absolute influence that biosolids incorporated with the soil may have on pathogen inactivation. The results from the present study suggested that biosolids do not 'protect' the pathogens nor prolong their survival. For this reason it may be possible that the addition of biosolids to soil may actually result in an increased rate of inactivation of pathogens in the soil, and this therefore, would be a positive argument for biosolids reuse.

9.8. Conclusions

Following the research conducted under this project, several objectives were addressed and key outcomes attained. The research objectives are discussed below:

- A method was developed to enable the decay times of enteric microorganisms (both indicator and pathogenic) to be examined across the growing season of wheat, from soil and biosolids-amended soil samples. While the external environmental conditions could not be exactly matched, sentinel chambers provided suitable microcosms to allow gaseous and moisture exchange to occur without the loss of microorganisms. This key outcome was demonstrated by similar decay patterns of *E. coli* inside the chambers (inoculated) with the *E. coli* naturally occurring in biosolids applied to the topsoil in the field (Figures 6-20 and 6-21). In addition, soil moisture changes inside the chamber were similar to the changes in soil moisture (topsoil) outside the chambers (Figures 6-10, 6-11, 6-14 and 6-15);
- The methodology for enumeration of enteric microorganisms in soil and biosolids samples was tested and initial decay times for *S. enterica* and MS2 were obtained. Similar decay times were found for *S. enterica* (T_{90}=25 d in glasshouse (Table 4-1) and 21 d and 25 d in the field (Table 6-6), both using Moora biosolids-amended soil) and MS2 (T_{90}=29 d in the glasshouse (Table 4-1) and 36 d and 29 d in the field (Table 6-6), both in Moora biosolids-amended soil) (Table 6-6), in the pot experiment (Chapter 4) compared with the field experiments (Chapter 6). This demonstrated the key outcome of this objective being a method capable of representing decay patterns both in field and glasshouse conditions;
- Decay times (T_{90}) are commonly used to describe time (d) for a one-$\log_{10}$ reduction of microorganisms in environmental samples. However, when decay patterns are non-linear, this line of regression can be inaccurate. Three statistical models were compared to determine the most appropriate model for obtaining decay times (T_{90}). For the purpose of this research, the quadratic equation was found to best describe decay patterns and provide a decay time (T_{90}) or days required for a one-$\log_{10}$ reduction to occur. This research objective was explored and further work is needed to refine the use of this model;

- The decay times of *E. coli* (indicator bacteria), *S. enterica* (pathogenic bacteria), bacteriophage MS2 (surrogate virus) and human adenovirus (pathogenic virus) in biosolids-amended agricultural soil were obtained. The key outcome of this objective was that the enteric microorganisms decayed faster in soils amended with biosolids compared with soil where no biosolids were present. This indicated that biosolids incorporated into soil increased the inactivation of pathogens. In addition, it was found that decay times are specific to microorganism type;
- It was observed that microorganism decay was correlated to the changes in soil moisture and soil temperature in the field. A key outcome from this objective found that enteric microorganism decay patterns are mostly influenced by declining soil moisture (particularly bacteria), and viruses are mainly influenced by increasing soil temperature;
- No particular effect of soil type or site was found to influence enteric microorganism decay patterns; however, the decay patterns can differ for each type of microorganism present in biosolids-amended soils;
- The effect of plant location (i.e. spikelet, leaves) on microorganism decay (on wheat plants at the flowering stage) was examined. The key outcome from this experiment demonstrated that microorganism decay was faster from the spikelet (grain-bearing region) than the leaves (less than 2 d) and thus the risk to humans or livestock at consumption was considered low. In addition, grain variety influenced microorganism decay times, along with microorganism type;
- The effect of threshing on enteric microorganisms inoculated onto mature wheat plants was examined. The outcomes of this objective were that threshing significantly reduced microorganism numbers, in particular *S. enterica;* and the highest level of microorganisms were found to be deposited onto the chaff post-threshing. The inactivation of microorganisms was, again, influenced by microorganism type as bacteriophage (surrogate virus) was more stable than the bacteria following the process of threshing. The key outcome of this experiment was that threshing reduced enteric microorganism numbers significantly and the risks of unsafe levels of bioaerosols in dust samples were considered to be low; and
- The presence/absence of bacteria - heterotrophic, *E. coli* and enterococci – in a mature wheat crop at harvest was examined. The outcomes of this objective were

that no *E. coli* were present at the site following biosolids application in the same season; bacteria levels (enterococci and heterotrophic bacteria) were highest on the chaff, particularly at the unamended site; the harvester generated higher numbers of bacteria into the dust than were found in the clean air samples; and these bacteria were higher in number at the biosolids-amended site compared with the unamended site. Overall, bacterial contamination in dust from biosolids application was not considered a high risk and the key outcome from these experiments was that microorganism numbers were highest on the chaff, and thus chaff could be sampled instead of air or dust samples to determine bioaerosol risk at crop sites.

From the studies presented in this thesis, it is concluded that pathogens from biosolids are of greatest risk to humans immediately following dispatch from the wastewater treatment plant. Microbial contamination levels are highest during this time and thus transport providers, handlers, spreaders, farmers and workers are at greatest risk of exposure to disease-causing pathogens. In addition, the climatic conditions common for southern Australia do not favour the prolonged survival of enteric pathogens in the soil, particularly from spring to summer, due to 1) decreasing soil moisture content (i.e. long dry periods with minimal rainfall events) and 2) increasing temperatures.

While biosolids are incorporated with the soil, the pathways to ingestion are low where withholding periods are maintained. Therefore, the main pathway to transmission may be more prevalent from poor hygienic practices such as food consumption following handling or the transfer of biosolids into vehicles or homes.

CHAPTER 10 REFERENCES

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