

ARE PATHOGENS PRESENT  
IN WHEAT AT HARVEST  
FOLLOWING THE LAND APPLICATION  
OF BIOSOLIDS?



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## **Declaration**

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

Signed:

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Dated:

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***“Half the secret of resistance is cleanliness, the other half is dirtiness.”***

***- Anonymous***

## Abstract

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A research project was undertaken at CSIRO Floreat in 2004 to study whether pathogens present in biosolids could survive the growing season and be of risk to human health. The aim of the study was to determine the decay rate of the indicator micro-organisms bacteria (*E. coli* and enterococci) and virus (bacteriophage), present in land-applied biosolids in a wheat field trial at Toodyay in Western Australia.

The field experiment consisted of four rates of biosolids (0, 4, 8 and 16 dry t/ha), arranged in a randomised block design, and replicated three times. Bacterial cell numbers were taken weekly from the soil for 8 weeks, then taken at weeks 10, 12, 15, 18 and 23. Soil samples were taken to determine soil moisture. Air temperatures were measured using Tiny-Talk temperature sensors and daily rainfall was measured using a tipping bucket rain gauge. The experiment was sampled for the presence of bacteria on plant leaves at week 15. Root samples were also tested for bacterial cell numbers at weeks 15 and 18. At harvest, grain samples from the 16 dry t/ha and nil plots were tested for the presence of bacteria.

The glasshouse experiment consisted of four treatments (0, 8, 16a and 16b dry t/ha equivalent) and replicated three times. The treatment containing no wheat (16b) was to compare the affect of shading on microbial survival. Biosolids were sterilised and then re-seeded with selected bacteria and a virus. Bacteria cell numbers and viral plaques were taken from the pot trial on days 0, 4, 7, 14, 21 and 28. Glasshouse temperatures were recorded daily using a thermo hydrograph. Soil temperatures were taken at sampling. Pots were kept at a constant moisture level without leaching throughout the experiment.

Results indicated that in the field bacteria survived in the soil for at least five months. Enterococci appeared to have died off earlier than *E. coli* but then reappeared after a rainfall event in December. Average *E. coli* cell numbers were slightly higher in the lower rate of biosolids (8 dry t/ha) than 16 dry t/ha, despite having no statistical significance. At weeks 10 and 12, *E. coli* cell numbers increased by two-log<sub>10</sub> across both treatments, possibly due to the influence of environmental conditions. *E. coli* cells present in root samples were approximately two-log<sub>10</sub> higher than the cell numbers

present in the adjacent soil. There were no bacterial cells present in the leaf samples taken at week 15. At harvest, there was no presence of bacteria on wheat grains.

Results indicated that in the glasshouse, bacteria and the virus that had been artificially seeded onto the sterilised biosolids survived for approximately one month. There was no statistical significance for survival across treatments. No other findings were reported.

Based on the results, there is no risk that the selected indicator micro-organisms – *E. coli*, enterococci and bacteriophage – could be present in grains at harvest time and therefore are not of risk to human health at consumption of grain products. However, the length of survival, higher levels of bacteria in the root zone and the ability for sudden increase in cell numbers are factors for consideration in the management of exposure pathways.

Results so far indicate that there is no reason to discontinue biosolids land application. However, more research is required to further determine absolute risk. It is recommended that further study be carried out on: the survival of other pathogens such as *Salmonellae*, *Clostridium perfringens*, *Cryptosporidium* and helminth on agricultural land in the wheatbelt; the risk of pathogen ingestion across the food chain due to animal-carriers; and food products grown using biosolids fertilisers — given the survival behaviour of bacteria, the shorter growing season and immediate consumption of the products.

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# Abbreviations

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The following abbreviations are used throughout this thesis. Additional abbreviations for chemicals are presented in Appendix 2.

ATCC	American Type Culture Collection
ACM	Australian Collection of Micro-organisms
cfu	colony forming unit
CSIRO	Commonwealth Scientific and Industrial Research Organisation
dd H <sub>2</sub> O	distilled, deionised H <sub>2</sub> O
DNase	deoxyribonuclease
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>Famp</i>	<i>E. coli</i> HS (p <i>Famp</i> ) R
GSWC	Gravimetric Soil Water Capacity
M	Molarity
MS2	bacteriophage or phage
NBRP	National Biosolids Research Program
Rpm	revolutions per minute
PB	phosphate buffer
<i>Ps. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
RNase	ribonuclease
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
t/ha	tonnes per hectare
TYGA	Tryptone Yeast Glucose Agar
TYGB	Tryptone Yeast Glucose Broth
USEPA	United States Environmental Protection Authority

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# **1 Introduction**

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## **1.1 Background**

At the National Biosolids Conference held in Brisbane in 2003 it was recognised that public perception of the safety of applying biosolids to agricultural land for use as a fertiliser was a major concern. As the main method of waste disposal in Western Australia is to spread it as a fertiliser in agriculture, the Water Corporation acknowledged the need for further information relating to the risk of infection from pathogens associated with land-applied biosolids, and have given their support to a pathogen project under the umbrella of the National Biosolids Research Program.

The first crop following the land application of biosolids is often cereal grain. Grains play a major role in the economy of Australia and are a main source of income for some producers in the agricultural industry. A large percentage of Australian grains are sold to customers in Asia, who require high quality products. Globally, agricultural industries are moving towards increased accountability and traceability in production, especially since experiencing recent disease outbreaks and health scares.

In Western Australia alone, Co-operative Bulk Handling (CBH) have recently released a new quality assurance program and have just announced a new joint venture with Indonesia's Salim Group in the formation of Pacific Agrifoods, thus making CBH a major stakeholder in one of Asia's largest flour milling operations, Interflour (Henderson 2004). In order to guarantee markets and to satisfy customers of Australian agricultural grain produce, suppliers will be required to comply with quality assurance programs that track information throughout the whole production and supply chain.

At present, there is minimal information on the microbial levels in harvested grains, following the application of biosolids. Consumers need confirmation that there is little or no health risks associated with crops grown using biosolids. Research data and scientific evidence needs to be gathered and made readily accessible in order to ensure that the national practice of using biosolids as a source of fertiliser is better understood.

## **1.2 Research problem**

To ensure public safety, both from handling biosolids to consuming products fertilised with biosolids, the question of how long pathogens (indicator micro-organisms) can survive on cereal crops under Australian conditions needs to be answered. To do this, the area of exposure needs to be assessed from spreading through to harvest and the decay rates of selected micro-organisms needs to be monitored in the soil, on the plant roots and on wheat grains at harvest.

## **1.3 Research aim**

The aim of this study is to determine the presence and level of selected microbial indicators — bacteriophage, enterococci and faecal coliform — in cereal grains from spreading to harvest, following the application of biosolids.

## **1.4 Research objectives**

As a result of the research aim, it is necessary to determine:

1. How long the selected micro-organisms can survive in the field compared to in the glasshouse;
2. If the application rate of biosolids affects the survival rate of the selected micro-organisms;
3. If the selected micro-organisms are able to survive on the cereal root system; and
4. If the selected micro-organisms are present on grains at harvest time.

## **1.5 Definitions**

A number of definitions are required before proceeding in order to describe unfamiliar terms and to further the reader's understanding.

**Bio-aerosols:** A system of living microbial colloidal particles dispersed in gas.

**Biosolids:** Wastewater sludge that has been altered to become a beneficial and useful resource (Liang et al. 2003; Spinose & Vesilind 2001). Sewage, primarily from domestic sources, is treated and processed to enable materials to be land applied and the solid, semi-solid or liquid materials are then termed 'biosolids' (Evanylo 1999; Sidhu 2000).

**Indicator micro-organisms:** Selected micro-organisms used to detect the presence of pathogens and to index the possible levels of contamination (Prescott et al. 2002).

- Ingestion:** To take micro-organisms into the body either through inhalation or contact with the mouth, the skin or the eyes.
- Pathogen:** Any disease-causing organism (Prescott et al. 2002).
- Pellet:** The solid, compact portion of bacterial culture that has been centrifuged.
- Spiking:** The deliberate seeding, or adding of, selected micro-organisms into a substance or liquid.

It should be noted that indicator micro-organisms have been used throughout this study to detect the level of pathogens present, as is the common practice in wastewater sampling.

## **1.6 Outcomes and benefits**

The outcomes that will be realised on completion of this Honours project will lead to a better understanding of:

1. The level of bacteria and viruses present at various stages of handling biosolids;
2. The decay rates of indicator micro-organisms found in biosolids under Western Australian wheatbelt conditions;
3. The areas to which humans are exposed to the greatest level of risk of ingesting pathogens from biosolids; and
4. Areas requiring further research.

Initially, the Water Corporation of Western Australia will use the information from this research. Regulators, public health and safety officers, researchers in wastewater and sludge, local government planners, managers, consultants and scientists will then be able to benefit from the information. By gaining actual data from field practice as to the behaviour and survival rate of indicator micro-organisms, the areas and levels of risk to humans can be better managed. This data could then be the basis of protection for consumers involved in the grain industry, should any concerns with the practice of disposing of biosolids onto agricultural land arise.

This dissertation is comprised of an introduction to the research problem (chapter 1), a review of relevant literature (chapter 2), a description of the materials used (chapter 3), discussion of the methodology (chapter 4) and results (chapter 5), followed by a discussion of significant findings, research limitations and opportunities for further research (chapters 6).

## 2 Review of Literature

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A review of literature was carried out to establish the possibility that pathogens could be present at harvest in cereal grains grown in land-applied biosolids. The pathways of exposure to pathogens, from biosolids to humans, needed to be understood along with the survivability of pathogens. An understanding of possible mechanisms for entry of pathogens into grains, either through plant uptake or bio-aerosols, assisted in the development of the research design.

This review formulated the hypothesis that the indicator micro-organisms — faecal coliform, enterococci and bacteriophage — will not be present in cereal (wheat) grains grown with biosolids fertiliser at harvest; and therefore, that at consumption of the grain, humans will be of no risk from these organisms.

### 2.1 Sludge processing

Sludge is required to be stabilised before it can be deposited onto land. This is done to reduce pathogenic micro-organisms and chemicals that could be a health hazard to humans; to reduce offensive odours; and to decrease the rate of putrefaction (Spinosa & Vesilind 2001; Switzenbaum et al. 1997). The most common technologies used to stabilise sludge to achieve the regulatory requirements are described in Table 2-1. These include anaerobic digestion, aerobic digestion, composting, alkaline stabilization and heat drying (Epstein 2003; Gerba et al. 2002; Sahlstrom et al. 2004; Sidhu 2000; Switzenbaum et al. 1997). These methods of stabilisation determine the classification and potential end-use of the biosolids. The three stabilization grades P1, P2 and P3 are derived from USEPA Part 503 Regulations (N. Penney [Water Corporation of Western Australia, Leederville] 2004, pers. comm., 28 May, 2004; Gibbs et al. 1994) and are described further in Appendix 1 Table A 1: Three biosolids classifications.

**Table 2-1: Major stabilisation processes**

STABILISATION METHOD	COMMENTS
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.

Sources: (Boost & Poon 1998; NRC 1996; Epstein 2003; Lewis-Jones & Winkler 1991; Liang et al. 2003; McFarland 2001; Pederson 1981; Sidhu 2000; Spinosa & Vesilind 2001; Switzenbaum et al. 1997; USEPA 1999).

The biosolids to be used in this study originated from the Woodman Point Wastewater Treatment Plant (WWTP). This plant uses anaerobic digestion to process sludge (Appendix 1: Table A2). Gibbs et al. (1994) state that sludge which has only been treated through the process of anaerobic digestion and dewatering presents risks which are too high to allow “unrestricted marketing” of the product, due to the high concentration of pathogens. It is for this reason that solids from this location have been selected for use in the trial; to provide a “worst-case” scenario.

The Australian regulations for the processing and application of biosolids are set under strict guidelines. These guidelines are based on the United States Environmental Protection Agency (USEPA) Part 503 Rule, established to decrease the level of risk to human health and the environment (N. Penney 2004, pers. comm., 28 May). Cameron et al. (1997) considered it risky to transfer guidelines from one waste disposal system to another due to the wide range of waste materials with differing physical, chemical and biological characteristics.

Switzenbaum et al. (1997) quoted Metcalf and Eddy's work from 1991 in noting that of all constituents removed by treatment, sludge is by far the largest in volume and its processing and disposal is perhaps the most complex problem facing the engineer in the field of wastewater treatment. Cameron et al. (1997) believed that the pressure to dispose of wastes onto land rather than into water often results in engineers being forced to design land treatment systems that have minimal scientific information as a guide.

To enable materials to be land applied, sewage, which is primarily from domestic sources, is treated and processed. The solid, semi-solid or liquid materials are then termed 'biosolids' (Evanylo 1999; Sidhu 2000). Biosolids is a term that is given to wastewater sludge that is destined for beneficial use and therefore has become a useful resource (Liang et al. 2003; Spinosa & Vesilind 2001). Switzenbaum et al. (1997) stated that one of the keys to successful marketing of biosolids products is quality and that quality is the basis for protection of human health and the environment.

Sidhu (2000) stated that the proper disposal of biosolids is essential to protect public health and to prevent contamination of the environment. Switzenbaum et al. (1997) believed the assessment of quality also depends on the desired final use or disposal of the product. Sahlstrom et al. (2004, p.1) believed that many of the pathogens potentially found in sludge are "zoonotic bacteria", which are important to the eco-cycle; perhaps not the best choice of words, as Prescott et al. (2002) defined "zoonosis" as a disease of animals that can be transmitted to humans.

Although processed, biosolids still contain human pathogens and complete elimination of pathogens would be expensive to achieve (Sidhu 2000). Pathogen concentrations for each category (Table A1) must be below the select criteria as specified in the guidelines (DEP, WRC & DOH 2002). The densities are measured by indicator micro-organisms such as faecal coliform, *Salmonella* sp. and enteric viruses (Spinosa & Vesilind 2001); however, other undetected pathogens of threat to human health may also be present in biosolids.

## **2.2 The presence of micro-organisms**

Some of the microbial organisms in sewage sludge or untreated biosolids are pathogenic (Evans 2001). After outlining factors that affect the survival of micro-organisms in sludge, Seviour and Blackall (1999) highlighted the fact that many organisms survive the processing system and therefore may pose a serious potential health hazard (Nasser et al. 2003; Rooklidge 2003; Sahlstrom et al. 2004; Sidhu et al. 2001; Spinosa & Vesilind 2001).

The micro-organisms that survive in biosolids which are of threat to humans include bacteria, viruses, protozoa cysts and helminth eggs (Armon et al. 1994; Brown et al. 1980; Cameron et al. 1997; Cliver 1980; NRC 1996; Dowd et al. 2000; Edmonds & Mayer 1979; Epstein 1998, 2003; Evans 2001; Evanylo 1999; Farrah et al. 1981; Krogmann & Boyles 2000; Lewis & Gattie 2002; Nell et al. 1983; Priestley 2004; Sidhu 2000; Smeal 1995; Smith & Farrell 2003; Sorber & Moore 1987).

Gibbs and Goen (1995) stated that the level of risk pathogens in biosolids products presented is still unknown. Spinosa and Vesilind (2001) similarly noted that there is little, if any evidence, that sludge disposal has caused public health problems, as it is difficult to distinguish between the sources and effects of pathogenic organisms in everyday life (Lewis & Gattie 2002).

The organisms present in biosolids are grouped in Table 2-2, which also summarises some of the pathogens that are found in biosolids, along with the associated health risks upon ingestion.

**Table 2-2: Principal pathogens of concern in domestic sewage and sewage sludge**

<b>ORGANISM</b>	<b>DISEASE AND/OR SYMPTOMS</b>
<b>BACTERIA</b>	
<i>Salmonella</i> sp.	Salmonellosis (food poisoning), Typhoid fever
<i>Escherichia coli</i> (enteropathogenic strains) <sup>1</sup>	Gastroenteritis
<i>Shigella</i> sp.	Bacillary dysentery, severe gastroenteritis
<i>Yersinia</i> sp.	Acute gastroenteritis (including diarrhoea, abdominal pain)
<i>Vibrio cholerae</i>	Cholera
<i>Campylobacter jejuni</i>	Gastroenteritis
<b>ENTERIC VIRUSES</b>	
Hepatitis A virus	Infectious hepatitis
Norwalk and Norwalk-like viruses	Epidemic gastroenteritis with severe diarrhoea
Rotaviruses	Acute gastroenteritis with severe diarrhoea
Enteroviruses	
Polioviruses	Poliomyelitis
Coxsackievirus	Meningitis, pneumonia, hepatitis, fever, cold-like symptoms etc.
Echoviruses	Meningitis, paralysis, encephalitis, fever, flu-like symptoms, diarrhoea etc.
Reovirus	Respiratory infections, gastroenteritis
Astroviruses	Epidemic gastroenteritis
Caliciviruses	Epidemic gastroenteritis
<b>PROTOZOA</b>	
<i>Cryptosporidium parvum</i>	Gastroenteritis
<i>Entamoeba histolytica</i>	Acute enteritis
<i>Giardia Lamblia</i>	Giardiasis (including diarrhoea, abdominal cramps, weight loss)
<i>Balantidium coli</i>	Diarrhoea and dysentery
<i>Toxoplasma gondii</i>	Toxoplasmosis
<b>HELMINTH WORMS</b>	
<i>Ascaris lumbricoides</i>	Digestive and nutritional disturbances, abdominal pain, vomiting, restlessness
<i>Ascaris suum</i>	May produce symptoms such as coughing, chest pain and fever
<i>Trichuris trichiura</i>	Abdominal pain, diarrhoea, anaemia, weight loss
<i>Toxacara canis</i>	Fever, abdominal discomfort, muscle aches, neurological symptoms
<i>Taenia saginata</i>	Nervousness, insomnia, anorexia, abdominal pain, digestive disturbances
<i>Necator americanus</i>	Hookworm disease
<i>Hymenolepis nana</i>	Taeniasis

<sup>1</sup>Pathogens in proposed study

Source: (USEPA 1999)

Gauthier and Archibald (2001) cited the work of Geldreich et al. from 1962, stating that micro-organisms from faecal indicator groups and enterococci, observed in sewage sludge, were frequently even higher than those typically found in faeces. However, Spinosa and Vesilind (2001) confirmed that aerobic digestion, air drying, anaerobic digestion, composting and lime stabilization bring about a decrease in potential pathogens of 100-1000 times.

Evans (2001) believed that the risk of pathogenic organisms in untreated sewage sludge is often exaggerated, as the content of *Salmonella*, for example, is generally low because most people, most of the time, do not have salmonellosis. However, Evans (2001) also pointed out that in the United Kingdom (UK) most people, most of the time, are not excreting virulent pathogens, but the risk may be increased on occasions as a result of outbreaks of infectious disease. Not taking any risks, Ahmed and Sorenson (1995, p. 143) stated that “to ensure the safety of the environment and human health, further reduction of pathogenic bacteria through proper treatment must be accomplished before biosolids are released to the public or applied to land”.

Chaney et al. (1996) noted that the worst area of risk from biosolids use or disposal was when individuals were sold or were given biosolids without controls, leading to uncontrolled rates of application and uncontrolled exposure which could then lead to human and/or environmental risk.

The Water Environment Research Foundation (WEF 2003) believed that we should be more concerned with the use of animal manures, due to the fact that indicator pathogen concentrations are higher in animal manures than in biosolids and their concentrations are not strictly regulated (Mawdsley et al. 1995; Stevens et al. 2002). In Jjemba (2002), it is claimed that animal manure is more highly concentrated and has a higher biochemical oxygen demand than treated sewage sludge. Evans (2001), working along similar lines, stated that with the emergence of new pathogens, animal manures have become a significant risk. However, Spicer (2002) stated that manures are more readily accepted than biosolids due to their having had a long-term, relatively problem-free history of use.

Evans (2001) suggested that, in general, inputs of risk materials vary from catchment to catchment and from country to country, but that it is still the responsibility of waste undertakers to consider likely hazards and to control any risks (Pillai & Ricke 2002).

Of all the pathogenic organisms, Lewis and Gattie (2002) stated that enteric viruses are of greatest risk to humans, due to their resistance to high pH and heat treatment, high infectivity and survivability (Gibbs et al. 1994; Lewis & Gattie 2002). Bacteria such as faecal coliform, *Listeria monocytogenes* and enterococci present in sludge are capable of surviving anaerobic digestion (Estrada et al. 2004; Gerba et al. 2002; Lewis-Jones & Winkler 1991; Sidhu 2000); thus their significance and choice for this research trial (Table 2.2). Regrowth may also occur, as with *Salmonella* sp., after waste treatment processes and land application (Awad et al. 1989; Gerba et al. 2002; Gibbs et al. 1994; 1997; Lewis & Gattie 2002; Sidhu 2000).

In the proposed study, the microbes to be studied are bacteriophage, enterococci and faecal coliform. These micro-organisms have been selected from the groups of bacteria, virus and protozoa, as indicators, to detect the presence and level of pathogens in stockpiled biosolids. Helminths were not selected for this study, as they are not a major problem in south-western Australia at present (S. Toze [CSIRO Land and Water, Floreat] 2004, pers. comm., 24 May, 2004).

### **2.3 Pathways of exposure**

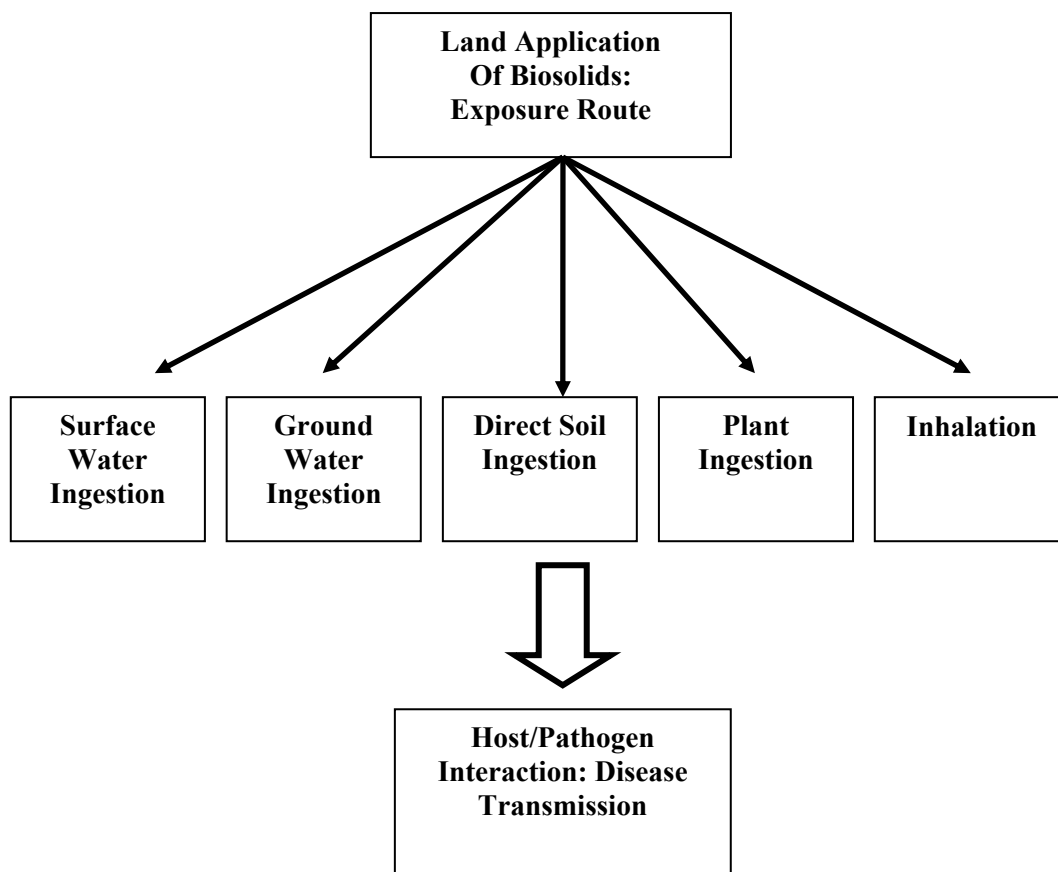
There is a risk of spreading diseases to people and animals if sludge containing pathogenic bacteria is spread onto arable land (Sahlstrom et al. 2004). The National Research Council (1996) believed that the final link in the transmission chain of infectious disease is the exposure of the susceptible human population to infectious agents.

The risk to public health from pathogens in biosolids that have been applied to land can depend upon: the treatment process used; how well the organism survives in the environment; the pathway for the organism to come into contact with the public; and the likelihood that the organism ingested will result in disease, which is dependant on the health and age of the exposed individual (Krogmann & Boyles 2000).

There are two approaches to assessing the potential hazard of viruses to humans from sludge applied to land. Firstly, the fate of the viruses from their environmental origin in faeces through to sludge processing and their exposure to environmental conditions can be assessed; secondly, the fate of viruses in sludge could be followed from land application until humans become exposed (Bertucci et al. 1987).

This study will follow the first approach, namely it will monitor decay rates; but it will also briefly cover the second approach with regard to possible areas of human exposure throughout the duration of the crop's growth and maturity. As illustrated in Figure 2-, the presence of pathogens in biosolids, following the land application, can be through: uptake by plants and entry into the food chain (Epstein 2003); movement through the soil and contamination of groundwater with potential contamination of drinking water (Epstein 2003; Gagliardi & Karns 2000); and runoff and erosion containing pathogens and contaminating surface water (Colford et al. 2003; Epstein 2003). Table A 3 (Appendix 1) is an additional summary of exposure pathways used in the risk assessment for land application of biosolids under USEPA Part 503 Rule.

**Figure 2-1: Relevant routes of exposure for land application of biosolids**



Source: Colford et al. 2003

Exposure to pathogens can occur through direct or indirect routes (Liu & Liptak 2000; Gibbs et al. 1994). The main groups at risk are further outlined in Table 2-3, where the routes are described as direct and indirect areas of exposure.

The direct contact pathways of pathogens to humans are hand, mouth and/or skin contact after handling biosolids (Gerba et al. 2002; Gibbs et al. 1995; Hu et al. 1996; Sidhu 2000); walking through an area to which sewage sludge has been applied (USEPA 1999); and inhalation of bioaerosols containing pathogens (Bertucci et al. 1987; Dowd et al. 2000; Epstein 1998; Lewis & Gattie 2002; McFarland 2001; Michalk et al. 1995; Ross et al. 1991; Sidhu 2000; USEPA 1999).

Indirect pathways of pathogen ingestion by humans potentially include: ingestion of plants, vegetables or fruit grown in biosolids amended soil (Ross et al. 1991; Cliver 1980; Gagliardi & Karns 2000); contamination of crops consumed by livestock that are used for human consumption (Brown et al. 1980; Mawdsley et al. 1995; Ross et al. 1991); contact with human, animal or insect carriers; and ingestion of surface water and groundwater containing pollutants from eroded biosolids (Ross et al. 1991; USEPA 1999). Gauthier and Archibald (2001) have found several reported cases whereby enterococci was present on insects, plants, and other natural sources in which faecal contamination was originally not considered likely.

**Table 2-3: Groups of people at risk from exposure to sewage sludge**

GROUP	PEOPLE AT RISK
Direct Exposure	<ul style="list-style-type: none"> <li>▪ Wastewater and sludge treatment plant workers</li> <li>▪ Sludge transporters and handlers</li> <li>▪ Member of the public using garden products containing sludge</li> <li>▪ Farm workers using sludge</li> <li>▪ Landscaping workers</li> </ul>
Indirect Exposure	<ul style="list-style-type: none"> <li>▪ Population groups consuming crops grown on sludge (i.e. raw sewage) amended soil</li> <li>▪ Population groups consuming meat or water indirectly contaminated from sludge</li> </ul>

Source: (Gibbs et al. 1994)

This research will concentrate on the indirect pathway of pathogen ingestion through plants (cereal grains) and soil; in the case of soil, the pathogens that are present in biosolids clumps remaining on the soil surface after application.

Nasser et al. (2003) stated that pathogenic micro-organisms may present a serious health risk to consumers of agricultural products. However, Epstein (2003) believed that food chain crops that are cooked or processed would have no potential for infection. Earlier, Epstein (1998, p.66) had suggested, “that there is very little danger to human health from the use of biosolids on field crops, fruit trees, processing crops, or other crops not used directly for human consumption since the material does not come in contact with the edible portion of the plant, or the food undergoes processing, to eliminate pathogens”. Epstein did not, however, provide any withholding periods.

According to Lewis and Gattie (2002), public exposure occurs after pathogens have been transported away from land application sites by air or water. Smeal (1995) believed that the levels of contamination of biosolids containing micro-organisms is low at the time of application and that these levels continue to decline under common field conditions. However, Farrah et al. (1981) stated that few researchers have studied the fate of indigenous microbes under field conditions, following sludge application to land, and that such studies should include research on the survival of viruses as well as the transport of viruses through the soil matrix.

Although stated over 20 years ago, Farrah et al. (1981) comments regarding the lack of data from studies carried out on the survivability of pathogens under field conditions are still relevant today, especially in Australia. Joshua et al. (1998, p. 137) supported this, stating, “that little information is available in Australian soils which are more acidic and less fertile than those used for biosolids studies in other parts of the world”. Therefore, this study will test pathogen survival on a selected soil-type in Western Australia.

## **2.4 Factors affecting survival**

Pathogens are removed by filtration, sorption, sedimentation and die-off (Rutledge & Wolf 1985). However, the existence of pathogens in soils and plants depends on their survival during wastewater treatment and biosolids processing, the method of land application, soil and environmental conditions (Epstein 2003). The survival of pathogens in soils is related to the biological, chemical and physical environment (Sidhu 2000; Sommers & Barbarick 1985).

The factors that can affect the survival of pathogens following the land application of biosolids include: the type (presence and quantity) of organism (Epstein 2003); the method of land application (surface application or incorporation); amendment solids content (applied as solids or liquid); climatic and microclimatic effects (temperature and moisture) (Gibbs et al. 1994; Mawdsley et al. 1995); the soil condition (infiltration, permeability, soil moisture content, soil pH (Mawdsley et al. 1995; Sommers & Barbarick 1985), organic matter, nutrient availability (Sidhu et al. 2001), sterility, chemical properties and texture); other soil microbial populations; and the plants' exposure to weather causing processes such as sunlight disinfection and desiccation (Awad et al. 1989; Brown et al. 1980; Edmonds & Mayer 1979; Epstein 1998; Gibbs et al. 1994; Lewis-Jones & Winkler 1991; Liang et al. 2003; Sommers & Barbarick 1985; Stevik et al. 2003; USEPA 1999; Watson 1980; WEF 2003).

Of these factors Awad et al. (1989) believed that temperature and moisture are the most influential (Cameron et al. 1997; Straub et al. 1993). Depending on the nature of the micro-organism, the longevity of pathogens is greater in moist soils in damp, shady areas (Estrada et al. 2004), or soils with a high moisture-holding capacity. Pathogenic micro-organisms survive longer at low temperatures and in soils with a pH range of 5.0 to 6.4 (CaCl<sub>2</sub>) (Estrada et al. 2004; Gerba et al. 2002; Sidhu 2000; Stevik et al. 2003). The soil to be used in both the project pot and field trials has a pH level of 5.1 (CaCl<sub>2</sub>) and a moisture-holding capacity of 16%, which should favour the survival of the indicator micro-organisms used in this study.

Krogmann and Boyles (2000) believed that many pathogens die because they do not have access to a host. However, Lewis-Jones and Winkler (1991) believed that bacteria are capable of survival and growth outside their animal host and that sewage sludge, in suitable conditions, provides a very good growth medium for many bacterial pathogens. The presence of other micro-organisms can reduce the numbers of pathogenic micro-organisms in the soil through competition. Organic matter can serve as a food and energy source to bacteria, therefore allowing it to increase (Epstein 2003).

Table 2-4 displays the potential survival time of indicator micro-organisms in the soil and on plant surfaces in the USA. Within two to three months following application, pathogenic bacteria and viral numbers are usually reduced to a minimum (Cameron et al. 1997). However, Dudley et al. (1980) recorded the survival of *Salmonella* organisms for up to 72 weeks (16 months) in sludges that were applied to land.

**Table 2-4: Survival times of pathogens in soil and on plant surfaces**

Pathogen Type	SOIL		PLANT	
	Absolute Maximum	Common Maximum	Absolute Maximum	Common Maximum
Bacteria	1 year	2 months	6 months	1 month
Viruses	1 year	3 months	2 months	1 month
Protozoan cysts	10 days	2 days	5 days	2 days
Helminth ova	7 years	2 years	5 months	1 month

Source: (USEPA 1999; Smith & Farrell 2003; Gibbs et al. 1994)

It can be seen by the data provided, that bacteria, viruses and helminth (*Ascaris*) ova pose the highest risk of infection due to their ability to survive in soil for longer periods of time. Gibbs et al. (1994) state that helminth (*Ascaris*) ova may survive for up to 15 years (USEPA 1992; Edmonds & Mayer 1979). However, the Water Environment Research Foundation (2003) claimed that researchers have found that many pathogens do not survive for extended periods.

Awad et al. (1989) stated that bacteria comprise the most numerous pathogens found in sewage sludge. Like viruses, they can survive and reproduce in sludge for several months in cool, moist conditions. In 1979, Edmonds and Mayer reported that bacteria could survive as long as 15 months and suggested that viruses had slightly less survival time than bacteria, despite having the ability to survive for a few hundred days.

However, Sidhu (2000) stated that pathogenic bacteria die quickly in comparison to viruses; viruses having the ability to survive up to six months in the soil. Edmonds and Mayer's study may have included the bacteria, *Salmonella*, which has been reported to survive in soil-amended with biosolids for up to seven months, although they do not specify this (Burge & Marsh 1978; Sidhu 2000). Bacteriophage (virus) has been shown to survive longer in soil and compost than poliovirus; however, Brown et al. (1980) believed varying conditions when carrying out testing may have influenced these results.

Pathogens below the soil surface persist for less than a year under most circumstances (Cliver 1980). Pathogens deposited on plant surfaces survive for less time than when in the soil (Edmonds & Mayer 1979; Epstein 1998). The sampling method in this research will involve samples being taken from the soil at a depth of 0-5 cm, with biosolids clumps being targeted in order to represent a "worst-case" scenario. Plant surfaces will also be tested in October (week 15).

Brown et al. (1980) suggested that the solids concentration of the sludge at the time of application may also influence the survival of micro-organisms. Sidhu (2000) stated that the potential risk associated with enteric pathogens such as *E. coli*, *Salmonella* and *Giardia* is expected to be higher at high rates of biosolids application. Therefore, in this research, four biosolids rates of 0, 4, 8 and 16 dry tonnes per hectare will be trialled to test the decay rates of pathogens at increasing application rates. The highest rate is double what is routinely applied, again, to examine a “worst-case” scenario.

For this research project, the decay rates of the selected indicator micro-organisms will be tried in soil, plants and grains over the five months of the cereal crop-growing season, in the central wheatbelt of Western Australia. Gibbs et al. (1994) pointed out that survival at the soil surface under dry, hotter conditions can cause the rapid inactivation of pathogens. This study will only test survival rates in the first 0-5cm of soil under Australia’s dry, hot conditions following incorporation with biosolids.

## **2.5 Pathogen presence in plants**

According to Epstein (1998; 2003), pathogens are not taken up from the soil by plant roots, as they are too large to enter the root system and be translocated to the leafy portions of the plant. Cliver (1980) believed that root crops may be subject to surface contamination by sludge-borne pathogens no matter how the sludge is applied. However, he also states that pathogens are unlikely to persist for less than one year, and that sludge tilled beneath the soil’s surface is unlikely to contaminate the aboveground parts of the plants.

By the time cereal plants come into grain fill, the majority of pathogens present would have been expected to have died off (Epstein 2003). Plants that have edible portions growing above the ground should only become directly contaminated if sludge is sprayed onto the standing crop (Cliver 1980). However, any pathogens found on the surface of the plant generally survive for a shorter period of time than those found in the soil — exposure to sunlight, ultraviolet light and desiccation reduce their ability to survive (Cliver 1980; Edmonds & Mayer 1979; Epstein 2003).

The public concern that pathogens may be taken up by the plant is based upon the possibility that chemical constituents or therapeutic agents (Jjemba 2002) can enter plants by the roots, be transferred to the edible portions of the plant, and be consumed by humans (NRC 1996; WEF 2003). If consumers have concerns about pathogens being present, commercial processing, such as thermal processing, and hermetically sealed food storage containers are an effective safeguard prior to consumption (Cliver 1980). Cliver (1980) considered anaerobic and

mesophilic digestion to be sufficient processing treatments in reducing pathogens in sludge used to fertilise grain consumed by meat animals.

The presence of pathogens on plant roots will be tested through the sampling of indicator bacteria on cereal grain root systems. Plants will be sampled in the soil from the field trial containing the highest level of biosolids, being 16 dry tonnes per hectare.

## 2.6 Similar studies

There have been many tests conducted on the concentrations of pathogens in stored biosolids, composts and wastewater applied to land, but minimal research has been carried out on pathogens present following the application of biosolids to agricultural land.

In 1980, Watson carried out research on the survival and decay rates of *Salmonellae* in cattle slurry applied to arable land. It was found that *Salmonellae* survived in low dosages for about six weeks in summer conditions. The period of survival was dependent upon the numbers of *Salmonellae* present in the sludge prior to spreading, and the environmental conditions that the organisms were exposed to.

In 1994, Armon et al. carried out a study on vegetable crops grown for immediate consumption, to test whether pathogens were present on or within lettuces, parsley, cabbages, onions, carrots, fennel, radishes and tomatoes, after being irrigated with highly polluted effluents. The research found that the level of risk was dependant on the quality of effluent used. Vegetable texture, such as that of foliage vegetables like lettuces, affected the level of susceptibility to contamination and the length of micro-organism survival.

In 1995, Eamens et al. investigated the concentrations and decay rates of four bacterial species found in two types of biosolids (lagooned and dewatered) applied to agricultural land. Indicator micro-organisms (bacteria) were tested and used as a basis for assessing the risk faced by humans and animals when using biosolids. The study found that the bacteria in the lagooned biosolids steadily declined over five to six months, whereas the bacteria in the dewatered biosolids survived for up to four to five months. It was found that the bacteria, *Cl. perfringens*, might survive in excess of seven months on agricultural land given favourable environmental conditions. Their studies showed that soil depth made minimal difference to the bacteria numbers. Of interest though, was that herbicide treatments at two to six months reduced bacterial numbers significantly.

In 1997, Gibbs et al. carried out studies on the concentrations of faecal coliform, faecal streptococci and *Salmonellae* in biosolids mixed into sandy soils. This study detected repopulation or regrowth occurrence of micro-organisms under favourable conditions, such as winter rainfall, and concluded that soil amended with biosolids could not be considered pathogen-free for at least one year following application. Also, the occurrence of regrowth needs to be considered when establishing the means by which to protect public health.

In 2003, Harapas et al. carried out a similar study to that of Armon et al. (1994), by growing vegetables on poultry litter. The presence and level of the micro-organisms *Escherichia coli*, *Salmonellae*, *Campylobacter* and *Listeria* were tested on lettuces, celery and coriander at harvest. The study resulted in low levels of *E. coli* present and no detection of *Salmonellae* or *Campylobacter* at harvest.

In 2004 Nicholson et al. (2004) carried out similar work in the United Kingdom to Watson (1980) by testing the survival of pathogens in livestock manure at storage and after land application. Results found that *E. coli* 0157, *Salmonella* and *Campylobacter* generally survived for up to three months in storage and up to one month in the soil. *Listeria* survived for up to six months in storage and more than one month in the soil.

## 2.7 Further research

The gaps in the research indicate more can be discovered about the presence of pathogens in the soil and the pathways to human exposure (Loehr & Alharthy 1985). To address some of these gaps this study monitored degradation rates over the growing season and tested the levels of bacteria in the soil, on the plants, in the root zone and on the grains. Climatic conditions were recorded in the field, and in the glasshouse, more controlled conditions were provided. This has lead to the following hypothesis: the indicator micro-organisms would not be present in cereal (wheat) grain at harvest following the application of biosolids at seeding; and therefore the grain will be considered safe for human consumption.

## 3 Materials

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### 3.1 Reagents and chemicals

The reagents and chemicals used in this study are listed with their suppliers in Appendix 2 (Table A4).

### 3.2 Reference bacterial strains

The reference bacterial strains used in this study along with their source are shown in Table 3-1 below.

**Table 3-1: Reference bacterial strains**

ORGANISM	SOURCE/STRAIN
<i>Escherichia coli</i>	ACM 1803
<i>E. coli</i> HS (pFamp) R	ATCC 700891
<i>Enterococcus faecalis</i>	ATCC 19433
<i>Pseudomonas aeruginosa</i>	ACM 495
<i>Staphylococcus aureus</i>	ACM 2434

### 3.3 Media and cultures

All media were made in distilled deionised water (ddH<sub>2</sub>O), unless otherwise indicated. Sterilizations were carried out by autoclaving at 121°C for 15 minutes, unless otherwise indicated.

#### 3.3.1 Bacterial

##### Coliform medium

Chromocult™ Coliform – Agar	26.5 g
ddH <sub>2</sub> O	1.0 L

##### Enterococci medium

Chromocult™ Enterococci – Agar	33.0 g
ddH <sub>2</sub> O	1.0 L

***E. coli* positive control**

TYGB (see Viral section 3.3.2)	100 mL
<i>E. coli</i> 1803	100 µL

Overnight cultures were grown in a shaking incubator set at 37°C for 12-18 hours.

***E. coli* negative control**

Nutrient broth	1.30 g
ddH <sub>2</sub> O	100 mL
<i>Ps. aeruginosa</i> <sup>1</sup>	100 µL

Nutrient broth (Oxoid) was made to a concentration of 13 g/L as per manufacturer's instructions.

***Enterococci* positive control**

Brain heart	3.70 g
ddH <sub>2</sub> O	100 mL
<i>E. faecalis</i> (ATCC 19433) <sup>1</sup>	100 µL

***Enterococci* negative control**

Brain heart	3.70 g
ddH <sub>2</sub> O	100 mL
<i>S. aureus</i> <sup>1</sup>	100 µL

**Slope culture preservation**

Bacterial culture	8.0 mL
Glycerol (20%)	2.0 mL

Solution was vortexed in a 15 mL centrifuge tube, put into 1.5 mL sterilised tubes using a pipette and placed into a -80°C freezer.

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<sup>1</sup> Strains were added after broths and H<sub>2</sub>O had been autoclaved and cooled. Cultures were grown in the shaking incubator at 37°C overnight.

### 3.3.2 Viral

#### **Tryptone Yeast Glucose Broth (TYGB)**

Host: *Famp*

Tryptone	10.0 g
Yeast extract	1.0 g
NaCl	8.0 g
ddH <sub>2</sub> O	1.0 L

pH: 7.2 ± 0.1

The following were added, per 100 mL, after autoclaving and before use:

200 µl (5x) of glucose/calcium chloride solution

200 µl (5x) of magnesium sulphate

1 mL of Ampicillin

Overnight hosts were grown in a shaking incubator set at 37°C for 12-18 hours.

#### **TYGB Exponential Host**

TYGB	100 mL
TYGB Overnight Host <i>Famp</i>	1000 µL
Calcium chloride (CaCl <sub>2</sub> )	200 µL
Magnesium sulphate (MgSO <sub>4</sub> )	200 µL
Nalidixic acid	400 µL
Ampicillin	1000 µL

Exponential cultures were grown in a shaking incubator set at 37°C for 3 to 3½ hours.

### **Tryptone Yeast Glucose Agar (TYGA)**

#### Base Plate Ingredients

##### *Broth:*

Tryptone	10.0 g
Yeast Extract	1.0 g
NaCl	8.0 g
ddH <sub>2</sub> O	1.0 L

##### *Plus Agar:*

Agar Bacteriological	14.0 g
Calcium chloride (CaCl <sub>2</sub> ) <sup>2</sup>	200 µL
Magnesium sulphate (MgSO <sub>4</sub> ) <sup>2</sup>	200 µL

### **Tryptone Yeast Glucose Single Strength Agar (TYGSSA)**

#### Overlay Agar Ingredients

##### *Broth:*

Tryptone	10.0 g
Yeast Extract	1.0 g
NaCl	8.0 g
ddH <sub>2</sub> O	1.0 L

##### *Agar:*

Overlay/semi-solid (ss):	0.7 g
Calcium chloride (CaCl <sub>2</sub> ) <sup>3</sup>	200 µL
Magnesium sulphate (MgSO <sub>4</sub> ) <sup>3</sup>	200 µL
Nalidixic acid <sup>3</sup>	400 µL
Ampicillin <sup>3</sup>	1000 µL

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<sup>2</sup> Ingredients were added after autoclaving broth and cooling it in the water bath at 50°C.

<sup>3</sup> Ingredients were added after autoclaving Agar and cooling it in a water bath at 50°C. Note that ingredient requirements are as per 100 mL not per litre.

### 3.4 Buffers and solutions

#### 0.2M Monobasic stock

Sodium dihydrogen orthophosphate	13.9 g
ddH <sub>2</sub> O	500 mL

#### 0.2M Dibasic stock

Sodium phosphate dibasic heptahydrate	53.65 g
OR Anhydrous form	28.40 g
ddH <sub>2</sub> O	1 L

#### 0.1M Phosphate Buffer (pH 7.2)

Monobasic stock	84 mL
Dibasic stock	216 mL

Stock and buffers were stored at room temperature or 4°C.

#### 0.1M Sodium Hydroxide (NaOH)

Sodium Hydroxide	20 g
ddH <sub>2</sub> O	500 mL

#### Nalidixic Acid<sup>4</sup>

Nalidixic Acid powder	250 mg
NaOH (1 M)	2 mL
ddH <sub>2</sub> O	8 mL

#### Ampicillin<sup>4</sup>

Ampicillin	150 mg
NaOH (1 M)	2 mL
ddH <sub>2</sub> O	8 mL

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<sup>4</sup> After dissolving solution, antibiotics were filtered through a 0.22 µm using a 10 cc/mL plastic syringe, then stored at -20°C in a freezer for no more than 6 months.

**Glucose/Calcium Chloride Solution (5x)<sup>5,6</sup>**

Calcium chloride (CaCl <sub>2</sub> ·2H <sub>2</sub> O)	7.5 g
Glucose	25 g
ddH <sub>2</sub> O	50 mL

**Magnesium Sulphate (5x)<sup>6</sup>**

Magnesium sulphate (MgSO <sub>4</sub> )	7.5 g
ddH <sub>2</sub> O	50 mL

**Microbial Seeding H<sub>2</sub>O**

Sodium Thiosulphate (Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> )	0.6 g
H <sub>2</sub> O (tap water)	6.0 L

Water was sterilized at 121°C in the autoclave for 1 hour and cooled before use.

**Sterilising watering cans**

At establishment of the pot trial, an estimated level of sodium hypochlorite (NaClO) was added to distilled, deionised water (ddH<sub>2</sub>O) used to fill watering cans, and left to settle for 20 minutes. After this time, watering cans were flushed at least five times in deionised water, to remove all traces of sodium hypochlorite.

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<sup>5</sup> Solution was heated gently to dissolve, filtered and stored at 4°C.

<sup>6</sup> Solutions were passed through 0.22 µm filters using a 60 cc/mL plastic syringe, placed into sterile tubes and stored at 4°C.

## 4 Methodology

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This research project aimed to determine the presence and level of selected microbial indicators — bacteriophage, enterococci and faecal coliform — in cereal grains from spreading to harvest after application of biosolids at seeding, given Western Australian conditions. By determining this, the Water Corporation may better be able to reduce risks of exposure to humans from processing to application. Experimental research was used both in the field trial and glasshouse trial to test the decay rates of indicator micro-organisms. The method of conducting this research was formulated through a combination of the National Biosolids Conference proceedings, consultation with microbiological experts and meeting with members of the National Biosolids Research Program.

The field and pot trial experiments were designed around the research objectives, these being to determine:

1. How long the selected micro-organisms can survive in the field compared to in the glasshouse;
2. If the application rate of biosolids affects the survival rate of the selected micro-organisms;
3. If the selected micro-organisms are able to survive on the cereal root system; and
4. If the selected micro-organisms are present on grains at harvest time.

### 4.1 Research method

Experimental research was used to measure progress and the amount of change between variables both in the field trial and the pot trial. This process manipulated one or more independent variables and measured their affect on one or more dependent variables (Malhotra 1999) in a cause-and-effect relationship. The dependent variables were *E. coli*, enterococci and bacteriophage, and the independent variables were the treatments. To do this, all factors were controlled where possible except the dependent variables that were the centre of investigation (Leedy & Ormrod 2001). Further to this, documented literature was reviewed to study what had already been discovered about the subject.

As well as identifying what had already been studied about the selected topic, the literature review gave a theoretical base on which to build a rationale for the study (Leedy & Ormrod 2001). Information was acquired using library searches covering such key

words as ‘biosolids’, ‘pathogens’, ‘sewage sludge’, ‘enterococci’, ‘faecal coliform’, ‘bacteriophage’, ‘risk’, ‘agricultural’ and ‘human health’. It was found that the most relevant studies have been carried out and documented over many years by researchers in the United States of America.

## 4.2 Experiment details

### 4.2.1 Field Trial

#### *Location and climate*

The experiment was conducted at Harcourt Lowline Stud (100 km east of Perth), Toodyay, Western Australia, from June to December 2004. The climate is typically dry in the summer, with the majority of rainfall (70%) received during winter and spring months. The average annual rainfall is 430 mm, with a mean daily maximum annual temperature of 25°C and an average wind speed of 12 km/hr. The site had several features that deemed it a typical representation of wheatbelt conditions: reliable rainfall, suitable loamy sand soil, a pH of 5.1 (CaCl<sub>2</sub>), a moisture-holding capacity of approximately 16%, the absence of salinity and minimal frost risk due to undulating topography. Such conditions are favourable to the survival of pathogens. Soil and air temperatures were measured using Tiny-Talk temperature sensors and daily rainfall was measured using a tipping bucket rain gauge throughout the duration of the experiment.

#### *Treatments*

The field trial consisted of four biosolids treatments: 16 dry t/ha (treatment 1); 8 dry t/ha (treatment 2); 4 dry t/ha (treatment 3); and nil dry t/ha (control), with each treatment in triplicate in a randomised block design to give a total of 12 plots (Table 4-1). Each plot was 2 m wide x 10 m long, with a 1 m buffer zone between plots.

**Table 4-1: Randomised block design for field trial**

*B <sup>4</sup> A	B <sup>8</sup> B	B <sup>0</sup> C
B <sup>0</sup> A	*B <sup>4</sup> B	B <sup>16</sup> C
B <sup>8</sup> A	B <sup>16</sup> B	*B <sup>4</sup> C
B <sup>16</sup> A	B <sup>0</sup> B	B <sup>8</sup> C

\* Treatment 3 eliminated

***Preparation***

The trial plot was sprayed with Roundup (Glyphosate) at 1L/ha on 14 May 2004 and cultivated with tines on 17 May 2004 to remove weeds. The field was sampled for bacterial cell numbers on 9 June 2004 to test for the presence of the selected bacterial organisms prior to spreading the biosolids. The biosolids from the stockpile (stored on a trailer) were also sampled on 9 June 2004. The trailer was divided into four portions for sampling with four grab samples taken from each quarter. Samples were placed into four 500 ml Jupiter containers, labelled, stored on ice and couriered to Analytical Reference Laboratory (WA) in Perth (external laboratory), to determine the faecal streptococci count/grams (dry basis) prior to application. The length of time they were stored before being processed is unknown.

Anaerobically digested biosolids from Woodman Point WWTP were applied to the site on 16 June 2004. Biosolids from this location have the highest level of pathogens of the three processing plants in the metropolitan area of Western Australia, which enabled the worst-case scenario to be tested.

***Soil properties***

Two field soil site samples were taken on 5 April 2004 for analysis through CSBP (Table 4.2). Site A was located at the upper end of a gentle slope and Site B towards the dam at the south end. Results indicated the site contained loamy sand with brown to dark brown soil and a gravel content of 5-25%. Soil pH levels were slightly acidic at 5.1 (CaCl<sub>2</sub>) and 5.75 (H<sub>2</sub>O). Organic carbon (%) was ideal at site A. The calcium to magnesium ratio was ideal at both sites. Levels of other nutrients and trace elements present are listed in Table 4-2.

**Table 4-2: Field trial results from CSBP soil analysis**

<b>CSBP ANALYSIS RESULTS</b>	<b>SITE A</b>	<b>SITE B</b>
Soil texture	Loamy sand	Loamy sand
Soil colour	Dark brown	Brown
Gravel (%)	25	5
pH (1:5 CaCl <sub>2</sub> )	5.1 Low	5.1 Low
pH (1:5 H <sub>2</sub> O)	5.8	5.7
Organic carbon % (Walkley Black)	2.27 Ideal	1.93
Nitrogen	Marginal	Sufficient
Phosphorous sorption	Ideal	Low
Potassium	High	High
Sulphur	Sufficient	Sufficient
Soil stability rating	Ideal	Ideal
Magnesium % cations	14.8 Ideal	17.2 Ideal
Sodium % cations	4.4 Ideal	7.8 High
Calcium: magnesium ratio	4.7 Ideal	3.8 Ideal
Chloride (H <sub>2</sub> O) (mg/kg)	54 Sufficient	109 Sufficient
Aluminium (CaCl <sub>2</sub> ) (mg/kg)	1.2 Ideal	1.1 Ideal

### ***Seeding***

Having been spread manually, the biosolids were incorporated into the subsurface soil within 36 hours, using a disc plough as per industry guidelines. Seeding took place, late in the season, on 17 June 2004 using a 12-row disc-seeder. Wheat (*Triticum aestivum* cv. Westonia) was sown at a rate of 57 kg/ha to a depth of 2-3 cm over all plots, as a representative cereal variety. It is a common industry practice for farmers to sow the first crop to either canola or wheat, following land application of biosolids. Wheat was selected due to its importance as a major export product, and its use in the production of flour for human consumption. The fertiliser was top-dressed according to district practice using 80 kg/ha Di-ammonium Phosphate (DAP) at seeding.

### ***Other applications***

Urea was applied at 55 kg/ha 21 days after sowing (DAS). The plot was sprayed on 24 July 2004 with Chlorpyrifos 500 (Anticholinestase) to control Red-legged earth mite (RLEM) and Lucerne flea numbers. All broadleaved-weeds were removed by hand. A fungicide, Bayleton (Triadimffon), was applied at 1 L/ha with 0.05% oil on 12 September 2004 to control stripe rust.

### **Field sampling**

Two soil samples were randomly taken per third of the plot, (six samples per plot) across all representations. Samples were taken at 0-5 cm depth, using modified sterilised 10cc/mL plastic syringes as corers (Plate 4-1). In order to obtain a “worst-case” reading of indicator micro-organisms, sampling was directed towards the biosolids clumps. Soil samples were placed directly into 50 mL sterile polypropylene (centrifuge) tubes, stored at 4°C and analysed within 24 to 36 hours of collection. Sampling took place on the fourth day after seeding, then weekly thereafter for eight weeks. After this time, sampling was carried out at weeks 10, 12, 15, 18 and 23.

**Plate 4-1: Soil sampling of biosolids in the field**



The soil temperature was taken at each sampling event, except for week 23. A soil probe was placed into the soil at 10 cm depth. Day temperature and weather conditions were also noted. An initial sample of 200 g topsoil was also taken in the first week, for later determination of Gravimetric Soil Watering Capacity for the pot trial (see Glasshouse trial 4.2.2 *Soil watering capacity*).

Following discussion regarding the workload and additional time required to process a high number of samples, the rate of 4 dry t/ha was eliminated from the sampling regime along with the protozoa *Cryptosporidium*; however, the plot was still maintained throughout the season in the event it should have been required (Table 4-1).

### ***Soil moisture***

Soil moisture samples were taken from the field at each sampling event, except for week 23. Approximately 200 g of soil was collected from the soil surface (randomly selected) using a hand trowel. Soil was wrapped tightly in a plastic bag and placed on ice while in transit to prevent moisture loss. At the laboratory, 100g of the wet soil was weighed out, placed into a soil tin with a lid and oven dried. The drying oven was set at 105°C for 48 hours. The soil moisture percentage was determined using the following formula (Equation 1):

#### **Equation 1: Soil moisture percentage**

$$\text{Soil moisture \%} = \frac{\text{SWW} - \text{SWD} \times 100}{\text{SWW (wet)}} \quad (1)$$

Where SWW is the wet soil weight, including the soil tin and lid, and SWD is the dry soil weight, including the soil tin and lid, after oven drying and cooling.

### ***Plant and root sampling***

On 30 September 2004, in week 15 of the field trial, three leaf and three root samples were taken from the 16 dry t/ha and nil plots. Should any micro-organisms have been present, they would have appeared in the 16 dry t/ha plot (higher rate), thus representing the 8 dry t/ha plots as well. Lower leaves were randomly selected from plants off the 16 dry t/ha plot for each representation. These leaves were assumed more susceptible to the presence of pathogens, being closer to the ground surface. Root samples were randomly taken from the same plots, from different plants. Plants and roots were selected from areas where biosolids clumps were prevalent.

At week 18 of the field trial, a more extensive root sample was carried out. Six random root samples were taken from each 16 dry t/ha representative plot (A, B and C), the sample labels corresponding to the soil samples. Soil samples were taken at 5-8cm from the root samples, so that bacterial survival levels could be compared. To obtain a sample, soil had to be loosened around the plant base with a hand trowel due to the dryness of the soil.

Excess soil was removed from the roots. Roots were cut from the plant stems, placed into labelled 50 mL polypropylene tubes and stored on ice during transit. In the laboratory, the tubes were weighed, and the samples suspended in 15 mL of phosphate buffer and vortexed for 5 minutes each. For each sample, 200  $\mu$ L was pipetted onto the titre tray then diluted across once only (20  $\mu$ L sample to 180  $\mu$ L phosphate buffer).

#### ***Harvest and grain analysis***

Fifty wheat heads were randomly removed across each replicate in the 16 dry t/ha plot and nil plots on 22 November 2004. Two days later, the total heads from each replicate were bulk-threshed in a grain thresher; the nil plots were threshed first followed by the samples from 16 dry t/ha plots. Three samples were randomly scooped from the grain, placed into labelled 50 mL polypropylene tubes and stored on ice during transit. In the laboratory, the tubes were weighed and the samples suspended in 15 mL of phosphate buffer and vortexed for 1 minute each. For each sample, 10  $\mu$ L was pipetted onto prepared Agar plates in replicates of four.

Soil samples were taken from the field at the same time as grain samples to test for bacterial levels on biosolids remains.

#### 4.2.2 Glasshouse trial

##### *Location*

A pot trial was conducted in Glasshouse # 48 at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Land and Water, Floreat, Western Australia to align with the field trial. Air temperature was maintained at 24°C by an air-conditioning system that ranged from 8°C to 30°C and a relative humidity that ranged from 29% to 95%. Pot soil temperatures averaged 22.20°C across the five sampling events, taken at sampling in the morning. Temperature and humidity were recorded using a thermo hydrograph. Soil moisture was kept well-watered without leaching (refer to *Soil watering capacity* in this section). The pot trial was designed to test the survival of micro-organisms in a more controlled environment, simulating favourable conditions for pathogen survival.

##### *Treatments*

The pot trial commenced two months after the field trial, on 22 September 2004, with four treatments; each treatment was triplicated to a total of 12 pots. Plastic pots TerraBoxes™ (Planterra) measuring 45 cm long by 13 cm deep and 12-16 cm wide were lined with paper towelling to prevent free drainage and leaching of micro-organisms. Pots were filled with 7 kg of air-dried sieved soil from the field, and weighed.

Approximately 30 kg (wet) of biosolids were collected from the Woodman Point WWTP in Perth, Western Australia on 21 April 2004 and sealed in a double-layered black garbage bag. Biosolids were autoclaved at 121°C for one hour and re-autoclaved at 121°C for one hour the following day, to destroy micro-organisms and to remove the presence of and competition between all micro-organisms. Biosolids were applied to pots and incorporated with the soil using a spatula on 12 May 2004. The four treatments were 0, 8 and 16 dry t/ha equivalent and no wheat (Table 4-3). Treatment 2 (no wheat), was spiked with indicator micro-organisms to compare the effects of shading on micro-organism survival.

**Table 4-3: Pot trial treatments for micro-organism decay rates**

Treatment	Description	Pot Code
1	Nil biosolids, seeded with microbes and wheat	P <sup>0</sup>
2	Biosolids 635 g/pot (equiv. 16 dry t/ha), spiked, no wheat	NW <sup>16</sup>
3	Biosolids 318 g/pot (equiv. 8 dry t/ha), spiked, wheat	P <sup>8</sup>
4	Biosolids 635 g/pot (equiv. 16 dry t/ha), spiked, wheat	P <sup>16</sup>
5	Nil (control) pot	0

The cultured indicator micro-organisms were seeded back onto the biosolids in pots at the commencement of the trial. Soil was not sterilised, so that any normal interactions with soil organisms could still occur within the pots.

Equivalent rates of fertiliser and seed were applied as per field trial rates. Di-ammonium Phosphate (crushed) was seeded at 0.54 grams per pot and Westonia wheat was seeded at 0.41 grams (10 grains) per pot. Urea was applied at 0.37 grams per pot (20 granules) approximately 21 days after sowing.

**Calculating applications**

Calculations were based on:

100 kg/ha	= 10 g/m <sup>2</sup>
1 t/ha	= 100 g/m <sup>2</sup>

Pot area 45 cm x 15 cm = 675 cm<sup>2</sup>  
 = 0.0675 m<sup>2</sup> pot area

Fertiliser at 80 kg/ha

80 kg/ha = 8.0 g/m<sup>2</sup>  
 8 g/m<sup>2</sup> x 0.0675 m<sup>2</sup> pot area = 0.54 g/pot

Urea at 55 kg/ha

55 kg/ha = 5.5 g/m<sup>2</sup>  
 5.5 g/m<sup>2</sup> x 0.0675 m<sup>2</sup> pot area = 0.37 g/pot

Seed at 60 kg/ha

60 kg/ha = 6.0 g/m<sup>2</sup>  
 6.0 g/m<sup>2</sup> x 0.0675 m<sup>2</sup> pot area = 0.41 g/pot

*Biosolids rates:*

Equivalent 4 dry t/ha = 400 g/m<sup>2</sup>  
 = 400 x 0.0675 m<sup>2</sup> pot area  
 Dry weight = 27 g per pot x [83% wet portion -100=17%]  
 = (17/27 = 0.63 or 27/17 = 1.59)  
 = 83 x 1.59 = 131.97 g  
 = 131.97 g + 27 g  
 Wet weight = 158.97 g

Equivalent 8 dry t/ha = 800 g/m<sup>2</sup>  
 = 800 x 0.0675 m<sup>2</sup> pot area  
 Dry weight = 54g per pot x [83% wet portion -100=17%]  
 = (17/54 = 0.31 or 54/17 = 3.18)  
 = 83 x 3.18 = 263.94 g  
 = 263.94 + 54 g  
 Wet weight = 317.94 g

Equivalent 16 dry t/ha	= 1600 g/m <sup>2</sup>
	= 1600 x 0.0675 m <sup>2</sup> pot area
Dry weight	= 108 g per pot x [83% wet portion -100=17%]
	= (17/108 = 0.16 or 108/17 = 6.35)
	= 83 x 6.35 = 527.05 g
	= 527.05 + 108 g
Wet weight	= <u>635.05 g</u>

### ***Soil watering capacity***

Soil from the field trial (refer Table 4-2) was sifted and used for the pot trial. Pots were watered to a gravimetric soil water capacity (GSWC) of 16% using deionised water. The GSWC of 16% was determined as the level of water remaining after the following test. Soil was saturated, lightly covered with plastic wrap to prevent evaporation and left overnight for 24 hours. The top 5 cm of soil, from 10 cm free draining pot, was sampled. The moisture content was then determined by oven-drying the soil sample at 105°C for 48 hours. Pots were watered to GSWC at least every second day throughout the period of the experiment.

### ***Seeding***

#### ***Growing up cultures***

Cultures were grown up and washed prior to seeding. To culture bacteriophage (MS2), an *E. coli* host was first grown overnight using 100 mL of Tryptone Yeast Glucose Broth (TYGB). TYGB was autoclaved for 15 mins at 121°C. After cooling, 100 µL of *E. coli* HS (pFamp)R was added and broth was placed in the shaking incubator at 37°C overnight.

The following day, an exponential culture was made up from the overnight bacteriophage *E. coli* pFamp culture, by placing 1000 µL of overnight host into a new TYGB solution and incubating in the shaking incubator at 37°C for 4-5 hours. To the exponential culture, 100 µL bacteriophage stock was added and placed in the static incubator at 37°C to enable it to grow up overnight.

To culture *E. coli*, Nutrient Broth was made to a concentration of 1.3 g in 100 mL in ddH<sub>2</sub>O and autoclaved for 15 mins at 121°C. After cooling, 100 µL of *E. coli* (1803) was added to the broth and placed in the shaking incubator at 37°C overnight.

To culture enterococci, Brain Heart broth was made to a concentration of 3.70 g in 100 mL in ddH<sub>2</sub>O and autoclaved for 15 mins at 121°C. After cooling, 100 µL of enterococci (*E. faecalis*) was added to the broth and placed in the shaking incubator at 37°C overnight.

#### *Washing cultures*

Cultures were placed into individual sterilised centrifuge tubes and centrifuged (spun) at 5000 rpm for 10 minutes. For bacteria, the supernatant portion was carefully poured off the bacterial ‘pellet’ before adding 20 mL of phosphate buffer using a 25 mL graduated pipette. The culture was vortexed to dissolve the pellet back through the phosphate buffer, and this ‘washing’ process was repeated twice. After the third wash, the culture was resuspended in 20 mL of phosphate buffer and stored in a sterile 50 mL polypropylene tube at 4°C. The number of colony forming units (cfu) was determined by drop-plating the stock (diluted to 10<sup>-7</sup>) onto Chromocult™ (Merck) Agar plates. Bacterial stock was stored for no longer than a week before pots were seeded.

For bacteriophage, the supernatant was retained and the pellet removed. Using a 25 mL graduated pipette, the supernatant was placed into a sterile 50 mL polypropylene tube, filtered through 0.45 µm, stored at 4°C and used within a week.

To prepare the water for seeding indicator micro-organisms, 0.1 g/L of sodium thiosulphate (Na<sub>2</sub>O<sub>3</sub>S<sub>2</sub>) was added to approximately 10 L of tap water, to dispel chlorine, and autoclaved at 121°C for 30 minutes. Water was cooled down completely before use.

Plastic watering cans were sterilised by filling the cans with ddH<sub>2</sub>O, adding sodium hypochlorite (NaClO) and leaving the solution to settle for 20 minutes. Watering cans were flushed at least five times in ddH<sub>2</sub>O before being used to remove any trace of NaClO.

At seeding, 1 L of prepared water was poured into the sterilised watering cans and 10 mL of bacteriophage, 1 mL *E. coli* (1803) and 1 mL of enterococci were added. Pots were seeded according to individual weights (see Equation 2) and water-holding capacity (between 300 mL to 500 mL) as calculated in Microsoft Excel 2000. Time-zero samples were taken within 30 minutes of seeding.

The projected level of microbial cells in the pots after accounting for dilution factors such as soil, watering and seeding was  $10^6$  for bacteria and  $10^5$  for virus. The formula used to determine this level of indicator micro-organisms for seeding was Equation 2.

**Equation 2: Spiking level**

$$\text{Spiking level} = \frac{\text{Inoculum}}{\text{Inoculum} + \text{soil/water level}} \quad (2)$$

The following example is based on 7000 mL of soil in a pot, a watering level of 500 mL and an *E. coli* inoculation of 10  $\mu\text{L}$ :

Calculation based on:

1000 $\mu\text{L}$	=	1.0 mL
100 $\mu\text{L}$	=	0.1 mL
10 $\mu\text{L}$	=	0.01 mL

$$\begin{aligned} \text{Dilution factor} &= \frac{0.01}{0.01 + (7000+500)} \\ &= \frac{0.01}{7500.01} \\ &= 0.0000013 \text{ or } 1.30\text{E}6 \end{aligned}$$

***Pot sampling***

Soil samples were taken from depths of 0-5 cm using modified sterilised plastic syringes. Three core samples were taken randomly across each pot. Samples were placed directly into sterile polypropylene centrifuge tubes and analysed within 8 hours (bacteria) and 24 hours (virus) of collection. Sampling took place at days 0, 7, 14, 21 and 28 to determine decay rates.

### 4.2.3 Laboratory Processes

#### *Preparation*

Sterile polypropylene centrifuge tubes were pre-weighed and labelled prior to each sampling event. Soil corers were made using modified 10 cc/mL plastic syringes, the ends removed with a Stanley trimmer. Corers were arranged into plastic boxes (18 to a box) and sterilised in the autoclave for 15 minutes at 121°C.

Phosphate buffer was made up to pH 7.2, by combining 84 mL of monobasic and 216 mL of dibasic. The buffer was sterilised in the autoclave for 20 minutes at 121°C. Monobasic stock was made up, by combining 13.9 g sodium phosphate monobasic and 500mL of distilled water (ddH<sub>2</sub>O). Dibasic stock was made up by combining 53.65 g of sodium phosphate dibasic heptahydrate (or 28.4 g anhydrous form) with 1 L of ddH<sub>2</sub>O.

#### *Bacteria:*

Plates for bacteria were made up using Chromocult™ media (Merck) by autoclaving 1 L of distilled water for 20 minutes at 121°C to sterilise. The media should never be autoclaved. After cooling, 26.5 g/L of Chromocult™ Agar was added (Coliform) or 33.0 g/L (enterococci), according to the required bacterial analysis. The media were dissolved gently to protect the dyes in the microwave and placed in a water bath to cool to approximately 50°C for 30 minutes prior to pouring. Plates were arranged in the laminar flow cabinet for pouring the media into the bases. Approximately 20 plates per 100 mL were attained. After allowing the media to set, the plates were labelled and packaged with the bases facing upward to reduce evaporation. The plates were stored at 4°C for no longer than 3 weeks.

#### *Virus:*

The day before analysis of the bacteriophage, 1 mL of host *E. coli* HS (pFamp)R (from a slope culture stored in 20% glycerol at -80°C) was inoculated in a sterile 250 mL stoppered conical flask containing 100 mL of TYGB, 200 µl of glucose/CaCl<sub>2</sub>·2H<sub>2</sub>O (5x), 200 µl of MgSO<sub>4</sub>·7H<sub>2</sub>O (5x) and 1 mL of ampicillin. The culture was incubated at 37°C, shaking overnight at 100±10 rpm (approximately 16 hours).

Base plates for the virus were prepared any time from 3 weeks prior to an hour before the exponential growth culture expired. These plates formed the base onto which the semi-solid growth lawn was layered. After autoclaving the TYGB + Agar (see TYGA Section

3.3.2), 200 µl of MgSO<sub>4</sub>.7H<sub>2</sub>O (5x) and 200 µl of glucose/CaCl<sub>2</sub>.2H<sub>2</sub>O (5x) were added per 100mL of autoclaved TYGB + Agar. If required, the basal media were maintained in a water bath at 48°C. The base plates were poured into Petri dishes (approximately 10 plates per 100 mL). Plates were dried thoroughly before use or storage at 4°C.

Semi-solid TYGB + Agar (ssTYGA) overlay could be prepared and autoclaved days before the analysis, then left in a dark place at room temperature. When ready to use the ssTYGA, the overlay was melted in a microwave until the entire solid was dissolved. The sample was allowed to cool to approximately 48°C and then 1 mL of glucose/CaCl<sub>2</sub>.2H<sub>2</sub>O (5x), 1 mL of MgSO<sub>4</sub>.7H<sub>2</sub>O (5x), 1 mL of ampicillin and 400 µL nalidixic acid was added. The solution was mixed and 3 mL aliquots of the overlay were dispensed into sterile 15 mL polypropylene tubes. The tubes were placed in a water bath set on 48°C until ready for use.

#### ***Analysis — bacteria***

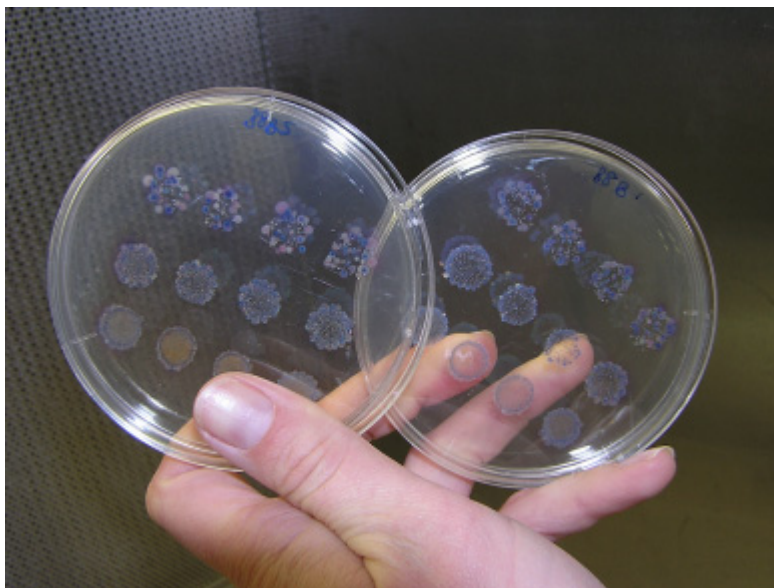
Samples were weighed prior to suspension in 15 mL of phosphate buffer. Tubes were vortexed for 1 minute, then 200 µl of each sample was pipetted into titre trays using modified tips; the ends being removed to accommodate for the viscous nature of the sludge. Samples were diluted up to 6 times across the titre tray according to Equation 3, to make counting of colony forming units (cfu) possible. Agar plates were then spot-plated using 10 µl of the sample per representation, replicated four to six times across each plate.

#### **Equation 3: Dilution factor**

$$\text{Dilution factor} = \frac{\text{Amount of micro-organisms transferred}}{\text{Amount transferred} + \text{amount present}} \quad (3)$$

After drying, plates were incubated at 37°C for up to 24 hours. Bacteria numbers were compared against positive and negative controls using the strains *E. coli* 1803 (positive) and *Ps. aeruginosa* (negative) and ATCC 19433 *E. faecalis* (positive) and *S. aureus* (negative) for enterococci. Upon enterococci evaluation, red colonies with a diameter or 0.5 to 2.0 mm were counted. Upon evaluation of *E. coli*, any visible dark-blue to violet colonies (Salmon-GAL and Xx-glucuronide reaction) were counted (Plate 4-2).

**Plate 4-2: Dark-blue to violet colonies of *E. coli* (cfu)**



***Analysis – virus***

Samples were weighed prior to suspension in 15 mL of phosphate buffer. Tubes were vortexed for 1 minute, then 200  $\mu$ L of each sample was pipetted into titre trays using modified tips; the ends being removed to accommodate for the viscous nature of the sludge. Samples were diluted up to 6 times across the titre tray according to Equation 3, to make counting of colony forming units (cfu) possible.

To each 15mL polypropylene tube containing the ssTYGA, 500  $\mu$ L of the host (*E. coli* HS(pFamp)R exponential growth) was added. The samples were mixed gently and poured onto labelled base plates. The plates were swirled to allow overlay to completely cover the basal surface. Of the sample to be tested, 10  $\mu$ L was drop-plated onto each plate and repeated in quadruplicates together with both positive and negative controls. Where spread-plating was used, 100  $\mu$ L of sample to be tested was added with 500  $\mu$ L of the host (*E. coli* HS(pFamp)R exponential growth). Samples were repeated in triplicate together with both positive and negative controls. When the overlaid plates had set, the plates were incubated at 37°C for 18 hours, the Agar side facing downwards.

After incubation, the plates were examined for viral plaques in the *E. coli* HS(pFamp)R growth lawn on the plates. The size and translucence of the plaques could be compared to the positive controls plated.

*Further experimentation:*

Phage plaques were difficult to count due to growth of background flora (indigenous or autochthonous bacteria) that grew up ahead of viruses after an incubation period of 15 hours at 37°C. Further experimentation was carried out in the laboratory to improve detection through the use of heat treatments, lower incubation rates and reduced incubation times.

Heat-treating samples at 60°C for 5 minutes resulted in a greater increase in background flora than was expected. This could be due to the cells that survived heat treatments being more vigorous. Incubation temperatures were lowered to 28°C in an attempt to discourage background indigenous flora. This resulted in no lawn growth at all; therefore no detection of virus or growth of indigenous micro-organisms occurred.

Incubation times kept as close to 15 hours as possible improved visibility of the plaques considerably. As phage plaque numbers in the pot trial reduced over time, detection and counting of plaques presented more of a challenge, largely due to the level of ‘dirt’ in the sample. The counting of plaques in diluted samples was difficult, but not impossible, as was detecting plaques in the neat (undiluted) sample. Further experimentation was carried out to compare the method of drop-plating 10 µl with spread-plating 100 µl of the neat sample. Therefore, in order to detect plaque levels in the neat sample (undiluted), more work, is required.

#### **4.2.4 Analysis of data**

All data was recorded and calculated in Microsoft Excel 2000. Data was transferred from Microsoft Excel 2000 into SPSS 12.0 for Windows for statistical analysis. The split file function was used to compare groups by experiment type — as either field trial data or pot trial data. Data was arranged using the average of the six samples taken from the field for each replicate of A, B and C. Pot trial data consisted of the average of three samples taken from each replicate of A, B and C.

Descriptive statistics were run first using the explore function to determine the mean, median, variance, minimum, maximum, range skewness and standard deviation. The dependant variable was the micro-organism for the week (eg. *ecoli7*), the independent variables being the treatments (eg. 0, 8, 16 dry t/ha). The explore test ran box-plots for each week’s survival counts across treatments. ANOVA was run to determine which variables were significantly different and to find out if there was a difference between the means. The post-hoc tests generated under multiple comparisons were Bonferroni, LSD

and Tukey HSD. No further testing was required due to minimal significance. A confidence interval of 95% was assumed with a 0.05% level of significance.

The decay rates  $\lambda$  (day<sup>-1</sup>) for each treatment in the field and pot trials were determined from each plot using equation (4) (Yates et al. 1990).

**Equation 4: Decay rates for indicator micro-organisms over time**

$$\lambda = -(\log_{10} ((A_t/A_0)) / (D_t - D_0)) \quad (4)$$

Where  $A_t$  = the final average cell numbers at day t,  $A_0$  = the average cell numbers at day 0,  $D_t$  = the final time period (days) and  $D_0$  = the commencement of the period of decay being tested. This equation gives the number of log reduction per day; therefore the following equation (5) was used to convert the decay rate to number of days for a 1 log<sub>10</sub> reduction.

**Equation 5: Decay rate conversion to number of days**

$$\text{No. of days} = 1 / \lambda \quad (5)$$

Where no. of days = the number of days for 1 x log<sub>10</sub> reduction. This is the equivalent of a reduction termed T90.

All data and results in Excel and SPSS 12.0 for Windows are available as 'Field Trial.xls', 'Pot Trial.xls', 'Statistics data.sav', 'Statistics1.spo' and 'Statistics2.spo' on the CD-ROM Data Files attached.

#### **4.2.5 Assumptions and limitations**

The study was carried out based on the following assumptions:

- That the sampling process and the viscous nature of biosolids did not influence cell numbers;
- That the conditions in the glasshouse trial remained constant;
- That the moisture levels in the pots remained constant;
- That the conditions in the field were representative of typical climatic conditions to wheat-growing regions; and
- That the method of sampling remained consistent throughout both experiments.

Factors that placed limitations on the study were:

- The time period available to carry out the research;
- Insufficient sampling experience and knowledge in microbiology; and
- Restricted time and labour, thus limiting the frequency and number of samples taken from each plot – increases in each could have reduced random error and variability between samples.

## 5 Results

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### 5.1 Survival of indicator micro-organisms

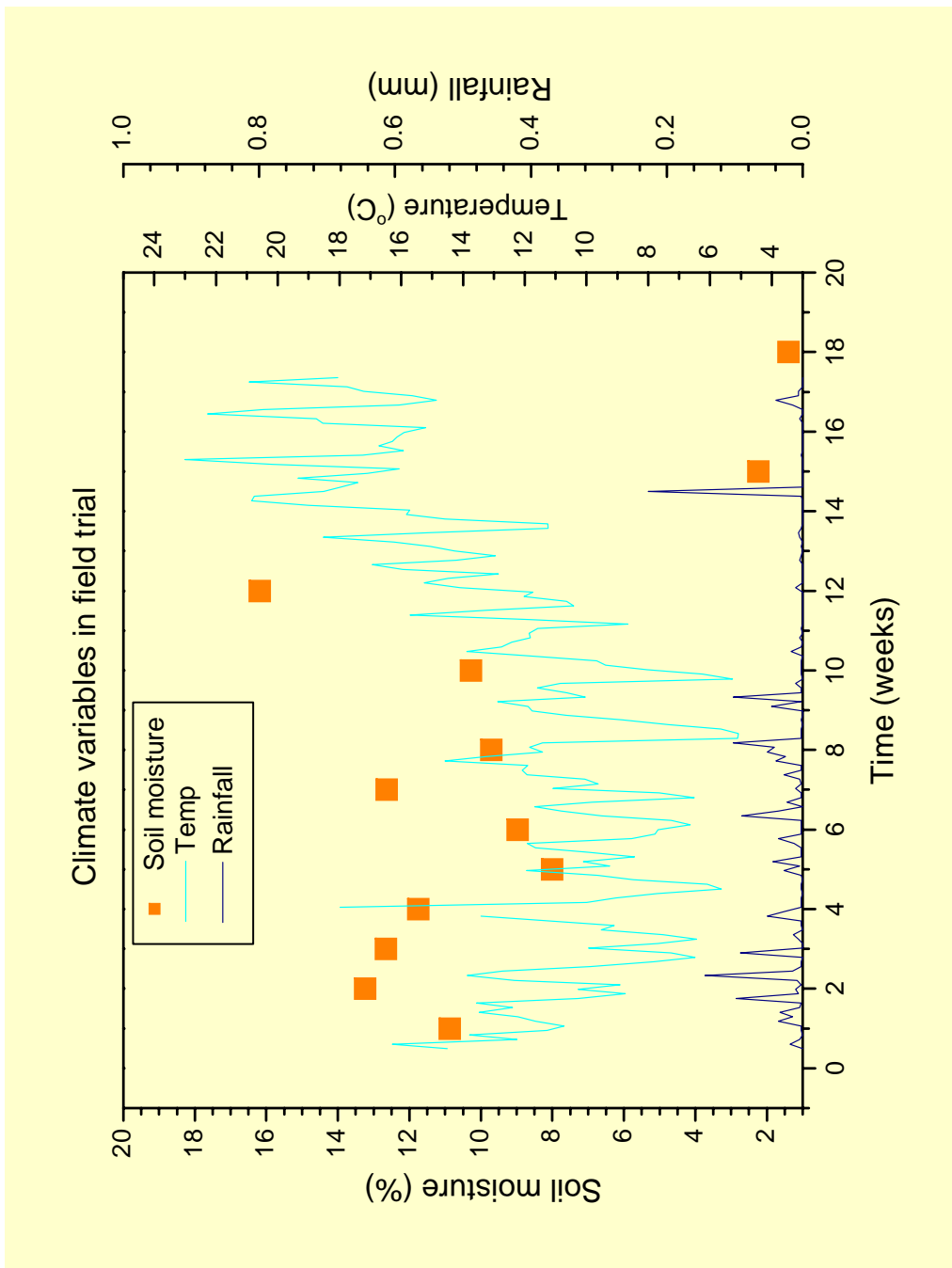
The length of survival of indicator micro-organisms was studied in the field trial and compared to their length of survival in the more controlled conditions in the glasshouse. All results are based on the means of three replicates.

#### 5.1.1 Field trial

Bacterial cell numbers from the stockpile were tested through an external laboratory. No bacteria were detected in soil samples taken from the field before biosolids were applied and therefore the results were not documented. Once the field trial had commenced, bacterial cell numbers declined over the five months of sampling across both bacteria (*E. coli* and enterococci) in the biosolids treatments: 16 dry t/ha and 8 dry t/ha.

Climatic conditions remained fairly constant from weeks 1 to 12 (17 June to 6 September 2004). From week 12 to week 18, day temperatures increased from 16°C to 23°C. Soil temperature increased from 16°C in week 10 to 37.50°C in week 18. Overcast days were observed at the time of sampling during this period. Soil moisture levels slightly increased from 10.30% in week 10 to 16.20% in week 12 after a dry spell occurred from week 8, when soil moisture was 9.71%. At 15 and 18 weeks soil moisture decreased to 2.23% and 1.39% respectively (Figure 5-1 and Table A12). A rainfall event occurred in weeks 22 and 23, when 5.0 mm and 8.20 mm (respectively) of moisture were received. Daytime temperature, soil temperature and soil moisture levels were not recorded at sampling in week 23.

Figure 5-1: Climatic variables in the field trial



***E. coli* survival trend**

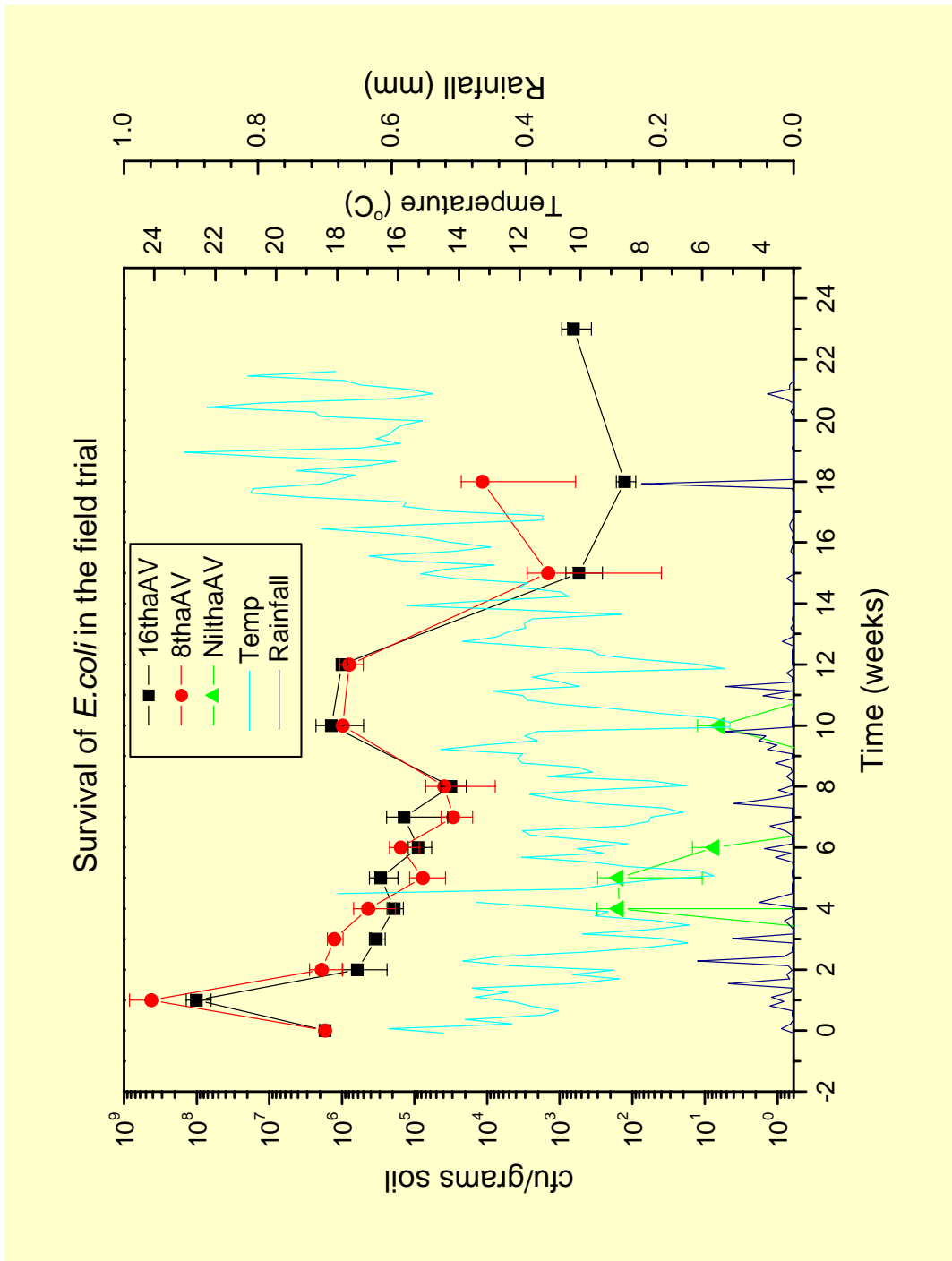
*E. coli* were detectable for five months in the field. It was observed that *E. coli* followed the same survival patterns in 16 dry t/ha and 8 dry t/ha treatment throughout the sampling period but this was not statistically significant ( $P>0.05$ ). *E. coli* cell numbers commenced at  $1.70E6$  cfu/grams soil from the stockpile and increased two- $\log_{10}$  by week one to  $1.01+E8$  cfu/grams soil (16 dry t/ha) and  $4.21E8$  cfu/grams soil (8 dry t/ha). Overall, *E. coli* cells lost three- $\log_{10}$  in both treatments over the following seven weeks to week 8.

As seen in Figure 5-2, cell numbers rapidly increased in both treatments at week 10. The cell numbers in treatment 1 (16 dry t/ha) increased two- $\log_{10}$  to  $1.39E6$  cfu/grams soil in week 10 and  $9.92E5$  cfu/grams soil in week 12. In treatment 2 (8 dry t/ha), *E. coli* cell numbers increased almost two- $\log_{10}$  from  $3.89E4$  cfu/grams soil to  $9.68E5$  cfu/grams soil in week 10 and then levelled to  $7.86E5$  cfu/grams soil by week 12. A loss of three- $\log_{10}$  occurred in treatment 1 to  $1.28E2$  cfu/grams soil between weeks 12 and 15. At week 18 a one- $\log_{10}$  loss occurred but cell numbers recovered back to  $6.48E2$  cfu/grams soil by week 23 (final week). In treatment 2, *E. coli* cells experienced a two- $\log_{10}$  loss in week 15, before increasing again to  $1.16E4$  cfu/grams soil by week 18. *E. coli* cells persisted at  $6.48E+02$  cfu/grams soil into the final sampling event of week 23 (24 November 2004).

In the nil plot, *E. coli* cells were detected in weeks 4 and 5 at two- $\log_{10}$  (approximately  $1.50E2$  cfu/grams soil) before decreasing to  $7.41E0$  cfu/grams soil in week 6. In week 10, *E. coli* was detected again at a level of  $6.35E0$  cfu/grams soil.

NB: standard error bars in the following figures are the standard deviation between the means of the three replicates.

Figure 5-2: E. coli cell numbers detected in the field trial



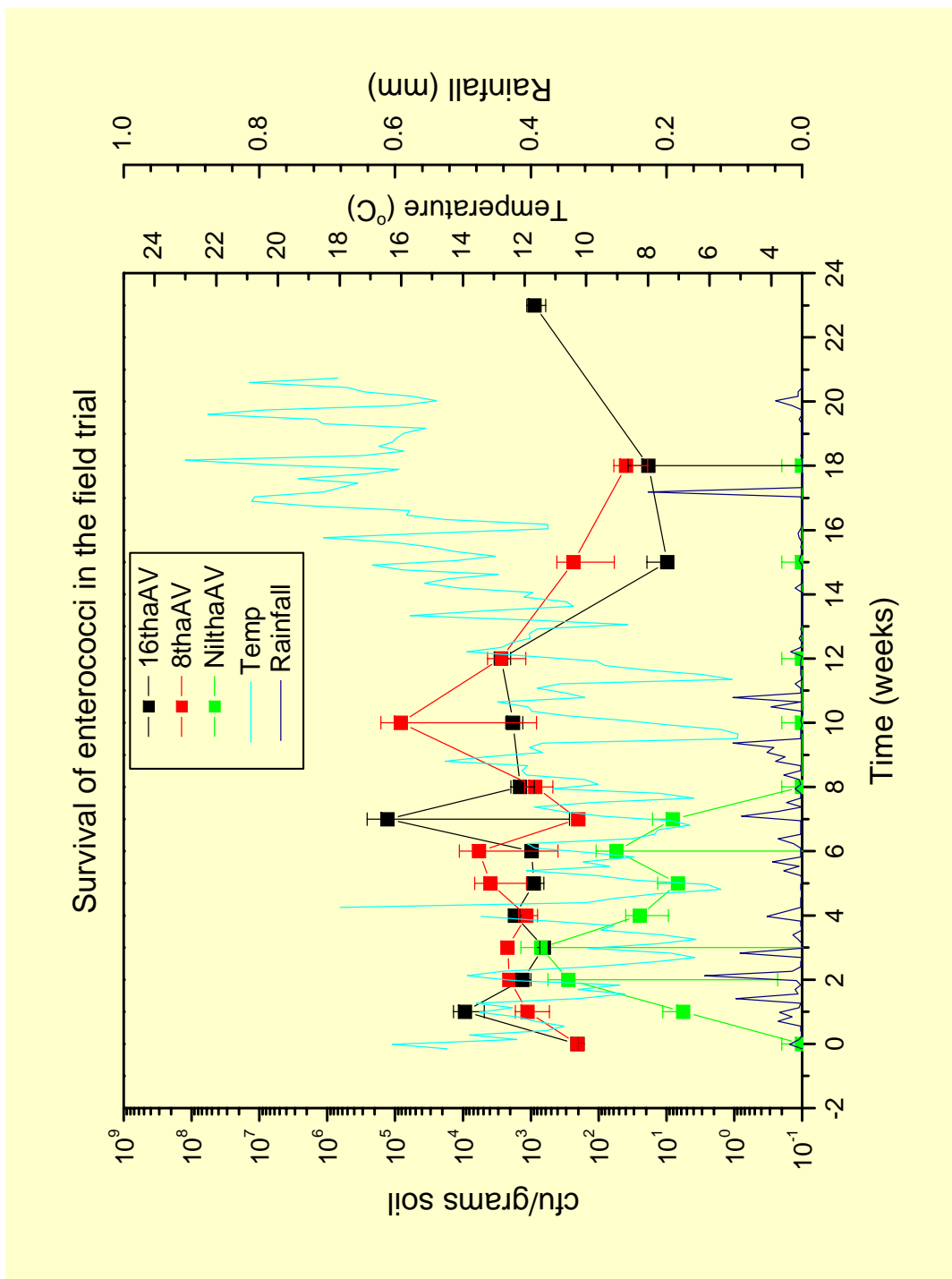
***Enterococci survival trend***

Enterococci cells were detected in the stockpile commenced at 2.03E2 cfu/grams soil for both treatments. As seen in Figure 5-3, enterococci cells in the 16 dry t/ha treatment increased almost two- $\log_{10}$  from spreading to week 1, then remained reasonably stable to week 12, except for a sudden increase of enterococci cells at week 7 to two- $\log_{10}$ . A three- $\log_{10}$  loss in enterococci cells occurred between week 12 and week 15 as temperatures increased and rainfall events decreased. The level of surviving cells was at 1.84E1 cfu/grams soil at week 18, and increased to 8.76E+02 by the final sampling event of week 23 (24 November 2004).

The enterococci cells detected in 8 dry t/ha biosolids increased one- $\log_{10}$  in the first six weeks from spreading. After a one- $\log_{10}$  loss, the cell numbers peaked at 8.17E4 cfu/grams soil in week 10. A three- $\log_{10}$  loss of cells occurred at the final week of sampling, when enterococci cell count was 3.93E1 cfu/grams soil (Figure 5-3).

Enterococci cells were detected in the nil plots from weeks 1 to 7. These cells had increased two- $\log_{10}$  in the first 21 days before a two- $\log_{10}$  loss occurred by week 5. In week 6, the cell numbers increased one- $\log_{10}$  and declined to 8.01E0 cfu/grams soil by week 7. After this period, enterococci cells fell below detection limits (Figure 5-3).

Figure 5-3: Enterococci cell numbers detected in the field



### 5.1.2 Decay rates

Indicator micro-organisms in the pot trial had faster rates of decay than organisms in the field trial. According to decay rates (equations 4 and 5), as seen in table 5-1, the time-frame in the field for a one- $\log_{10}$  reduction to occur in 16 dry t/ha treatment was 29.7 days for *E. coli* and 20.5/22.3 days for enterococci. In 8 dry t/ha treatment the time-frame for a one- $\log_{10}$  reduction to occur was 11.7 days for enterococci and 3.7 days for *E. coli*. Bacteriophage (MS2) was not tested in the field.

In the glasshouse (pot trial) the time-frame for a one- $\log_{10}$  reduction to occur in 16 dry t/ha (equivalent) treatment was -29.7/3.8 days for bacteriophage (MS2), 6.8 days for enterococci and 4.9 days for *E. coli*. In 8 dry t/ha (equivalent) the time-frame for a one- $\log_{10}$  reduction to occur was 11.1 days for bacteriophage, 2.4/13.1 days for *E. coli* and 5.3 days for enterococci. In 0 dry t/ha (equivalent), seeded with indicator micro-organisms, the time-frame for a one- $\log_{10}$  reduction to occur was 14.3 days for bacteriophage, 8.8 days for *E. coli* and 4.2 days for enterococci. In the pots containing no wheat (16 dry t/ha equivalent biosolids) the time-frame for a one- $\log_{10}$  reduction to occur was 9.6 days for bacteriophage, 59.6/2.8 days for *E. coli* and 3.7 days for enterococci (Table 5-1).

**Table 5-1: Decay rates (# days for a 1  $\log_{10}$  loss) indicator micro-organisms across treatments in the field and pot trials**

Indicator micro-organism	FIELD TRIAL (over 161 days)		POT TRIAL (over 28 days)			
	16 t/ha (days)	8 t/ha (days)	16 t/ha (days)	8 t/ha (days)	0 t/ha (days)	No wheat 16 t/ha (days)
<i>E. coli</i>	29.7	3.7	4.9	2.4, 13.1 <sup>a</sup>	8.80	2.8, 59.6
Enterococci	20.5, 22.3 <sup>a</sup>	11.7	6.8	5.3	4.20	3.7
MS2	-	-	-29.7, 3.8 <sup>a</sup>	11.1	14.3	9.6

<sup>a</sup>Two or more decay rates given for single trial indicators as first-order decay.

### 5.1.3 Pot trial

Bacterial cell numbers and bacteriophage plaques declined rapidly over 28 days in the pot trial. During this time, glasshouse temperatures increased and humidity levels decreased (Figures 5-4, 5-5 and 5-6). Soil moisture levels remained constant throughout the duration of the experiment (4.2.2 Glasshouse trial – *Soil watering capacity*). Soil temperatures were taken at each sampling event (4.2.2 Glasshouse trial), gradually declined to 19°C at day 14 and then increased to 27°C by day 28.

#### *E. coli survival trend*

As seen in Figure 5-4, the treatment 1 (nil biosolids) cell numbers commenced at 8.06E5 cfu/grams soil at seeding. By day 28, a three-log<sub>10</sub> loss had occurred. In the first four days, a one-log<sub>10</sub> loss occurred. A sharp trough occurred at day 14, where nil bacterial cells were detected; however, cell numbers at two-log<sub>10</sub> were again detectable by days 21 and 28.

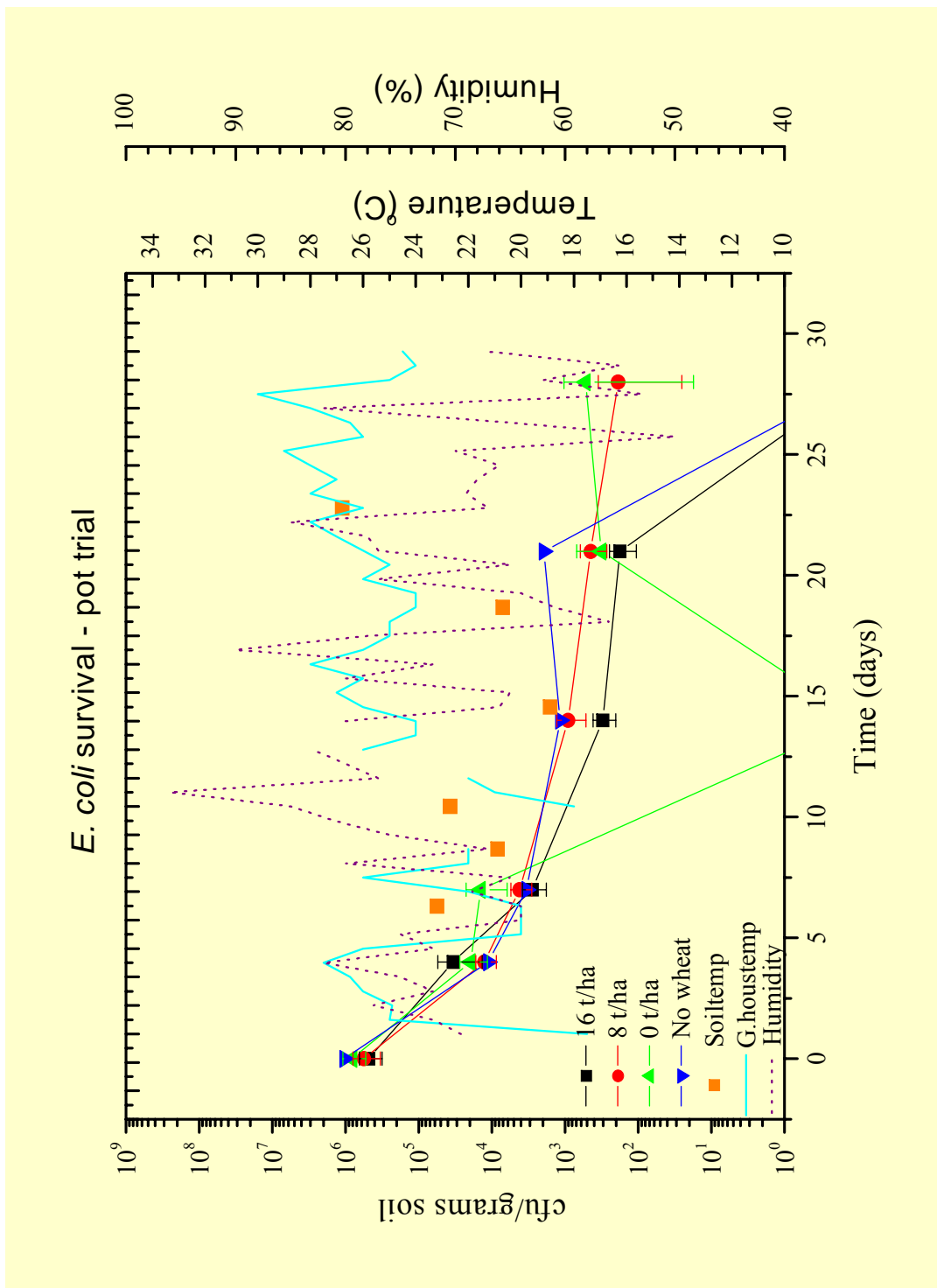
In treatment 2 (no wheat, 16 dry t/ha equivalent), *E. coli* cell numbers were at 1.04E6 cfu/grams soil at seeding (Figure 5-4). By day 21, a three-log<sub>10</sub> loss had occurred and cell numbers fell below detection limits by day 28. In the first four days, a two-log<sub>10</sub> loss occurred and cell detection limits stabilised to three-log<sub>10</sub> from day 7 to day 21.

In treatment 3 (8 dry t/ha equivalent) the cell numbers at seeding was 5.62E5 cfu/grams soil. By day 28, a three-log<sub>10</sub> loss had occurred; with a one-log<sub>10</sub> loss from day 0 to day 4, a one-log<sub>10</sub> loss between day 4 to day 7 and a one-log<sub>10</sub> loss between day 7 to day 14 (Figure 5-4).

In treatment 4 (16 dry t/ha equivalent) the cell numbers at seeding was 4.80E5 cfu/grams soil. By day 21, a three-log<sub>10</sub> loss had occurred and cell numbers had fallen below detection limits by day 28. A one-log<sub>10</sub> reduction occurred consistently across sampling points from day 0 to day 14 (Figure 5-4).

The main treatment effect was not significant ( $P>0.05$ ). The interaction between both bacteria and the virus, and days for survival versus treatments was also not significant ( $P>0.05$ ).

Figure 5-4: *E. coli* cell numbers detected in the pot trial



***Enterococci survival trend***

As seen in Figure 5-5, in treatment 1 (nil biosolids) the cell numbers commenced at  $1.05E5$  cfu/grams soil at seeding. By day 14, a four- $\log_{10}$  loss had occurred, and cell numbers had fallen below detection limits by day 21. A one- $\log_{10}$  reduction consistently occurred across sampling points from day 0 to day 7, with a two- $\log_{10}$  loss occurring from day 7 to day 14.

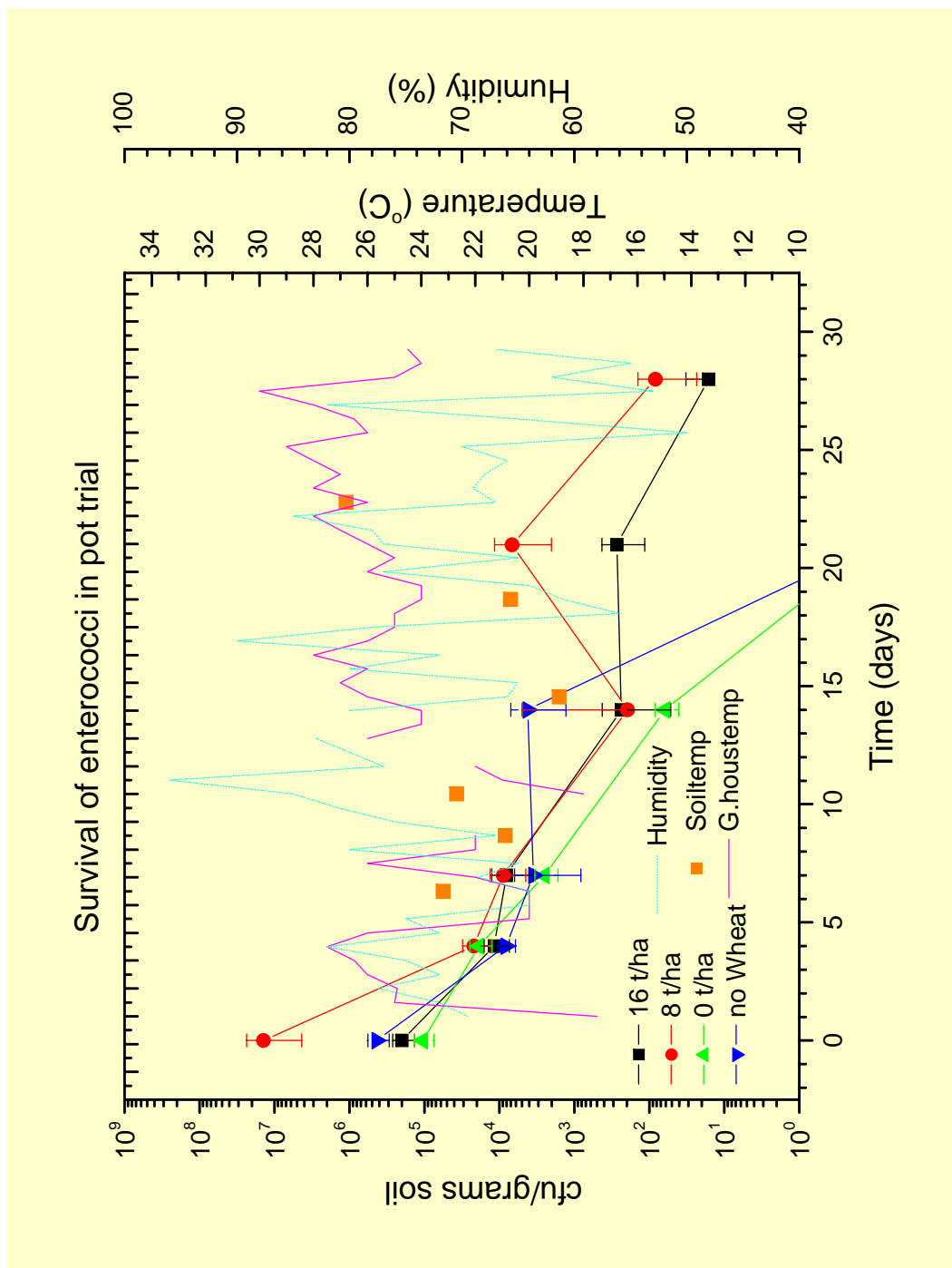
In treatment 2 (no wheat, 16 dry t/ha equivalent) the cell numbers at seeding was  $4.32E5$  cfu/grams soil. A two- $\log_{10}$  loss occurred from day 0 to day 4. By day 14, a further two- $\log_{10}$  loss had occurred. Cell numbers fell below detection limits by day 21 (Figure 5-5).

The treatment 3 (8 dry t/ha equivalent) cell numbers at seeding was  $1.40E7$  cfu/grams soil. By day 28, a six- $\log_{10}$  loss had occurred. A three- $\log_{10}$  loss occurred from day 0 to day 4. A one- $\log_{10}$  reduction occurred between days 4 to 7 and then cell counts fluctuated across the next 14 days (Figure 5-5).

The treatment 4 (16 dry t/ha equivalent) cell numbers at seeding was  $2.00E5$  cfu/grams soil. By day 28, a four- $\log_{10}$  loss had occurred after a consistent rate of decay (Figure 5-5).

The main treatment effect was not significant ( $P>0.05$ ). The interaction between both bacteria and the virus, and days for survival versus treatments was also not significant ( $P>0.05$ ).

Figure 5-5: Enterococci numbers detected in the pot trial



***Bacteriophage survival trend***

As seen in Figure 5-6, for treatment 1 (nil biosolids) the level of plaques detected was 1.67E5 cfu/grams soil at seeding. By day 28, a two-log<sub>10</sub> loss had occurred after a consistent rate of decay.

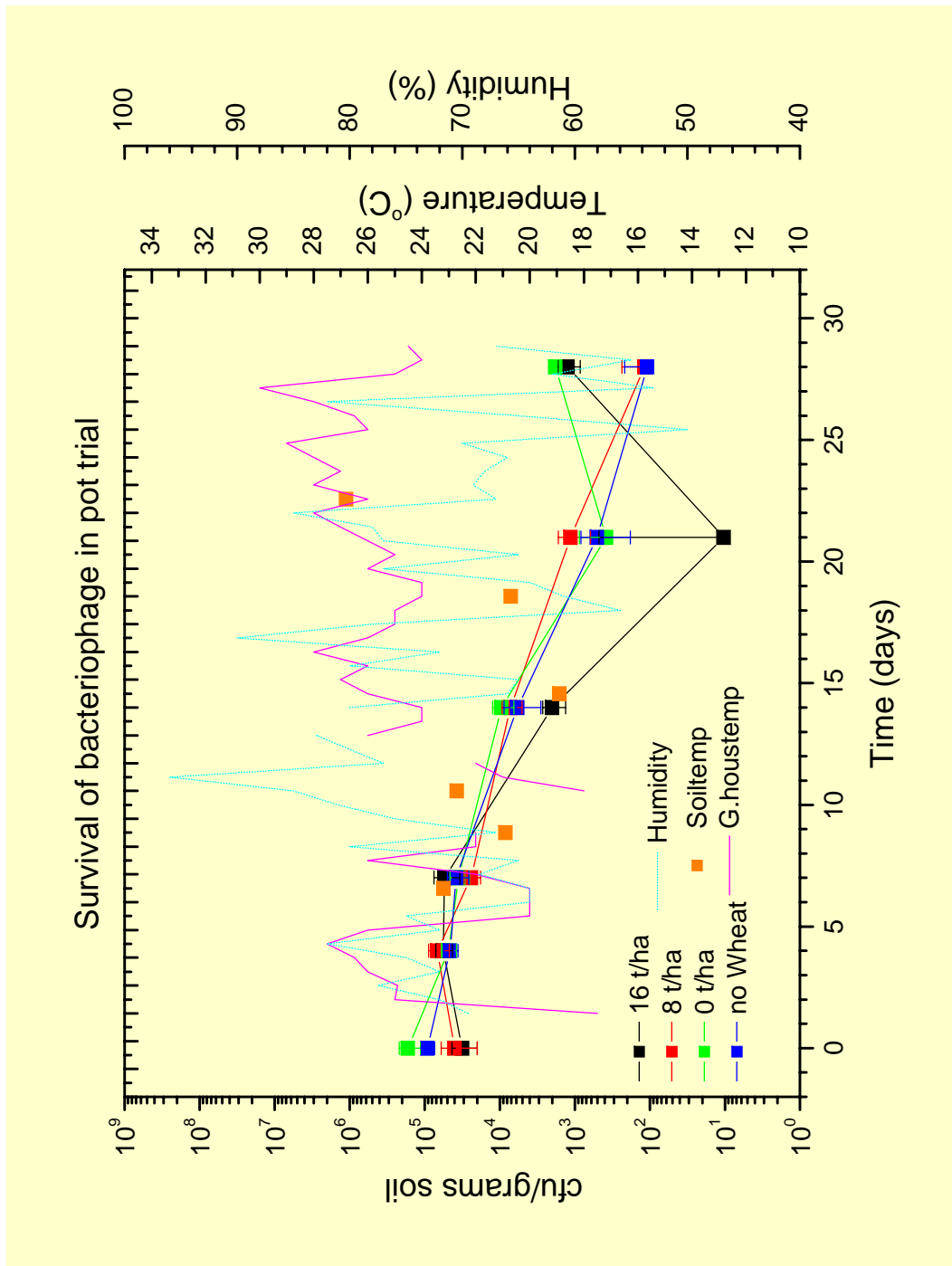
In treatment 2 (no wheat, 16 dry t/ha equivalent), the level of plaques detected was 9.25E4 cfu/grams soil at seeding. By day 28, a two-log<sub>10</sub> loss had occurred after a consistent rate of decay. Between days 21 and 28 an upward spike of one-log<sub>10</sub> had occurred (Figure 5-6).

In treatment 3 (8 dry t/ha equivalent) the level of plaques detected was 4.01E4 cfu/grams soil at seeding. By day 28, a two-log<sub>10</sub> loss had occurred after a consistent rate of decay (Figure 5-6).

In treatment 4 (16 dry t/ha equivalent) the level of plaques detected was 3.17E4 cfu/grams soil at seeding. By day 28, a one-log<sub>10</sub> loss had occurred, after a trough which took plaque detection numbers down to 1.04E1 cfu/grams soil at day 21. Between days 21 and 28 and upward spike of two-log<sub>10</sub> had occurred (Figure 5-6). The standard-error bar at this point was 4.66E2 (Pot.Trial.xls on CD-ROM Data Files).

The main treatment effect was not significant ( $P>0.05$ ). The interaction between both bacteria and the virus, and days for survival versus treatments was also not significant ( $P>0.05$ ).

Figure 5-6: Bacteriophage plaque numbers detected in the pot trial



## Treatment affect on survival

The affect of the various biosolids rates, or treatments, across time on the survival of indicator micro-organisms was tested using SPSS.

### 5.1.4 Field trial

There was no significance in the mean sampling results between treatments 16 dry t/ha and 8 dry t/ha ( $P>0.05$ ). However, in Table 5-2 it can be seen that the survival rates were, by observation, higher in 8 dry t/ha treatment than in the 16 dry t/ha treatment.

**Table 5-2: Comparison of change in time of bacterial numbers between the biosolids application rates**

Week	<i>E. coli</i> (cfu/grams soil)		Enterococci (cfu/grams soil)	
	8 dry t/ha	16 dry t/ha	8 dry t/ha	16 dry t/ha
0	1.70E+06	1.70E+06	2.03E+02	2.03E+02
1	<b>4.21E+08</b>	1.01E+08	1.11E+03	<b>9.36E+03</b>
2	<b>1.88E+06</b>	6.10E+05	<b>2.06E+03</b>	1.33E+03
3	<b>1.27E+06</b>	3.35E+05	<b>2.21E+03</b>	6.44E+02
4	<b>4.34E+05</b>	1.90E+05	1.15E+03	<b>1.70E+03</b>
5	7.68E+04	<b>2.93E+05</b>	<b>3.94E+03</b>	9.00E+02
6	<b>1.54E+05</b>	8.97E+04	<b>5.80E+03</b>	9.73E+02
7	2.94E+04	<b>1.38E+05</b>	2.00E+02	<b>1.29E+05</b>
8	<b>3.89E+04</b>	3.16E+04	8.51E+02	<b>1.43E+03</b>
10	9.68E+05	<b>1.39E+06</b>	<b>8.17E+04</b>	1.83E+03
12	7.86E+05	<b>9.92E+05</b>	<b>2.75E+03</b>	2.72E+03
15	<b>1.43E+03</b>	5.41E+02	<b>2.35E+02</b>	9.69E+00
18	<b>1.16E+04</b>	1.28E+02	<b>3.93E+01</b>	1.84E+01

— Treatment for which organism's survival rate was greatest

Analysis of variance (ANOVA) was carried out across treatment groups for *E. coli* and enterococci at each sampling week. For *E. coli*, the results indicated significance between treatment groups at weeks 3 and 12 ( $P < 0.05$ ). For enterococci, the results indicated significance between treatment groups ( $P < 0.05$ ) at weeks 2 and 4 (Table 5-3).

**Table 5-3: Significance levels (ANOVA) between treatments in field trial**

Week	<i>E. coli</i>		Enterococci	
	F value	Sig. ( $P < 0.05$ )	F value	Sig. ( $P < 0.05$ )
1	0.811	0.488	3.848	0.084
2	2.856	0.134	9.047	<b>0.015</b>
3	12.319	<b>0.008</b>	3.641	0.092
4	2.173	0.195	11.121	<b>0.010</b>
5	4.000	0.079	1.623	0.273
6	3.247	0.111	0.979	0.429
7	1.482	0.300	1.001	0.422
8	1.141	0.380	3.585	0.095
10	1.947	0.223	0.999	0.422
12	8.275	<b>0.019</b>	2.518	0.161
15	0.786	0.498	1.696	0.261
18	1.090	0.395	1.545	0.288

Post-hoc tests (multiple comparisons) were carried out in SPSS for *E. coli* and enterococci in the field trial across treatment groups 0, 8 and 16 dry t/ha at each sampling week. The significant results ( $P < 0.05$ ) are displayed in Table 5-4 and summarised in Table 5-5. All the other multiple comparisons were not statistically significant ( $P > 0.05$ ).

**Table 5-4: Matrix of significant combinations (P<0.05) for *E. coli* and enterococci**

Week	Nil dry t/ha	8 dry t/ha	16 dry t/ha
<i>E. coli:</i>			
3	✗	✓	✓
3	✓	✓	✗
5	✓	✗	✓
6	✓	✓	✗
12	✓	✓	✗
12	✓	✗	✓
<i>Enterococci:</i>			
2	✓	✓	✗
2	✓	✗	✓
4	✓	✓	✗
4	✓	✗	✓
8	✓	✗	✓

✓ Significant treatments (P&lt;0.05)

✗ Non-significant treatments (P&gt;0.05)

**Table 5-5: Significant results (P<0.05) only of Post Hoc test run for the field trial**

Dependent variable	(I) Treatment (t/ha)	(J) Treatment (t/ha)	Tukey HSD	LSD	Bonferri
Entero wk 2	0	8	0.013	0.005	0.016
	0	16		0.047	
<i>E. coli</i> wk 3	8	16	0.029	0.012	0.037
	0	8	0.007	0.003	0.009
Entero wk 4	0	8	0.048	0.021	
	0	16	0.009	0.004	0.011
<i>E. coli</i> wk 5	0	16		0.034	
<i>E. coli</i> wk 6	0	8		0.044	
Entero wk 8	0	16		0.037	
	0	16	0.020	0.008	0.025
<i>E. coli</i> wk 12	0	8	0.051	0.022	
	0	16	0.020	0.008	0.025

### 5.1.5 Pot trial

An analysis of variance (ANOVA) was carried out across treatment groups 0, 8, 16 dry t/ha and no wheat (16 dry t/ha) for *E. coli*, enterococci and bacteriophage for each sampling event. Results indicated no significance between any of the groups for any of the indicator micro-organisms ( $P>0.05$ ). The ANOVA table of results can be found in Appendix 3 (Table A28).

Pot experiments were carried out to test the effects of shading on the survival of indicator micro-organisms. The pots containing wheat versus no-wheat (both at 16 dry t/ha equivalent) indicated no significance between treatment ( $P>0.05$ ).

Post-hoc tests (multiple comparisons) were carried out in SPSS for *E. coli*, enterococci and bacteriophage in the pot trial across treatment groups 0, 8, 16 dry t/ha and no wheat (16 dry t/ha) at each sampling event. There was no significance across any of the treatments ( $P>0.05$ ). Results from this test are available in the SPSS files on the CD-ROM Data Files attached.

## 5.2 Survival on the root system

Bacterial numbers on the cereal root system were tested in comparison to the bacterial numbers contained in the soil. *E. coli* in the root zone was two- $\log_{10}$  higher at both weeks 15 and 18 (Table 5-6). Enterococci in the root zone were one- $\log_{10}$  higher at week 15 (Table 5-7). Soil samples were not analysed for the presence of enterococci in the root zone at week 18.

**Table 5-6: Survival of *E. coli* on the root system vs. soil (16 dry t/ha)**

Week	Roots (mean cfu/grams)	Soil (mean cfu/grams)
15	<b>1.48E+04</b>	5.41E+02
18	<b>4.76E+04</b>	1.28E+02

**Table 5-7: Survival of enterococci on the root system vs. soil (16 dry t/ha)**

Week	Roots (mean cfu/grams)	Soil (mean cfu/grams)
15	<b>4.07E+01</b>	0.00E+00
18	Not tested	5.51E+01

### 5.3 Presence on grains at harvest

Grains were tested in the field at harvest in week 23 for the presence of bacteria. No *E. coli* cells were detected on the grains. Enterococci cells were detected on grains in replicate A from the 16 dry t/ha plot at a level of 3.97E+02 cfu/grams grain (Table 5-8 and Figure A4).

**Table 5-8: Bacterial presence in grains at harvest**

Sample ID	<i>E. coli</i>	Enterococci
G16A1	0.00E+00	<b>3.20E+02</b>
G16A2	0.00E+00	<b>7.67E+02</b>
G16A3	0.00E+00	<b>1.05E+02</b>
G16B1	0.00E+00	0.00E+00
G16B2	0.00E+00	0.00E+00
G16B3	0.00E+00	0.00E+00
G16C1	0.00E+00	0.00E+00
G16C2	0.00E+00	0.00E+00
G16C3	0.00E+00	0.00E+00
G0A1	0.00E+00	0.00E+00
G0A2	0.00E+00	0.00E+00
G0A3	0.00E+00	0.00E+00
G0B1	0.00E+00	0.00E+00
G0B2	0.00E+00	0.00E+00
G0B3	0.00E+00	0.00E+00
G0C1	0.00E+00	0.00E+00
G0C2	0.00E+00	0.00E+00
G0C3	0.00E+00	0.00E+00

## 6 Discussion

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Of the total 218, 264 dry tonnes of biosolids produced annually in Australia, 48% is applied to agricultural land. Stevens et al. (2002) stated that the remainder is used for forestry or goes to landfill and composting. Although biosolids are stabilised they may still contain a certain level of pathogens of potential harm to human health.

The aim of this project was to study the persistence of bacteria and a virus following the application of biosolids as a fertiliser to a wheat crop. The study covered the presence and level of selected microbial indicators — faecal coliform (*E. coli*), enterococci and bacteriophage — from the stockpile to land application and across the season until the grains were removed at harvest.

To determine the level of selected indicator micro-organisms, soil samples were taken regularly from the field trial and the pot trial, across the growing stages of a wheat crop, so that decay rates could be observed. The results the research are intended to provide the Water Corporation in Western Australia and other wastewater reuse programs with adequate data concerning the level of risk involved to humans in the agricultural land application of biosolids.

From here, regulators, public health and safety officers, researchers in wastewater and sludge, local government planners, managers, consultants and scientists will use the information. By gaining actual data from field practice as to the behaviour and survival of pathogens, the areas and level of risk to humans can be better managed. This data will then be basis of protection for consumers involved in the grain industry should any concerns arise with the practice of the use of biosolids onto agricultural land. The results however have been collected from indicator micro-organisms and therefore the actual persistence of bacteria, viruses and other pathogens is still unknown.

## 6.1 Survival factors in the field trial vs. pot trial

### 6.1.1 Decay rates

Decay rates were determined so that the length of persistence of indicator micro-organisms in biosolids applied to dark-brown loamy-sand soil (Table 4-2) could be more easily predicted. Note that decay rates in biosolids applied to other soil types and conditions may vary. The trends for the average cell counts, as plotted on the graphs in Figures A20, A21 and A22, were classified as zero-order or first-order when determining the decay rates (Table 5-1).

#### *Field trial*

Data was plotted on a logarithmic scale to demonstrate whether zero-order or first-order decay occurred (Figure A20). From the decay rates in Table 5-1, it is evident that *E. coli* from the field from 16 dry t/ha plots had the lowest rate of decay as compared to the other indicator micro-organisms in the trial. *E. coli* had a decay rate of 29.7 days with a zero-order (linear) decay rate. Enterococci in the field from 16 dry t/ha plots had a first-order decay rate as the decay trend was not completely linear and tailed off without dropping below detection limits. From weeks 1 to 12 the rate of decay was 20.5 days as bacterial numbers showed a gradual decline (Figure A20) before slightly increasing across weeks 12 to 23, for which the rate of decay was 22.3 days.

In the 8 dry t/ha treatment the *E. coli* decay rate was 3.7 days for a one- $\log_{10}$  reduction to occur, whereas enterococci took 11.7 days for a one- $\log_{10}$  reduction to occur (Table 5-1).

#### *Pot trial*

Bacteriophage plaque numbers in the 16 dry t/ha (equivalent) treatments had a first-order decay rate (Figure A21). From the decay rates in Table 5-1, it is evident that bacteriophage in the pots had the lowest rate of decay as compared to the other indicator micro-organisms in the trial. A lag occurred from days 0 to 7 and thus the reason for the decay rate of -29.7 days for a one- $\log_{10}$  reduction to occur. From days 7 to 21 the decay rate was 3.8 days for a one- $\log_{10}$  reduction to occur. A sudden increase occurred in plaques detected at day 28. This figure was not included amongst the calculations, even though the standard error bar from day 21 would have bridged this gap somewhat (Table 5-1 and Figure A21).

*E. coli* cell numbers in the 8 dry t/ha (equivalent) treatment had a first-order decay rate. Bacterial cell numbers from days 0 to 4 had a faster decay rate of 2.4 days for a one-log<sub>10</sub> reduction to occur, whereas the decay rate from days 4 to 28 was 13.1 days for a one-log<sub>10</sub> reduction to occur (Table 5-1 and Figure A21).

*E. coli* cell numbers in the no-wheat treatment (16 dry t/ha equivalent) had a first-order decay rate. Bacterial cell numbers from days 0 to 7 had a faster decay rate of 2.8 days for a one-log<sub>10</sub> reduction to occur; however, the decay rate from days 7 to 21 was 59.6 days for a one-log<sub>10</sub> reduction to occur (Table 5-1 and Figure A22). This rate of 59.6 days was across three sampling points and is high due to little variation in bacterial cell numbers in this period. At sampling on day 28, bacterial cell counts fell below detection limits. However, the sampling error bar at day 21 makes cell decay more linear.

All other decay rates for indicator micro-organisms (including the nil t/ha treatment) across the various treatments in both experiments were reasonably linear (Figures A21 and A22) and therefore, were assumed zero-order decay rates.

## 6.1.2 Bacteria

### *Length of survival*

Cameron et al. (1997) stated that within two to three months following application, pathogenic bacteria and virus numbers were usually reduced to a minimum. The bacteria tested in livestock manure applied to land by Nicholson et al. (2004) survived for a month in the soil (refer to 2.6 Similar studies). A study in the United States of America by Islam et al. (2004), testing bacteria numbers in animal manures applied to vegetable fields, reported the survival of *E. coli* for up to 6½ months. The USEPA guidelines (Table 2-4) indicate that bacteria can survive for a maximum of 1 year in the soil and a maximum of 6 months on plant surfaces. However, the common maximum is 2 months in the soil and 1 month on the surface of plants. The means and conditions under which the time-frames for the USEPA guidelines were derived were not described.

In this study, bacteria were detectable in the field for at least five months and one month (bacteria and bacteriophage) in the pot trial (Section 5.1.1 Field trial). At harvest, *E. coli* was detected at an average of 6.48E+02 cfu/grams soil and enterococci cell numbers increased up to 8.76E+02 cfu/grams soil. The reason for bacteria surviving on in this case, or increasing as in the case of enterococci, could be the rainfall events which occurred around the time of sampling. A total of 13 mm of moisture was received in the

fortnight prior to sampling and it was observed that summer temperatures had declined during this period.

Although survival rates for the field have been determined in this study, it must be taken into consideration that these rates are based on indicator micro-organisms used to detect the presence of bacteria therefore the true level of pathogens present in the field has not yet been documented. Differences in length of survival could also be due to the variation in trial methods under which the experiments were carried out, along with other possible factors that will be discussed in this section.

In the pot trial, biosolids were sterilised and applied four months prior to application. They were then re-seeded or spiked using laboratory-cultured bacteria and a surrogate virus (Section 4.2.2 Glasshouse trial). This procedure was carried out in an attempt to provide more controlled conditions under a “worst-case” scenario, as it was expected that conditions would provide indicator micro-organisms with optimal conditions for survival in the pots. If biosolids had been applied un-sterilised to the pots, interaction between other microbes would have been expected to occur as per normal, thus reducing the survival rates through natural interactions. However, the results were the opposite of those which were anticipated. Indicator micro-organisms in the pot trial survived for less time than was expected and bacteria in the field survived for longer than was expected. In the field, biosolids were applied directly from the stockpile, therefore indicator micro-organism numbers were un-manipulated (Section 4.2.1 Field trial — *Preparation*).

### ***Microbial competition***

Loehr (1974) found that when organisms in a waste enter the soil they face competition for food supply. He believed that competition and predation was greatest in the surface soil layer, since oxygen was more abundant and rates of decomposition were greater. Epstein (2003) backed this finding in saying that the presence of other micro-organisms can reduce the numbers of pathogenic micro-organisms in the soil through competition. Cameron et al. (1997) found that the initial rate of degradation is generally fast, due to a flush of biological activity. They also believed that the wastes themselves can contribute a considerable number of micro-organisms to the soil microbial pool. However, Sidhu (2000) expected that there was a higher risk of increased micro-organisms associated with higher applications of biosolids.

In the current study, natural interactions in the field were allowed to occur between other micro-organisms detectable in the applied biosolids and in the soil. In the pot trial, natural interactions were allowed to occur only between introduced indicator micro-organisms and the soil but between any micro-organisms that would normally be present in biosolids.

In the field it appears that initially there was a rapid decay of *E. coli* in the first two weeks followed by a steady decline of cell numbers across the following six weeks (Figure 5-2). This suggests that a flush of biological activity occurred, as Cameron et al. (1997) had described, as the micro-organisms were introduced. In the current study, as this activity reduced after a period of competition, bacterial levels stabilised (Figure 5-2).

In a study by Banning, Toze and Mee (2003) it was suggested that an increase in available nutrients may have reduced the potential for *E. coli* to survive in biofilms either due to the occurrence of enhanced competition for nutrients or enhanced antagonism by the indigenous microbial population. In groundwater studies, Gordon and Toze (2003) found that the most influential factor affecting the decay of *E. coli* and viruses was the presence of groundwater micro-organisms and that temperature, the presence of oxygen and nutrient levels indirectly influence indicator micro-organism decay rates by influencing the activity of the groundwater micro-organisms. In the pot trial, indigenous micro-organisms in the soil may have competed with the indicator micro-organisms for nutrients, water and oxygen therefore influencing the decay of indicator micro-organisms. In the field trial, a certain level of competition may have influenced indicator micro-organism decay rates however increased decay was evident with climatic changes such as increased daytime temperatures and decreased rainfall events resulting in rapid soil drying.

### ***Organism vigour***

Biosolids for the field trial originated from Woodman Point WWTP, where sludge is processed using anaerobic digestion (Section 2.1 Sludge processing). According to Boost and Poon (1998), this process uses fermentation by bacteria of organic material in the absence of free oxygen (Table 2-1). It is possible that indicator micro-organisms naturally detectable in the field experiment were those having more vigour; perhaps some were resistant, having survived the stabilisation process and therefore having better survivability than the micro-organisms that were cultured in the laboratory for the pot trial. This could explain the greater length of persistence of indicator micro-organisms in the field trial as compared to the glasshouse trial.

### ***Soil pH levels***

Biosolids dispatched from the WWTP have pathogens surviving in pH7.76 (average figure in H<sub>2</sub>O), equivalent to approximately pH7.0 (CaCl<sub>2</sub>) (Water Corporation 2002). As found in the literature (Section 2.4 Factors affecting survival), pathogens survive longer in soils with a pH range of 5.0 to 6.4 (CaCl<sub>2</sub>). The differences in pH levels may not have been significant enough to have had a major influence on pathogen survival rates. In this trial, the field soil was pH5.1 (CaCl<sub>2</sub>) or pH5.75 (H<sub>2</sub>O); as indicated in Table 4-2; however, the effects of pH changes on survival were not tested in this experiment.

### ***Temperature***

Higher temperatures in the glasshouse as compared to the field could have contributed to the increased rate of decay that was experienced in the pot trial as was also found by Boost and Poon (1998) and Mawdsley et al. (1995). Temperatures in the glasshouse reached 30°C (Figure 5-4) in the first four weeks of the pot trial as compared to day temperatures around 18°C in the first four weeks of the field trial (Figure 5-1). This was due to the pot trial commencing in September as compared to the field trial commencing in June.

Even though micro-organisms favour temperatures of up to 37°C, it seems that the glasshouse conditions observed in this trial such as fast drying, limited nutrition sources and full sunlight may not have been ideal for the survival of indicator micro-organisms that had been cultured in nutrient broths in the laboratory. Humidity levels would have been different in the field as compared with the glasshouse due to the trials commencing at different months of the year. For this reason also, the field trial had lower temperatures to the pot trial. As was discussed earlier, Gordon and Toze (2003) found that temperature, nutrient levels and the presence of oxygen may indirectly influence *E. coli* and viral decay by influencing the activity of other indigenous micro-organisms.

Results indicated that in the field trial between weeks 12 and 15, a rapid decay of micro-organisms occurred (Figures 5-2 and 5-3). This may have been due to soil temperatures and day temperatures rapidly increasing and soil moisture rapidly decreasing over this period (Figure A7) as was also found by Edmonds and Mayer (1979). Ahmed & Sorensen (1995) and Awad et al. (1989) found that a rapid decline in soil moisture affected microbial numbers. Estrada et al. (2004) emphasised that micro-organisms survive longer at low temperatures in damp, shady areas as was also stated by Cameron et al. (1997), Epstein (1998), Gerba et al. (2002) and Stevik et al. (2004). Liang et al. (2003) argued that moisture was more important for microbial activity than temperature.

However, Estrada et al. (2004) reported that the variation in micro-organism numbers detected depends more on their ability to survive on soil nutrients rather than on climatic conditions.

### ***Drying***

Humidity levels were recorded in the glasshouse trial but not in the field trial. The rate of evaporation in the pot trial was high, as it was observed that pots in the glasshouse required approximately 250 ml of water per day. This faster rate of drying may have impacted on the survival rate of pathogens in the pot trial. It is interesting to note that in Spinosa and Vesilind (2001), Nell et al. (1983) and Pederson (1981) air-drying has been used in treatment plants as a technique to reduce the level of pathogens in sludge (Table 2-1). For further field experimentation, it is recommended that humidity levels be recorded and the effects of drying on pathogen survival tested.

### ***Herbicides***

Eamens et al. (1995) found that herbicide treatments at two to six months reduced bacterial numbers significantly. In this study, the use of herbicides during the crop growth stages was avoided so that this factor would not impact on the results. The only chemicals applied to the crop once seeded were pesticides and fungicides. Broadleaf weeds were removed by hand (4.2.1 Field trial – *Other applications*).

### ***Organic matter***

Soil sampling was directed more towards the clumps of biosolids in the field and in the pots (4.2.1 Field trial – *Field sampling* and 4.2.2 Glasshouse trial – *Pot sampling*). In the pot trial, biosolids underwent a certain level of decomposition prior to seeding and their structure changed considerably, becoming more broken-down in nature. This difference in structure may have had a number of implications. Because the biosolids in the field were more ‘clumpy’, it is possible they were more capable of holding nutrients, organic matter, moisture and warmth – all of which are suited to the survival of pathogens. Also, in the field the topsoil contained a certain level of organic matter, whereas organic matter in the pot trial was removed through the process of sifting. Estrada et al. (2004) reported that an increase in organic matter content in the soil improved the survival of coliform. Epstein (1998; 2003) found that organic matter served as a source of food and energy to bacteria, thus allowing it to increase. Therefore, this factor may have contributed to the faster rate of decay in the pots than in the field.

### **Regrowth**

Results indicated an increase in *E. coli* populations at week 10 (late August) and week 12 (early September) and an increase in enterococci populations from weeks 18 to 23 (late November) in the field trial. Climatic conditions during these periods saw an increase in soil moisture due to rainfall events, lower daytime temperatures, some possible overcast weather and an increase in soil temperatures at sampling (Figures A1, A4 and A7). Brown et al. (1980) found that rainfall reduces microbial populations only slightly and that it is possible that rainy, humid weather (as also occurred in their trial in September) may have actually resulted in increased bacterial populations and prolonged survival until the weather cleared. Edmonds and Mayer (1979) found that after initial decay, the survival pattern of faecal coliform was a declining series of die-offs and regrowth. Therefore, it appears that regrowth of bacteria, given the right conditions, is not uncommon.

### **Growth time**

Plant growth occurred at a faster rate in the glasshouse, given its optimal conditions for growth. By day 14, the plants from treatment 3 (8 dry t/ha equivalent) and treatment 4 (16 dry t/ha equivalent) were at the five-leaf stage, whereas plants grown in the field usually take approximately five weeks to reach the same stage (Anderson & Garlinge 2000). This 'sped-up' environment may have also contributed to an increased rate of decay in the pot trial as compared to the field.

## **6.1.3 Virus**

### **Survival**

Observations from the pot trial indicated that at the time of final sampling (day 28), for all treatments except the 8 dry t/ha (equiv.), the levels the levels of bacteriophage plaques were slightly higher than those of *E. coli* or enterococci. This was the opposite of what Baker andHerson (1999) found when referring to the work of Miller et al. (1998). Miller et al. (1998) evaluated male-specific bacteriophage plaque assays in samples taken from a pork slaughterhouse wastewater treatment plant. They found that the numbers of phage plaque-forming units per gram were usually lower than standard indicators; and this included total coliform or *Escherichia coli* numbers.

Nasser et al. (2003) found that phage persisted for long periods in saturated soil and that its survival was not affected by soil microbial activity. In this study, pots in the glasshouse were kept at saturated conditions and natural microbial activity in the soil would have occurred, as the soil was not sterilised prior to seeding. However, according

to the statistical analysis, data from the pot trial was considered too minimal to obtain results of any significance; therefore, more study is required on the external factors that influence viral behaviour and survival both under glasshouse (pot) and field conditions.

### ***Detection***

Cliver (1980) believed that there was no doubt that viruses were often detectable in sludge, even after the sludge had been digested. In the current study, it was difficult to determine whether the virus was being absorbed by the sludge or was just shielded by surrounding dirt particles. These difficulties in detection methods for the virus were due to the viscous nature of the biosolids. Edmonds and Mayer (1979) supported this contention in saying that no viruses had been detected in sludge, but better detection techniques were required. The experience of this study suggests this gap in processing is still a limiting factor requiring further work.

From the abovementioned differences, it appears evident that results from the pot trial and the field trial cannot be justly correlated. In keeping with the use of data from the “worst-case” scenarios, it is recommended that data from the field trial be used over data from the pot trial, as indicator micro-organisms in the field experiment survived for a longer period of time.

For future establishment of a pot trial, it is recommended that the trial be carried out in the winter; that fresh biosolids be applied to the pots at the commencement of the trial (seeding); and that pathogen decay rates from un-manipulated biosolids be compared to sterilised, re-seeded biosolids. The results of survival rates for the pot trial in a shaded greenhouse compared with a glasshouse would also be interesting to trial.

## 6.2 Variation across treatments

**The significance of data across treatment groups in ANOVA for *E. coli* in the field trial at weeks 3 and 12, and enterococci at weeks 2 and 4 (Table 5-3), appears to correspond with the results of the post-hoc test in**

Table 5-5 ( $P < 0.05$ ). Statistical significance mainly occurred between the nil plots and main biosolids rates of 8 t/ha and 16 t/ha, as would be expected ( $P < 0.05$ ). These results proved that *E. coli* was not detectable in the field prior to the application of biosolids.

The significance between treatments 8 t/ha and 16 t/ha in *E. coli* at week 3 has no possible explanation, except it can be noted that *E. coli* in the lower biosolids rate (8 t/ha) was also higher in this week ( $P < 0.05$ ). The fact that there was generally no significance between the biosolids rates 16 dry t/ha and 8 dry t/ha in the field indicates that an increase in biosolids application will not necessarily result in a higher level of bacterial cells ( $P > 0.05$ ). In fact, it was observed in the field experiment that it may actually reduce bacterial numbers, possibly due to factors such as increased competition or increased pH levels in the biosolids. These results do not preclude the change of numbers of enteric viruses or protozoa. Further studies would be required in order to determine this.

This would be an interesting factor to consider in further research work, as the plant, root and grain samples were carried out on the higher treatment (16 dry t/ha) — under the assumption that this rate contained a higher level of bacteria. Also, biosolids are currently applied at 8 dry t/ha; therefore, determining the effect this level of treatment has on bacterial survival in the environment may have been more appropriate.

There was no significance across treatments in the pot trial ( $P > 0.05$ ). Tests run from SPSS indicated that the sample size was too small, as raw data from the trials was condensed into mean figures for each of the three replicates.

## 6.3 Pathogen presence in and on plants

Results indicated a nil presence of bacteria on the lower leaves of the plant samples taken in week 15 (Section 4.2.1 Field trial - *Plant and root sampling*). In general, Edmonds and Mayer (1979) found that survival time for pathogens deposited on plant surfaces was less than in the soil. According to Brown et al. (1980), the survival time of pathogenic organisms on the foliage of plants is influenced by environmental factors such as temperature, humidity, rainfall and exposure to sunlight. In saying this they were referring to leaves coated with sludge, and the fact that pathogenic micro-organisms can

persist for extended periods of time according to the solids concentration of sludge at the time of application. They also stated that any presence of micro-organisms on the leaves of a plant could be a possible health hazard to livestock ingesting the contaminated foliage. Armon et al. (1994) found that it was the texture of the foliage that influenced the plant's susceptibility to contamination and prolonged micro-organism survival. They were, however, working with effluent applied to vegetables, not biosolids that had been land-applied to a cereal crop. The results found in this study were as expected — no bacteria detected on the plant leaves.

#### **6.4 Root zone**

The root zone was sampled for the presence and level of *E. coli* at weeks 15 and 18 in the field. Results indicated that *E. coli* cell numbers were two- $\log_{10}$  higher (Table 5-6) and enterococci cell numbers one- $\log_{10}$  higher (Table 5-7) at week 15 in the soil sampled 5-10 cm from the root zone. This indicates that the root zone cannot be disregarded when assessing the risks associated with applying biosolids to food crops. This zone is cooler and typically facilitates growth due to production of exudates such as nutrients, moisture and oxygen than is contained in the surrounding soil — conditions which bacteria favour for survival. In the pot trial, two treatments were spread with 16 dry t/ha (equivalent) of biosolids. One contained seeded wheat; the other contained no wheat. This was designed to test the effects of shading on the survival of indicator micro-organisms. However, in future research it could prove more viable to test the factors that influence organism survival under the soil (root zone) as opposed to plant portions above the soil surface.

Cliver (1980) believed that it was reasonable to be concerned about the use of sludge on root crops, except where the sludge was sprayed directly onto a standing crop in the field. However, he also stated that the expected processing of the after product needed to be taken into consideration when expressing concerns over pathogen presence in food crops. Regarding the risk of higher numbers of bacteria around the root zone having the ability to get up into the plant, Cliver (1980) believed that the translocation of human pathogens within the tissue of the plant seemed to only be a concern in the case of viruses. And it is on such viruses that further research is required.

## 6.5 Pathogen presence on grains

In this study, grains were seeded in the field trial at the latest recommended date for the growing season. This contributed towards a “worst-case” scenario, in the expectation that plant height at harvest would be reduced and that the harvester comb would need to operate closer to the ground (as we had originally planned to harvest with a machine), thus increasing the risk of biosolids getting into the grains through biosolids clumps remaining on the soil’s surface at the time of harvest.

Results indicated that enterococci were at  $3.97E+02$  cfu/grams grain on grain samples obtained from replicate A of the 16 dry t/ha treatment in the field at harvest time. However, because the other eight plots tested contained no presence of bacteria, this result has been considered an outlier at this stage, possibly resulting from some form of contamination, until further testing is carried out. Given more available time, further sampling would have been conducted. Epstein (2003) stated that by the time cereal plants entered the stages of grain-fill, it was expected that most the pathogens detected would have died off. This was also the case in this study, namely no bacteria were expected in the grains.

As mentioned, it is likely that some form of contamination may have occurred, either by buckets or bags that were not sterile or from rodent droppings that may have been present in the thresher, even though the samples from the three nil plots passed through the thresher first. It was also observed that in the field, the week prior to sampling, the parrots were starting to feed off the ripening grain from plots in replicate A; therefore, it is possible that some contamination was introduced in this way. There was no presence of bacteria on the nil plot in replicate A, but that plot contained less wheat and more weeds as it experienced a delayed start at tillering due to insect damage.

In considering whether bacteria could be detectable on grains, Cliver (1980) stated that one might first consider whether the crop is being grown for food, feed, or fibre. Issac and Boothroyd (1996) found that many people in the agricultural sector were reluctant to use biosolids where crops for human consumption were being grown. Cliver (1980) confirmed that the risk of pathogen ingestion in food products was highest when crops were unlikely to be cooked before being eaten. Epstein (1998) refers to these as ready-to-eat crops, such as salads and fruits. Cliver (1980) was focusing on vegetable crops grown on sludge-amended soils when stating that there are not likely to be pathogens in a food that has been thermally processed in a hermetically sealed container to the point of being

shelf-stable at room temperature. Dudley et al. (1980) stated that food crops that undergo heat processing, fibre crops or acreage set aside for forest products can reasonably be used for land disposal of digested sludge.

In Australia, after crops are harvested, the wheat is then passed through augers and conveyor belts. It is transported by truck, rail and ship, and is stored at receival points whilst awaiting sale. Prior to consumption, most wheat is further processed through means such as grinding or rolling and in most cases is heated by cooking or steaming. Therefore, the likelihood that any pathogens detected could survive is minimal. Apart from this, Cliver (1980), Epstein (2003) and Edmonds and Mayer (1979) have found that as soon as pathogens are exposed to sunlight and ultraviolet light, desiccation occurs, and the chances of microbial survival, are further reduced.

## 6.6 Suitability of selection of indicator micro-organisms

This study sought to represent pathogens from three of the five groups found in biosolids — bacteria, viruses and protozoa. *E. coli* was selected due to its frequent use as an indicator micro-organism in detecting environmental faecal pollution in water as was stated by Mawdsley et al. (1995). Prescott et al. (2002) believed that indicator bacterium should survive longer than the hardiest enteric pathogen. This brings into question the use of *E. coli* as a microbial indicator in biosolids. The results of this study indicated that *E. coli* and enterococci survived up to five months, as compared to bacteria such as Salmonellae, which have been reported by Awad et al. (1989), Dudley et al. (1980) and Edmonds and Mayer (1979) to have survived for up to 2 years.

From this, the possibility of further work with Salmonellae survivability in land-applied biosolids arises. A report from Broos (2004) on the recent work carried out by Dr Jacqui Horswell from the Institute of Environmental Science and Research (ESR) in New Zealand indicate that *Salmonella* spp. survive much better than *E. coli* in biosolids applied to land, thus bringing into question the usefulness of *E. coli* as an indicator of pathogen survival.

This study has indicated that the faecal coliform bacteria, *E. coli* and enterococci, appeared to have undergone re-growth from weeks 10 to 12 and weeks 18 to 23 respectively. Gibbs et al. (1995) found that repopulation occurred in Salmonellae and faecal coliform on land where biosolids had been applied. Prescott et al. (2002) believe that indicator bacterium should not reproduce (in contaminated water) or produce an

inflated value, thus indicating that the selection of microbial indicators for use in biosolids should be further reviewed. In evaluating the usefulness of total coliform, faecal coliform and faecal streptococci as indicators, Baker and Herson (1999) found that total coliform gave a better correlation with the presence of *Salmonella* spp. and *S. aureus* than either of the two faecal groups of organisms.

Results indicated that phage almost fell below detection limits after one month in the pot trial. Cliver (1980) believed that amongst the pathogens that might occur in sludge, viruses might be overrated and parasites might be underrated as hazards. Cameron et al. (1997) found that the highest risk of disease transfer by micro-organism survival on crops was presented by intestinal nematodes. They believed that the risks with viruses were insignificant and that there were lower risks with bacteria than previously thought.

The study of *Cryptosporidium*, described by Prescott et al. (2002) as the smaller protozoan parasite of greater concern than *Giardia* was withdrawn from this study. Awad et al. (1989) stated that *Cryptosporidium* and *Giardia* were capable of existing for prolonged periods in the soil. Carey et al. (2004) stated that *Cryptosporidium* infect the gastrointestinal tract of animals and humans. A study by Gerba et al. (2002) in Arizona reported that *Cryptosporidium* were unlikely to survive the temperatures from anaerobic digestion and did not survive well under low moisture conditions (Joshua et al. 1998). Therefore it is recommended that further work relating to the survival of protozoa in Australian soils be carried out along with the possibility of protozoa transferring through livestock to humans (from land-applied biosolids).

## 6.7 General matters

### *Stockpile results*

Biosolids stockpiled for the field experiment were sampled for bacterial cell numbers prior to being applied to the trial plot. These samples were processed by an external laboratory (4.2.1 Field trial – *Preparation*); therefore, the difference in bacterial cell numbers recorded could be due to different methods of laboratory processing and possible processing delays which may have caused a reduction of surviving bacterial cells. Therefore, time-zero figures in the data should be discounted.

### *Statistical analysis*

Given more time, a statistical analysis using raw data obtained from core samples taken in the field and pot experiments would prove valuable. Average figures from the replicates were entered into SPSS and as a result the program considered the data too minimal for an accurate analysis.

R<sup>2</sup> values were not used in this study as the aim of both experiments was to predict the length of survival of the indicator micro-organisms given variable conditions for survival. The strength of the data and correlations was to be determined through the use of statistics.

## 6.8 Further studies

The selections of pathogens for this study and their use as indicators, along with the purposes for which biosolids are to be used, were all considered in terms of further research.

### 6.8.1 Pathogen types

#### ***Bacteria***

It is recommended that further work be carried out on the survival of bacteria *Salmonella* spp. further to the work of Watson (1980). Awad et al. (1989) stated that *Salmonella* spp. is considered the most persistent bacteria alongside *Clostridium perfringens*. Eamens et al. (1995) stated that *C. perfringens* has the ability to survive for prolonged periods of up to 6 months and has behavioural aspects such as spores that can increase under warm, wet conditions. For these reasons determining the decay rate of *C. perfringens* is recommended.

*Campylobacter* is a major pathogen/micro-organism in water has been suggested for use as an indicator in the revised Water Reuse Guidelines as an indication of risk from pathogenic bacteria in wastewater. Therefore it is recommended that the persistence of *Campylobacter* be tested in biosolids applied to land, as compared to wastewater, along with the prospect that these bacteria may prove a suitable indicator micro-organism.

#### ***Protozoa and virus***

Determining the decay rate of *Cryptosporidium* (protozoa) and the further testing of bacteriophage in biosolids applied to agricultural land is recommended. The rationale for selection *Cryptosporidium* has already been discussed in this chapter. Further work in detection methods for bacteriophage is required.

#### ***Helminths***

The study into the survival and translocation of helminth parasites from livestock to humans is recommended. Johnson et al. (1995) carried out work on the survival of tapeworm (*Taenia hydatigena*) and roundworm (*Ascaris suum*) eggs in processed biosolids. They found that roundworm eggs were resistant to high environmental temperatures and could survive for long periods in damp conditions in the soil. It could be possible that these favourable conditions are apparent around the root zone of the plant, and it becomes more likely that transfer to humans through contact with animals or the helminth eggs could occur, as was discovered in this work.

The helminth of greatest concern in Australia is the hookworm *Ancylostoma duodenale* as it is currently a major problem in the northern region of Australia. Therefore determining the behaviour and survival cycle of this helminth in biosolids applied to land is recommended.

It seems that pathogens (indicators) at greatest risk of transfer through biosolids, rather than indicator micro-organisms used in wastewater, should be further considered. The appropriate selection of indicator micro-organisms is required in order to be able to monitor more accurate pathogen levels in biosolids. This will allow the risks associated with the land application of biosolids to be properly gauged. Therefore a more practical approach to facing issues specific to pathogen persistence in biosolids/soils across varying locations is recommended.

### **6.8.2 Pathogen transfer and livestock**

Cameron et al. (1997) stated that in Australia approximately two-thirds of land surface is used for farming activities; the largest proportion being farming land utilised for the grazing of animals. They stated, along with Chaney et al. (1996) and Gagliardi and Karns (2000) that the soil ingestion pathway was recognised by the USEPA as an area for risk assessment, specifically the potential transfer of contaminants to humans and livestock from sewage sludge-treated soils. Cliver (1980) believed that sludge should only be applied to land that is then planted for the use of animal feed crops. He thought that biosolids that have undergone anaerobic or mesophilic digestion would be quite sufficient to fertilise grains that would be consumed by meat animals. However, Awad et al. (1989) warn that human roundworms, tapeworms and liver flukes, detected in sewage sludge applied to agricultural land, could pass back into the food chain and infect livestock and the community; this statement is also backed by Cameron et al. (1997).

### **6.8.3 Leaching**

Cameron et al. (1997) found that the risk of pathogen leaching is greatest when macro-pore flow occurs, since this allows transport through the soil profile with minimal interaction with the soil matrix. The current study's methodology has indicated that indicator micro-organisms were susceptible to leaching through the soil profile, especially in the pot trial, even though pots were kept watered under controlled conditions to the GSWC of 16%. The indicative level of watering (GSWC) could not be completely assured, especially as the plant demand for water increased with plant growth. In the field, leaching does occur through the profile in most soils given the right conditions;

therefore, the reduced level of indicator micro-organisms detected in the pots should not have been primarily be due to this factor. Work is required in this area to determine the effects of leaching on detection of micro-organisms.

#### **6.8.4 Recovery of viruses from sludge**

For further study, it is recommended that the methods for detecting viruses in sewage sludge be developed. Farrah et al. (1981) reported that few investigators have studied the fate of indigenous viruses during sludge application to land under field conditions. And, as already discussed, Edmonds and Mayer (1979) believed that better detection techniques were required.

#### **6.8.5 Other areas for further research**

Other areas arising from this study requiring further research are:

- The occurrence of increased numbers of pathogens around the root zone;
- Whether herbicides, pesticides, fungicides or commercial fertilisers affect the survival rate of pathogens detected in the soil;
- The effect that different soil types and conditions have on pathogen survival;
- The survival rate of indicator micro-organisms (in biosolids) in a greenhouse as compared to a glasshouse — to compare whether the shady conditions in the greenhouse will prolong the length of survival of micro-organisms. Brown et al. (1980) used a greenhouse to study the influence of simulated rainfall on residual bacteria and virus on grass treated with sewage sludge; comparing greenhouse and glasshouse environments could prove useful.

In conclusion, the National Research Council (2002, p. 368) stated that “the reliability of United States Environmental Protection Authorities (USEPA) prescribed treatment techniques should be better documented using current pathogen technology, and more research on environmental persistence is needed to verify that current management controls for pathogens are adequate to maintain minimal exposure concentrations over an extended period of time”, suggesting that there is support for further research at the highest levels.

## 7 Conclusion

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From the results, it is clear that decay rates in the field trial and the glasshouse (pot) trial were vastly different, due to a difference in the conditions at each location. This indicates that the methods used in other studies should be reviewed before their results are compared to the results of this study. There were no bacteria detected in the field prior to the application of biosolids. The bacterial numbers in the stockpile were lower than the numbers detected in the field after spreading and this may be due to the samples being processed in an external laboratory. Bacteria were still detectable in the field after five months. Other studies have found that bacteria are able to survive for up to six months in the field. The indicator micro-organisms, seeded into the pots for the glasshouse trial, fell below detection limits after only one month. A regrowth of *E. coli* cells occurred in the field at weeks 10 and 12. Enterococci also increased in number between weeks 18 and 23. Studies have shown that bacteria are capable of regrowth given favourable conditions.

There was nil significance of treatment affect ( $P>0.05$ ) across the survival rates of various indicator micro-organisms, except between treatments 8 dry t/ha and 16 dry t/ha at week 3 in the field. There was significance in the comparisons between the nil plots and treatments in the field, as would be expected. There were nil bacteria detected on the lower leaves of the wheat plants sampled in the field at week 12.

*E. coli* cells were detected around the root zone of the wheat in the field trial. Cell numbers were two- $\log_{10}$  higher in *E. coli* at weeks 15 and 18 than the cells detected in the adjacent soil. Enterococci cells were 1  $\log_{10}$  higher at week 15.

Enterococci were detected in the grain samples taken from replicate A of the 16 dry t/ha plot in the field. However, all other replicates from the 16 dry t/ha and 0 t/ha treatments (15 samples) resulted in no bacteria being detected; therefore, this data has been treated as an outlier (contamination issue) at this stage, until further testing is undertaken.

Based on the results of this study, the null hypothesis is accepted; namely, that the indicator micro-organisms are not detected in wheat at harvest following the application of biosolids. Results so far indicate no reason to discontinue biosolids land application to wheat crops; however, further research is required in order to determine the absolute risk.

## 8 Reference List

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## Appendix 1: Biosolids

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**Table A 1: Three biosolids classifications used in the USA**

CATEGORY	DESCRIPTION
P1 Primary treatment	<p>Very Limited Exposure.</p> <p>Includes disposal practices such as land-filling or limited access mine site rehabilitation.</p> <p>Removal of insoluble particulate materials by settling, screening, addition of alum and other coagulation agents, and other physical procedures.</p>
P2 Secondary treatment	<p>Limited Exposure.</p> <p>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.</p> <p>Biological removal of dissolved organic matter (trickling filters, activated sludge, lagoons, extended aeration systems and anaerobic digesters).</p>
P3 Tertiary treatment	<p>Unrestricted Exposure.</p> <p>For unrestricted marketing of biosolids where the possibility of public exposure is high.</p> <p>Biological removal of inorganic nutrients, chemical removal of inorganic nutrients, virus removal/inactivation and trace chemical removal</p>

Source: (Prescott, Harley & Klein 2002; Gibbs & Goen 1995)

**Table A 2: Description of wastewater treatment plants (Western Australia)**

<b>TREATMENT STAGES</b>	<b>*Woodman Point WWTP</b>	<b>Beenyup WWTP</b>	<b>Subiaco WWTP</b>
Input to plant	82.5 mL/day	59.2 mL/day	56.25 mL/day
Pre-chlorination (odour control)	Dosed in summer as needed at 6 mg/L	15 mg/L average	25 mg/L average
Primary treatment	Yes	Yes	Yes
<b>Raw sludge sample collected</b>	Yes	Yes	Yes
Secondary treatment (activated sludge)	No	Yes	Yes
Dissolved air flotation (DAF) of sludge	No	Yes	Yes
<b>Secondary sludge sample collected</b>	No	Yes	Yes
Raw sludge and secondary DAF sludge mixed	No	Yes	13 days, 35°C
Primary anaerobic digestion (heated)	16 days, 35°C	27 days, 35°C	30 days, open digesters, ambient temperature
Secondary anaerobic digestion (no heating)	6 days, closed digester 30°C	17 days, closed digesters, 30°C	Approximately ambient temperature
<b>Digested sludge sample collected</b>	Yes	Yes	Yes
Dewatering: Filter belt presses production	No	70 m <sup>3</sup> /day	80 m <sup>3</sup> /day
KMnO <sub>4</sub> addition for H <sub>2</sub> S control	No	55 g/m <sup>3</sup> of sludge	4.3 g/m <sup>3</sup> of sludge
Centrifuge production	30-40 m <sup>3</sup> /day	Yes	No
Sand drying beds	150 m <sup>3</sup> /day	No	No
<b>Dewatered sludge sample collected</b>	Yes	Yes	Yes

*\*Biosolids from Woodman Point WWTP to be used in this study*

Source: (Gibbs et al. 1994)

**Table A 3: Summary of exposure pathways used in risk assessment for land application of biosolids**

PATHWAY	DESCRIPTION OF HIGHLY EXPOSED INDIVIDUAL
1. Biosolids–Soil–Plant–Human	Human (except home gardener) lifetime ingestion of plants grown in biosolids-amended soil. General food chain.
2. Biosolids–Soil–Plant–Human	Human (home gardener) lifetime ingestion of plants grown in biosolids-amended soil.
3. *Biosolids–Human	Human (child) ingesting biosolids as residential soil or biosolids product on soil surface.
4. Biosolids–Soil–Plant–Animal–Human	Human lifetime ingestion of animal products (animals raised on forage grown on biosolids-amended soil).
5. Biosolids–Soil–Animal–Human	Human lifetime ingestion of animal products (animals ingest biosolids directly).
6. *Biosolids–Soil–Plant–Animal	Animal lifetime ingestion of plants grown on biosolids-amended soil such as livestock fed feed forages and grains 100% of which are grown on biosolids-amended land.
7. Biosolids–Soil–Animal	Animal lifetime ingestion of biosolids of animals grazing on biosolids sprayed biosolids.
8. *Biosolids–Soil–Plant	Plant toxicity due to taking up biosolids pollutants when grown in biosolids-amended soils such as ‘crops’ or vegetables in strongly acidic biosolids-amended soils.
9. Biosolids–Soil–Soil–Organism	Soil organism ingesting biosolids/soil mixture such as earthworms, slugs, bacteria, fungi in biosolids-amended soil.
10. Biosolids–Soil–Soil–Organism–Soil–Organism–Predator	Predator of soil organisms that have been exposed to biosolids-amended soils such as birds eating earthworms affected by biosolids.
11. *Biosolids–Soil–Airborne Dust–Human	Adult human lifetime inhalation of particles (dust) (e.g. tractor driver tilling a field).
12. Biosolids–Soil–Surface Water–Human	Human lifetime drinking surface water and ingesting fish containing pollutants in biosolids.
13. *Biosolids–Soil–Air–Human	Human lifetime inhalation of pollutants from biosolids. Farm households.
14. Biosolids–Soil–Ground Water–Human	Human lifetime drinking well water containing pollutants from biosolids leached from soil to ground water.

\*3, 6, 8, 11 & 13 are possible pathways relating to this study

Source: (Ahmed & Sorenson 1995; Chaney et al. 1996; Gibbs & Goen 1995; McFarland 2001).

## Appendix 2: Chemicals

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**Table A 4: Reagents and chemicals**

REAGENT/CHEMICAL	SUPPLIER
Agar Bacteriological (Agar No.1)	<i>Oxoid, England</i>
Ampicillin	<i>Boehringer Mannheim</i>
Brain Heart Broth	<i>Mercks reagents, Germany</i>
Calcium chloride (CaCl <sub>2</sub> .2H <sub>2</sub> O)	<i>Merck, Australia</i>
Chromocult™ Coliform — Agar	<i>Mercks reagents, Germany</i>
Chromocult™ Enterococci — Agar	<i>Mercks reagents, Germany</i>
D-(+)-Glucose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )	<i>BDH, Australia</i>
Ethanol	<i>BDH, England</i>
Magnesium sulphate (MgSO <sub>4</sub> .7H <sub>2</sub> O)	<i>BDH, Australia</i>
Nalidixic Acid	<i>Sigma, Germany</i>
Nutrient Broth	<i>Oxoid, England</i>
Sodium chloride (NaCl)	<i>BDH, Australia</i>
Sodium dihydrogen orthophosphate (NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O)	<i>Biolab (Aust) Limited, Australia</i>
Sodium hydroxide-pellets (NaOH)	<i>Ajax Chemicals, Australia</i>
Sodium hypochlorite (NaClO)	<i>APS, Australia</i>
Sodium phosphate, Dibasic, Anhydrous (Na <sub>2</sub> HPO <sub>4</sub> )	<i>J. T. Baker, USA</i>
Sodium thiosulphate (Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> )	<i>BDH Laboratory Supplies, England</i>
Tryptone	<i>Oxoid, England</i>
Yeast Extract	<i>Oxoid, England</i>

Source: (CSIRO 2004)

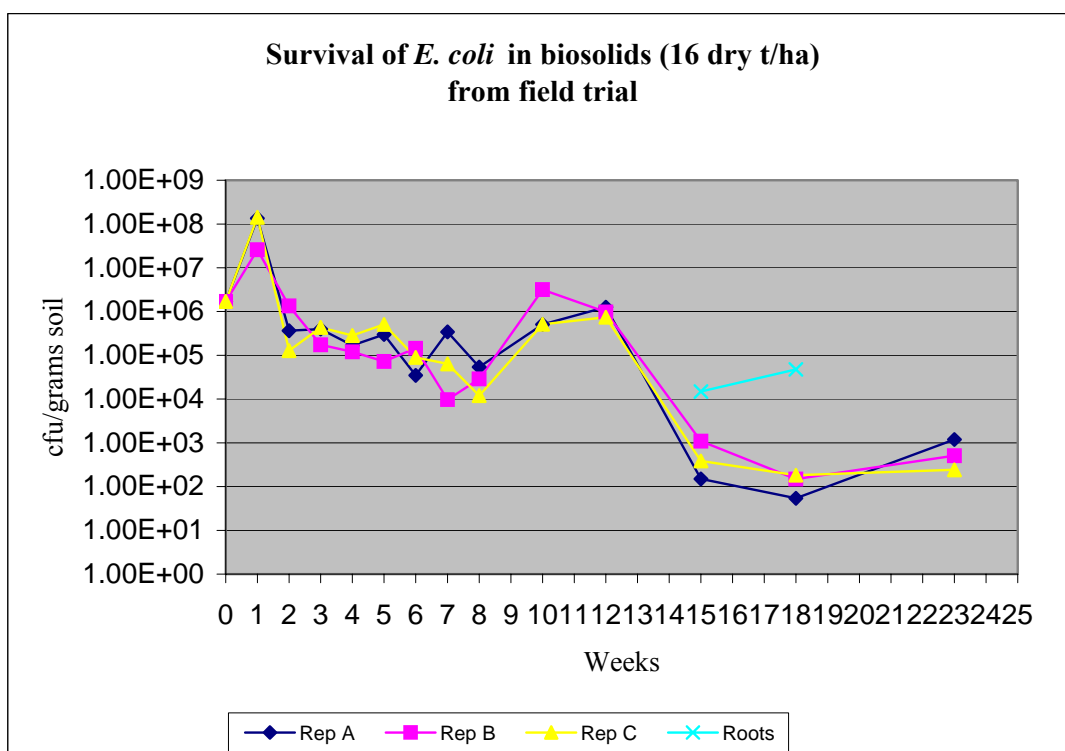
## Appendix 3: Sampling Data

Table A 5: *E. coli* survival from the field trial (16 dry t/ha)

**FIELD TRIAL DATA SUMMARY**  
*E. coli* cfu/grams soil at 16 dry t/ha

Weeks	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	1.70E+06	1.70E+06	1.70E+06	1.70E+06	0.00E+00	0.00E+00
1	1.35E+08	2.58E+07	1.43E+08	1.01E+08	6.54E+07	3.78E+07
2	3.64E+05	1.34E+06	1.28E+05	6.10E+05	6.41E+05	3.70E+05
3	3.98E+05	1.73E+05	4.33E+05	3.35E+05	1.41E+05	8.12E+04
4	1.68E+05	1.20E+05	2.84E+05	1.90E+05	8.41E+04	4.85E+04
5	2.99E+05	7.25E+04	5.07E+05	2.93E+05	2.17E+05	1.25E+05
6	3.48E+04	1.45E+05	8.95E+04	8.97E+04	5.50E+04	3.17E+04
7	3.41E+05	9.70E+03	6.36E+04	1.38E+05	1.78E+05	1.03E+05
8	5.41E+04	2.87E+04	1.20E+04	3.16E+04	2.12E+04	1.22E+04
10	5.08E+05	3.16E+06	5.11E+05	1.39E+06	1.53E+06	8.85E+05
12	1.26E+06	9.75E+05	7.43E+05	9.92E+05	2.58E+05	1.49E+05
15	1.50E+02	1.09E+03	3.84E+02	5.41E+02	4.90E+02	2.83E+02
18	5.41E+01	1.49E+02	1.82E+02	1.28E+02	6.62E+01	3.82E+01
23	1.19E+03	5.09E+02	2.42E+02	6.48E+02	4.91E+02	2.83E+02

Figure A 1: Survival of *E. coli* (16 t/ha) from field trial replicates

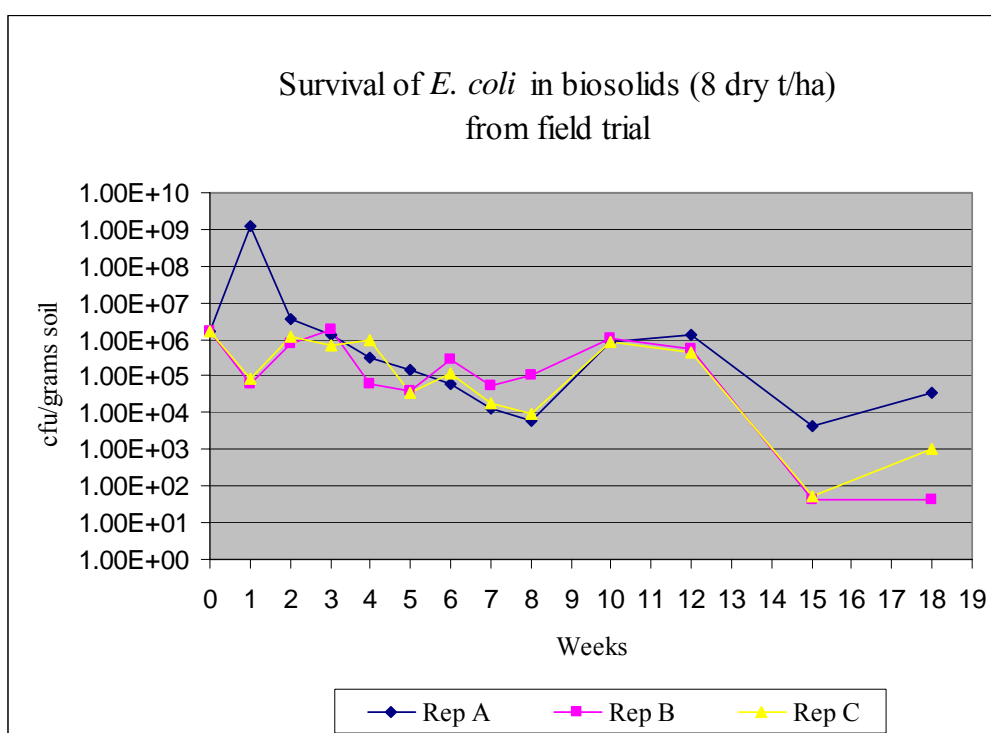


**Table A 6: *E. coli* survival from field trial (8 dry t/ha)**

**FIELD TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams soil) at 8 dry t/ha**

Weeks	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	1.70E+06	1.70E+06	1.70E+06	1.70E+06	0.00E+00	0.00E+00
1	1.26E+09	6.19E+04	8.82E+04	4.21E+08	7.28E+08	4.21E+08
2	3.69E+06	7.76E+05	1.18E+06	1.88E+06	1.58E+06	9.11E+05
3	1.27E+06	1.81E+06	7.23E+05	1.27E+06	5.42E+05	3.13E+05
4	3.26E+05	6.34E+04	9.11E+05	4.34E+05	4.34E+05	2.51E+05
5	1.55E+05	4.09E+04	3.45E+04	7.68E+04	6.78E+04	3.92E+04
6	6.02E+04	2.85E+05	1.18E+05	1.54E+05	1.17E+05	6.75E+04
7	1.31E+04	5.62E+04	1.88E+04	2.94E+04	2.34E+04	1.35E+04
8	5.93E+03	1.01E+05	9.40E+03	3.89E+04	5.41E+04	3.12E+04
10	8.94E+05	1.10E+06	9.05E+05	9.68E+05	1.18E+05	6.83E+04
12	1.34E+06	5.61E+05	4.61E+05	7.86E+05	4.79E+05	2.76E+05
15	4.21E+03	4.22E+01	5.25E+01	1.43E+03	2.40E+03	1.39E+03
18	3.36E+04	4.41E+01	1.06E+03	1.16E+04	1.91E+04	1.10E+04

**Figure A 2: Survival of *E. coli* (8 t/ha) from field trial replicates**

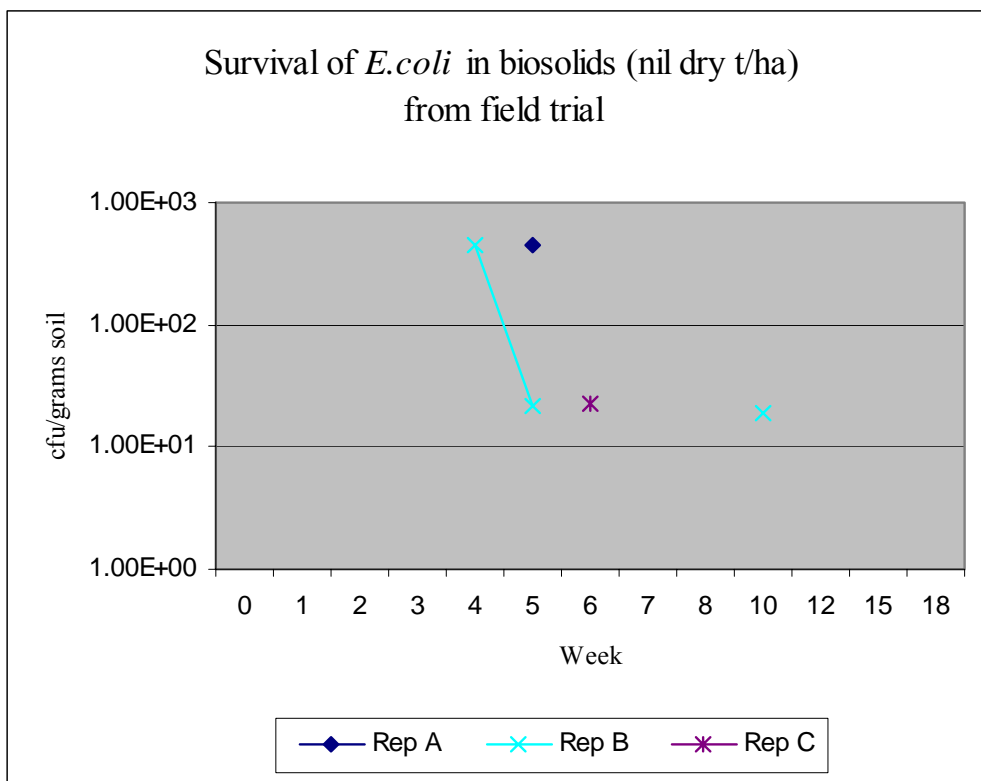


**Table A 7: *E. coli* survival from field trial (nil dry t/ha)**

**FIELD TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams soil) at nil dry t/ha**

Weeks	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
2	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	0.00E+00	4.58E+02	0.00E+00	1.53E+02	2.65E+02	1.53E+02
5	4.46E+02	2.18E+01	0.00E+00	1.56E+02	2.51E+02	1.45E+02
6	0.00E+00	0.00E+00	2.22E+01	7.41E+00	1.28E+01	7.41E+00
7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
8	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
10	0.00E+00	1.91E+01	0.00E+00	6.35E+00	1.10E+01	6.35E+00
12	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 3: Survival of *E. coli* (nil t/ha) from field trial replicates**

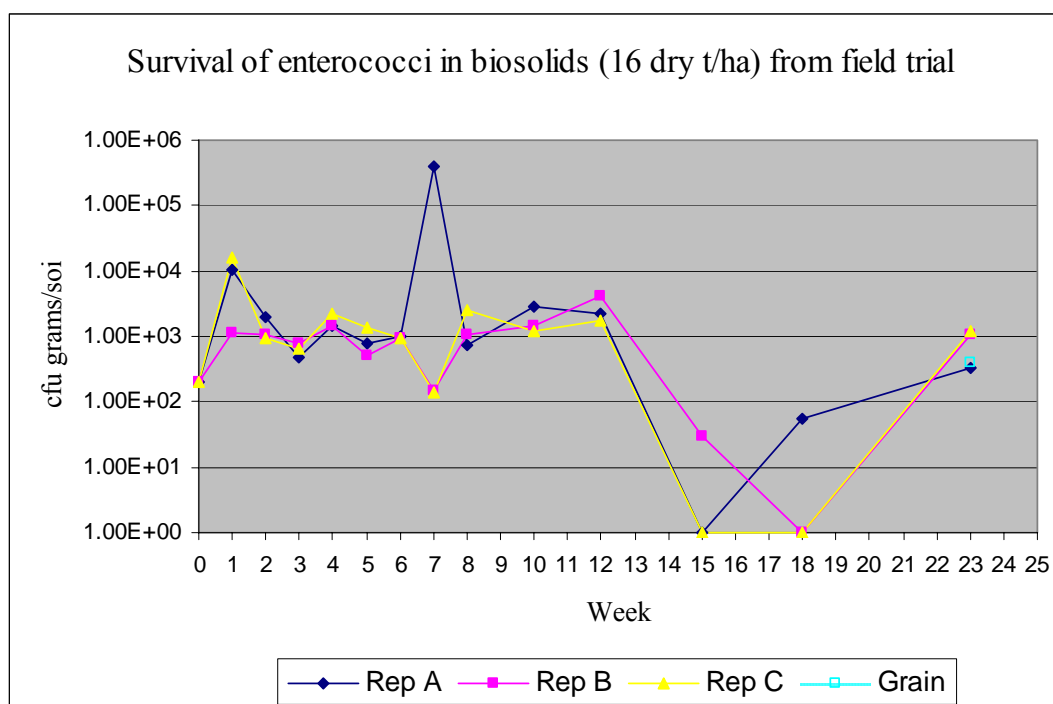


**Table A 8: Enterococci survival from the field trial (16 dry t/ha)**

**FIELD TRIAL DATA SUMMARY**  
**Enterococci cfu/grams soil at 16 dry t/ha**

Weeks	Rep A	Rep B	Rep C	Average	Std Dev	Std Error
0	2.03E+02	2.03E+02	2.03E+02	2.03E+02	0.00E+00	0.00E+00
1	1.04E+04	1.13E+03	1.65E+04	9.36E+03	7.76E+03	4.48E+03
2	2.00E+03	1.07E+03	9.12E+02	1.33E+03	5.90E+02	3.40E+02
3	4.76E+02	7.97E+02	6.60E+02	6.44E+02	1.61E+02	9.31E+01
4	1.45E+03	1.44E+03	2.22E+03	1.70E+03	4.46E+02	2.57E+02
5	7.97E+02	5.07E+02	1.40E+03	9.00E+02	4.54E+02	2.62E+02
6	1.01E+03	9.63E+02	9.42E+02	9.73E+02	3.75E+01	2.17E+01
7	3.86E+05	1.48E+02	1.41E+02	1.29E+05	2.23E+05	1.29E+05
8	7.22E+02	1.07E+03	2.48E+03	1.43E+03	9.33E+02	5.39E+02
10	2.85E+03	1.46E+03	1.19E+03	1.83E+03	8.87E+02	5.12E+02
12	2.19E+03	4.18E+03	1.79E+03	2.72E+03	1.28E+03	7.37E+02
15	0.00E+00	2.91E+01	0.00E+00	9.69E+00	1.68E+01	9.69E+00
18	5.51E+01	0.00E+00	0.00E+00	1.84E+01	3.18E+01	1.84E+01
23	3.39E+02	1.06E+03	1.23E+03	8.76E+02	4.73E+02	2.73E+02

**Figure A 4: Survival of enterococci (16 t/ha) from field trial replicates**

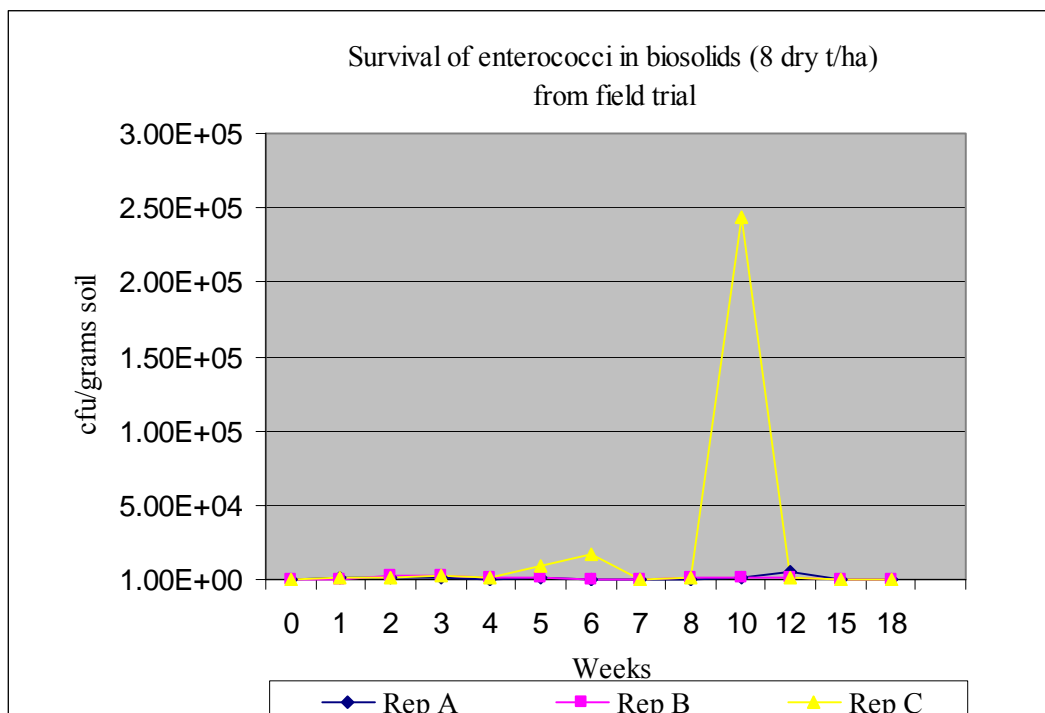


**Table A 9: Enterococci survival from the field trial (8 dry t/ha)**

**FIELD TRIAL DATA SUMMARY**  
**Enterococci cfu/grams soil at 8 dry t/ha**

Weeks	Rep A	Rep B	Rep C	Average	Std Dev	Std Dev
0	2.03E+02	2.03E+02	2.03E+02	2.03E+02	0.00E+00	0.00E+00
1	1.39E+03	0.00E+00	1.94E+03	1.11E+03	1.00E+03	5.77E+02
2	1.87E+03	2.60E+03	1.72E+03	2.06E+03	4.74E+02	2.74E+02
3	1.63E+03	2.05E+03	2.95E+03	2.21E+03	6.75E+02	3.89E+02
4	5.81E+02	1.05E+03	1.82E+03	1.15E+03	6.23E+02	3.59E+02
5	1.08E+03	1.22E+03	9.53E+03	3.94E+03	4.84E+03	2.79E+03
6	3.44E+02	4.48E+02	1.66E+04	5.80E+03	9.35E+03	5.40E+03
7	2.33E+02	1.48E+02	2.20E+02	2.00E+02	4.61E+01	2.66E+01
8	1.67E+02	1.46E+03	9.24E+02	8.51E+02	6.51E+02	3.76E+02
10	9.74E+02	7.11E+02	2.43E+05	8.17E+04	1.40E+05	8.09E+04
12	5.80E+03	1.80E+03	6.60E+02	2.75E+03	2.70E+03	1.56E+03
15	0.00E+00	1.24E+02	5.80E+02	2.35E+02	3.05E+02	1.76E+02
18	6.80E+01	0.00E+00	4.99E+01	3.93E+01	3.52E+01	2.03E+01

**Figure A 5: Survival of enterococci (8 t/ha) from field trial replicates**

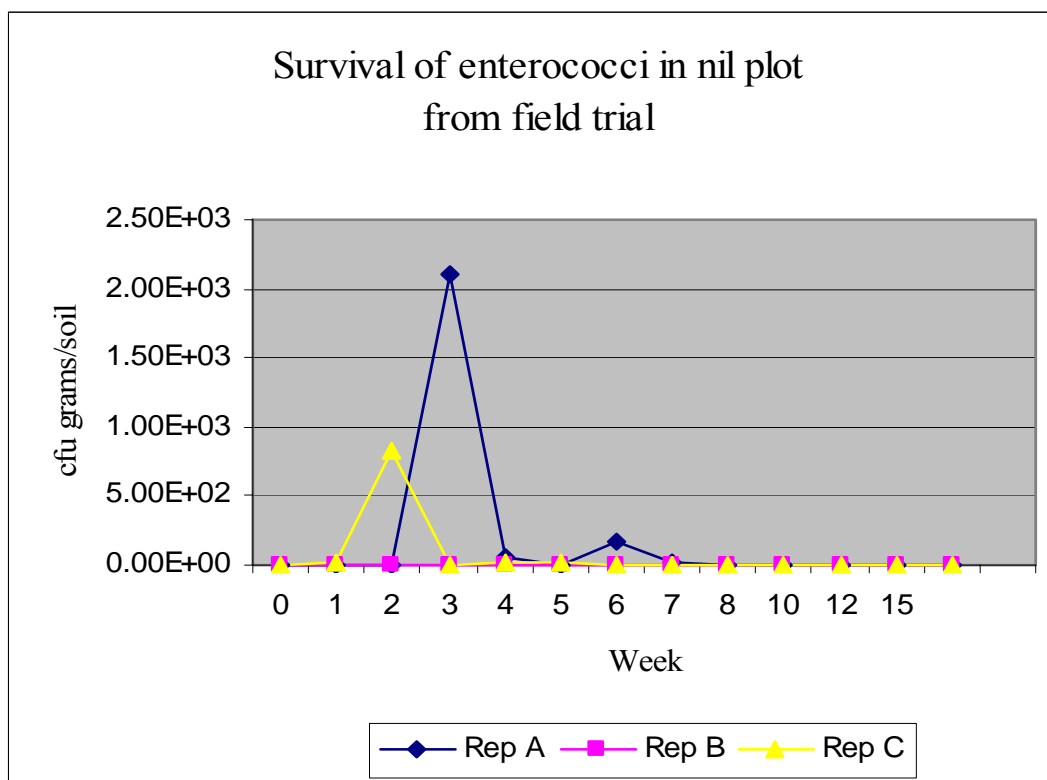


**Table A 10: Enterococci survival from the field trial (nil dry t/ha)**

**FIELD TRIAL DATA SUMMARY**  
**Enterococci cfu/grams soil at nil dry t/ha**

Weeks	Rep A	Rep B	Rep C	Average	Std Dev	Std Error
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	0.00E+00	0.00E+00	1.69E+01	5.65E+00	9.79E+00	5.65E+00
2	0.00E+00	0.00E+00	8.36E+02	2.79E+02	4.83E+02	2.79E+02
3	2.10E+03	0.00E+00	0.00E+00	7.01E+02	1.21E+03	7.01E+02
4	5.26E+01	0.00E+00	2.14E+01	2.47E+01	2.65E+01	1.53E+01
5	0.00E+00	0.00E+00	2.02E+01	6.73E+00	1.17E+01	6.73E+00
6	1.63E+02	0.00E+00	0.00E+00	5.42E+01	9.39E+01	5.42E+01
7	2.40E+01	0.00E+00	0.00E+00	8.01E+00	1.39E+01	8.01E+00
8	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
12	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 6: Survival of enterococci (nil t/ha) from field trial replicates**



**Table A 11: Excel data for *E. coli* from field sampling (\*Week 2)**

sample id	tube wt	total wt	sample wt	cell count	/mL	buffer sol	cfu/extract	dry wt	multiplication	cfu/g dryweight	average	std dev	std err
B16A1	12.20	23.58	11.38	1175	1.18E+05	15.00	1.76E+06	11.38	0.0879	1.55E+05			
B16A2	12.18	21.46	9.28	2200	2.20E+05	15.00	3.30E+06	9.28	0.1078	3.56E+05			
B16A3	12.23	25.02	12.79	1275	1.28E+05	15.00	1.91E+06	12.79	0.0782	1.50E+05			
B16A4	12.20	23.27	11.07	9250	9.25E+05	15.00	1.39E+07	11.07	0.0903	1.25E+06			
B16A5	12.40	26.16	13.76	1025	1.03E+05	15.00	1.54E+06	13.76	0.0727	1.12E+05			
B16A6	12.38	25.32	12.94	1375	1.38E+05	15.00	2.06E+06	12.94	0.0773	1.59E+05	3.64E+05	4.44E+05	1.81E+05
B16B1	12.18	21.96	9.78	1150	1.15E+05	15.00	1.73E+06	9.78	0.1022	1.76E+05			
B16B2	12.15	21.59	9.44	19250	1.93E+06	15.00	2.89E+07	9.44	0.1059	3.06E+06			
B16B3	12.20	24.19	11.99	14250	1.43E+06	15.00	2.14E+07	11.99	0.0834	1.78E+06			
B16B4	12.41	24.47	12.06	9000	9.00E+05	15.00	1.35E+07	12.06	0.0829	1.12E+06			
B16B5	12.26	23.37	11.11	13500	1.35E+06	15.00	2.03E+07	11.11	0.0900	1.82E+06			
B16B6	12.10	25.52	13.42	600	6.00E+04	15.00	9.00E+05	13.42	0.0745	6.71E+04	1.34E+06	1.13E+06	4.62E+05
B16C1	12.23	23.60	11.37	2075	2.08E+05	15.00	3.11E+06	11.37	0.0880	2.74E+05			
B16C2	12.23	24.35	12.12	1300	1.30E+05	15.00	1.95E+06	12.12	0.0825	1.61E+05			
B16C3	12.26	23.19	10.93	550	5.50E+04	15.00	8.25E+05	10.93	0.0915	7.55E+04			
B16C4	12.38	26.65	14.27	1250	1.25E+05	15.00	1.88E+06	14.27	0.0701	1.31E+05			
B16C5	12.23	22.01	9.78	700	7.00E+04	15.00	1.05E+06	9.78	0.1022	1.07E+05			
B16C6	12.24	25.54	13.30	163	1.63E+04	15.00	2.44E+05	13.30	0.0752	1.83E+04	1.28E+05	8.67E+04	3.54E+04
B8A1	12.37	24.72	12.35	22750	2.28E+06	15.00	3.41E+07	12.35	0.0810	2.76E+06			
B8A2	12.16	24.02	11.86	1150	1.15E+05	15.00	1.73E+06	11.86	0.0843	1.45E+05			
B8A3	12.37	21.19	8.82	1375	1.38E+05	15.00	2.06E+06	8.82	0.1134	2.34E+05			
B8A4	12.19	24.81	12.62	155000	1.55E+07	15.00	2.33E+08	12.62	0.0792	1.84E+07			
B8A5	12.11	26.79	14.68	3500	3.50E+05	15.00	5.25E+06	14.68	0.0681	3.58E+05			
B8A6	12.16	22.89	10.73	1500	1.50E+05	15.00	2.25E+06	10.73	0.0932	2.10E+05	3.69E+06	7.29E+06	2.98E+06
B8B1	12.86	26.86	14.00	1375	1.38E+05	15.00	2.06E+06	14.00	0.0714	1.47E+05			
B8B2	12.46	21.74	9.28	180	1.80E+04	15.00	2.70E+05	9.28	0.1078	2.91E+04			
B8B3	12.41	24.75	12.34	2150	2.15E+05	15.00	3.23E+06	12.34	0.0810	2.61E+05			
B8B4	12.54	25.44	12.90	2425	2.43E+05	15.00	3.64E+06	12.90	0.0775	2.82E+05			
B8B5	12.54	23.75	11.21	15750	1.58E+06	15.00	2.36E+07	11.21	0.0892	2.11E+06			
B8B6	12.45	24.53	12.08	14750	1.48E+06	15.00	2.21E+07	12.08	0.0828	1.83E+06	7.76E+05	9.33E+05	3.81E+05
B8C1	12.51	22.20	9.69	500	5.00E+04	15.00	7.50E+05	9.69	0.1032	7.74E+04			
B8C2	12.52	25.72	13.20	3600	3.60E+05	15.00	5.40E+06	13.20	0.0758	4.09E+05			
B8C3	12.89	23.38	10.49	238	2.38E+04	15.00	3.56E+05	10.49	0.0953	3.40E+04			
B8C4	12.64	24.50	11.86	1325	1.33E+05	15.00	1.99E+06	11.86	0.0843	1.68E+05			
B8C5	12.53	26.53	14.00	52500	5.25E+06	15.00	7.88E+07	14.00	0.0714	5.63E+06			
B8C6	12.50	27.88	15.38	7750	7.75E+05	15.00	1.16E+07	15.38	0.0650	7.56E+05	1.18E+06	2.19E+06	8.96E+05
B0A1	12.54	23.90	11.36	0	0.00E+00	15.00	0.00E+00	11.36	0.0880	0.00E+00			
B0A2	12.92	28.01	15.09	0	0.00E+00	15.00	0.00E+00	15.09	0.0663	0.00E+00			
B0A3	12.41	28.59	16.18	0	0.00E+00	15.00	0.00E+00	16.18	0.0618	0.00E+00			
B0A4	12.43	24.40	11.97	0	0.00E+00	15.00	0.00E+00	11.97	0.0835	0.00E+00			
B0A5	12.42	23.80	11.38	0	0.00E+00	15.00	0.00E+00	11.38	0.0879	0.00E+00			
B0A6	12.90	27.09	14.19	0	0.00E+00	15.00	0.00E+00	14.19	0.0705	0.00E+00	0.00E+00	0.00E+00	0.00E+00
B0B1	12.90	20.43	7.53	0	0.00E+00	15.00	0.00E+00	7.53	0.1328	0.00E+00			
B0B2	12.50	25.60	13.10	0	0.00E+00	15.00	0.00E+00	13.10	0.0763	0.00E+00			
B0B3	12.55	23.62	11.07	0	0.00E+00	15.00	0.00E+00	11.07	0.0903	0.00E+00			
B0B4	12.49	24.25	11.76	0	0.00E+00	15.00	0.00E+00	11.76	0.0850	0.00E+00			
B0B5	12.53	24.27	11.74	0	0.00E+00	15.00	0.00E+00	11.74	0.0852	0.00E+00			
B0B6	12.53	26.36	13.83	0	0.00E+00	15.00	0.00E+00	13.83	0.0723	0.00E+00	0.00E+00	0.00E+00	0.00E+00
B0C1	12.53	25.47	12.94	0	0.00E+00	15.00	0.00E+00	12.94	0.0773	0.00E+00			
B0C2	12.45	24.68	12.23	0	0.00E+00	15.00	0.00E+00	12.23	0.0818	0.00E+00			
B0C3	12.48	26.25	13.77	0	0.00E+00	15.00	0.00E+00	13.77	0.0726	0.00E+00			
B0C4	12.53	23.50	10.97	0	0.00E+00	15.00	0.00E+00	10.97	0.0912	0.00E+00			
B0C5	12.44	26.37	13.93	0	0.00E+00	15.00	0.00E+00	13.93	0.0718	0.00E+00			
B0C6	12.53	24.67	12.14	0	0.00E+00	15.00	0.00E+00	12.14	0.0824	0.00E+00	0.00E+00	0.00E+00	0.00E+00

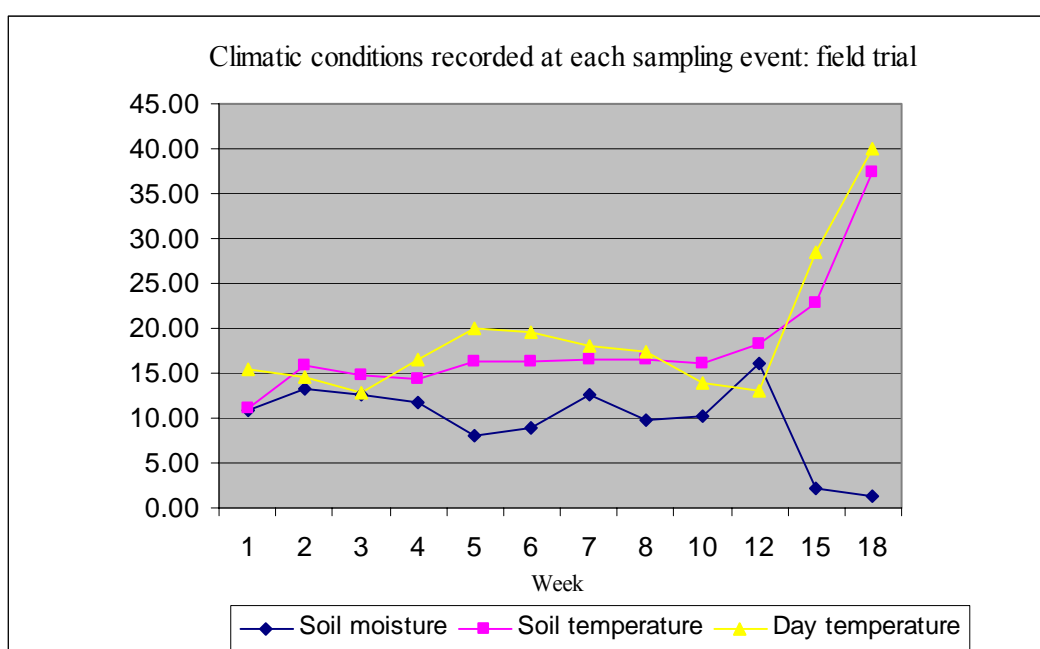
\*Excel spreadsheets were produced for weeks 1-8, 10, 12, 15, 18 and 23 are available as Field Trial.xls data on CD-ROM Data Files attached to document.

**Table A 12: Climatic conditions at each sampling event in the field**

**FIELD TRIAL DATA**  
**Conditions at sampling**

Week	Soil moisture	Soil temperature	Day temperature	Time of day
0	18.27	0	0	
1	10.87	11.00	15.40	9.00am
2	13.23	15.80	14.60	4.30pm
3	12.65	14.70	12.80	4.30pm
4	11.75	14.30	16.50	3.30pm
5	8.00	16.20	20.10	3.30pm
6	8.97	16.40	19.60	3.10pm
7	12.63	16.50	18.00	3.30pm
8	9.71	16.50	17.30	4.50pm
10	10.28	16.10	14.00	5.50pm
12	16.18	18.30	13.00	3.30pm
15	2.23	22.80	28.40	2.45pm
18	1.39	37.50	39.90	2.45pm

**Figure A 7: Climatic conditions at each sampling event in field**

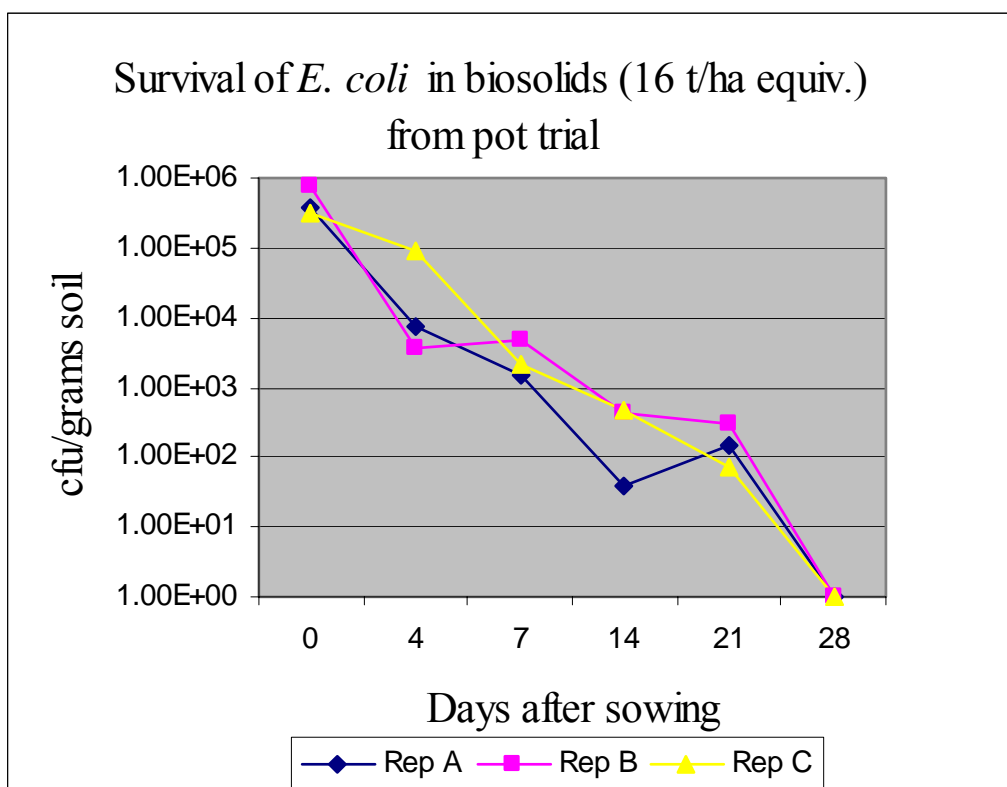


**Table A 13: *E. coli* survival from the pot trial (16 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams) at 16 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	3.77E+05	7.41E+05	3.23E+05	4.80E+05	2.85E+05	1.65E+05
4	7.40E+03	3.79E+03	9.11E+04	3.41E+04	3.57E+04	2.06E+04
7	1.48E+03	4.75E+03	2.21E+03	2.81E+03	1.76E+03	1.01E+03
14	3.76E+01	4.13E+02	4.70E+02	3.07E+02	1.82E+02	1.05E+02
21	1.46E+02	3.12E+02	7.53E+01	1.77E+02	1.24E+02	7.15E+01
28	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 8: Survival of *E. coli* (16 t/ha) from pot trial replicates**

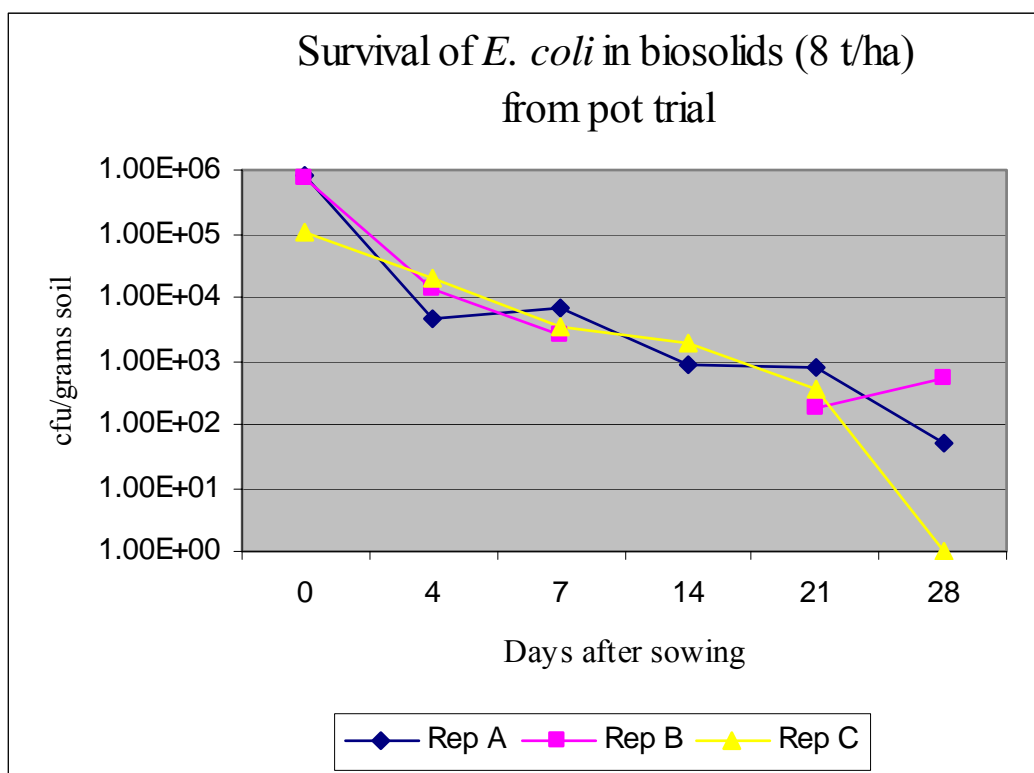


**Table A 14: *E. coli* survival from the pot trial (8 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams) at 8 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	8.08E+05	7.73E+05	1.05E+05	5.62E+05	3.92E+05	2.26E+05
4	4.58E+03	1.32E+04	1.99E+04	1.26E+04	6.83E+03	3.94E+03
7	6.46E+03	2.53E+03	3.53E+03	4.17E+03	2.29E+03	1.32E+03
14	8.38E+02	0.00E+00	1.89E+03	9.08E+02	6.79E+02	3.92E+02
21	7.85E+02	1.84E+02	3.58E+02	4.42E+02	2.99E+02	1.73E+02
28	5.27E+01	5.12E+02	0.00E+00	1.88E+02	2.82E+02	1.63E+02

**Figure A 9: Survival of *E. coli* (8 t/ha) from pot trial replicates**

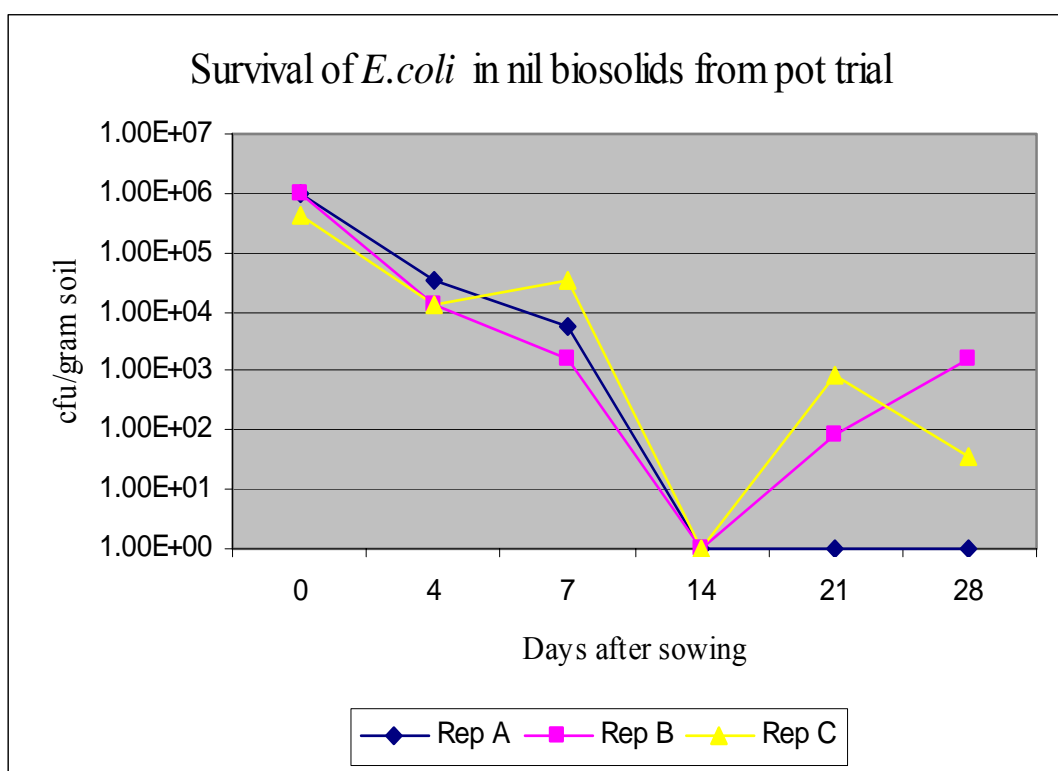


**Table A 15: *E. coli* survival from the pot trial (nil biosolids)**

**POT TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams) at nil biosolids**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	9.45E+05	1.03E+06	4.38E+05	8.06E+05	4.77E+05	2.75E+05
4	3.21E+04	1.38E+04	1.25E+04	1.95E+04	1.39E+04	8.00E+03
7	5.89E+03	1.54E+03	3.54E+04	1.43E+04	1.40E+04	8.10E+03
14	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.54E+02	2.05E+02
21	0.00E+00	7.96E+01	8.80E+02	3.20E+02	6.46E+02	3.73E+02
28	0.00E+00	1.54E+03	3.46E+01	5.26E+02	8.81E+02	5.08E+02

**Figure A 10: Survival of *E. coli* (nil biosolids) from pot trial replicates**

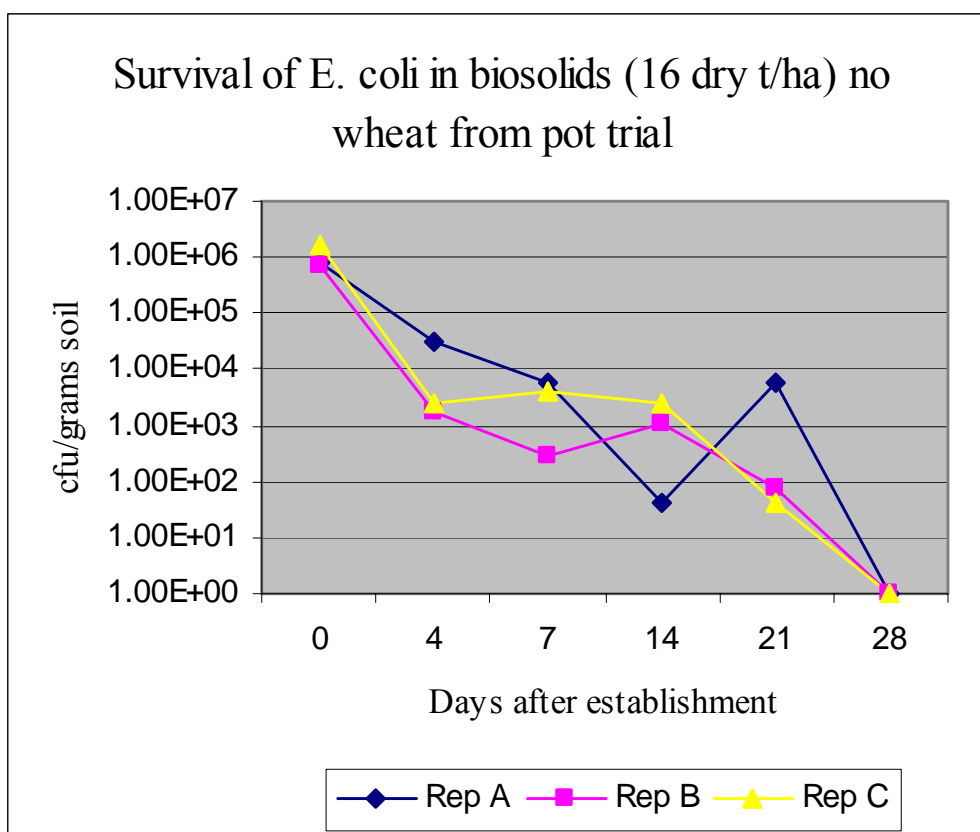


**Table A 16: *E. coli* survival from the pot trial (no wheat)**

**POT TRIAL DATA SUMMARY**  
***E. coli* (cfu/grams) at no wheat (16 dry t/ha equiv.)**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	7.93E+05	6.87E+05	1.65E+06	1.04E+06	6.57E+05	3.79E+05
4	2.96E+04	1.66E+03	2.47E+03	1.12E+04	1.11E+04	6.39E+03
7	5.68E+03	2.74E+02	4.14E+03	3.36E+03	2.26E+03	1.31E+03
14	4.23E+01	1.04E+03	2.44E+03	1.17E+03	2.26E+03	1.30E+03
21	5.76E+03	8.03E+01	3.99E+01	1.96E+03	2.34E+03	1.35E+03
28	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 11: Survival of *E. coli* (16 t/ha) no wheat from pot trial replicates**

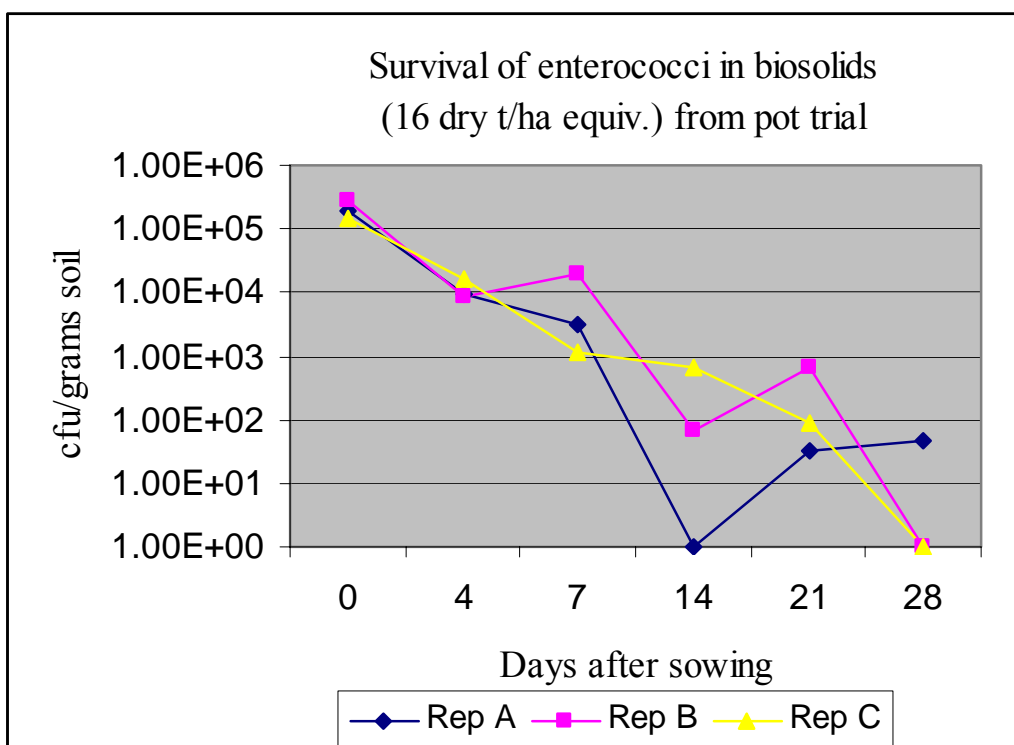


**Table A 17: Enterococci survival from the pot trial (16 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
**Enterococci (cfu/grams) at 16 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	1.85E+05	2.70E+05	1.44E+05	2.00E+05	1.11E+05	6.39E+04
4	9.60E+03	8.60E+03	1.70E+04	1.17E+04	7.46E+03	4.31E+03
7	3.09E+03	2.01E+04	1.18E+03	8.11E+03	7.87E+03	4.54E+03
14	9.90E-01	6.86E+01	6.40E+02	2.37E+02	3.20E+02	1.85E+02
21	3.30E+01	6.89E+02	8.95E+01	2.71E+02	2.69E+02	1.56E+02
28	4.83E+01	0.00E+00	0.00E+00	1.61E+01	2.79E+01	1.61E+01

**Figure A 12: Survival of enterococci (16 t/ha) from pot trial replicates**

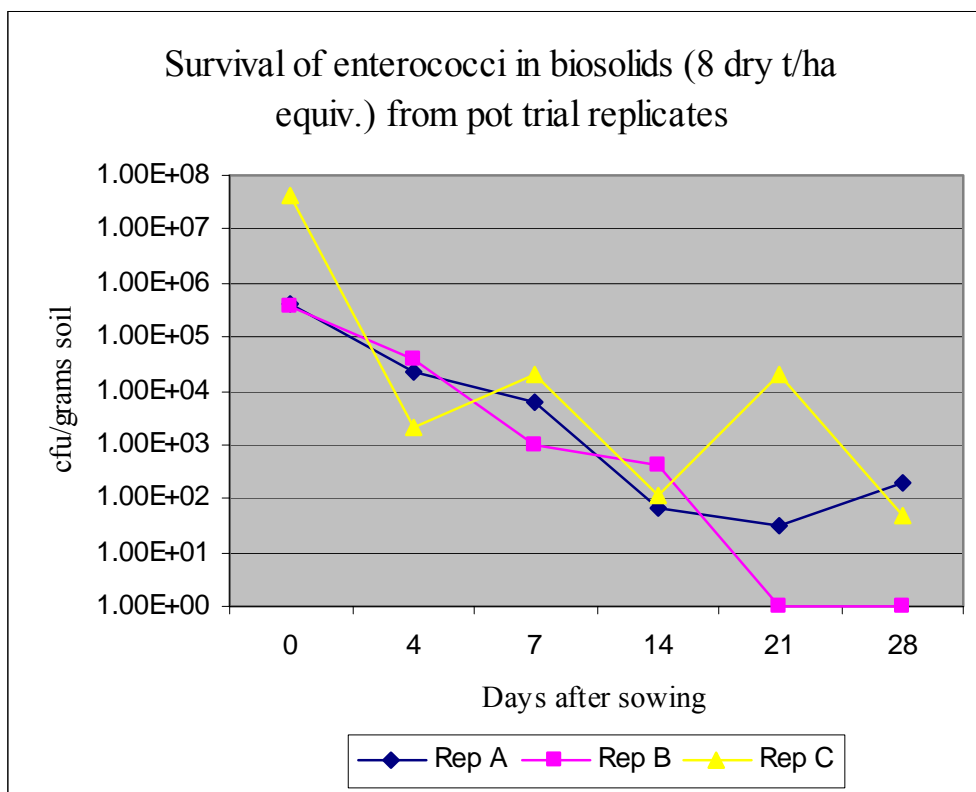


**Table A 18: Enterococci survival from the pot trial (8 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
**Enterococci (cfu/grams) at 8 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	4.03E+05	3.79E+05	4.12E+07	1.40E+07	1.68E+07	9.68E+06
4	2.36E+04	4.02E+04	1.99E+03	2.19E+04	1.53E+04	8.82E+03
7	6.31E+03	1.00E+03	1.93E+04	8.87E+03	7.61E+03	4.40E+03
14	6.44E+01	4.07E+02	1.13E+02	1.95E+02	8.30E+03	4.79E+03
21	3.08E+01	0.00E+00	2.04E+04	6.82E+03	8.32E+03	4.80E+03
28	1.97E+02	0.00E+00	5.00E+01	8.25E+01	1.03E+02	5.93E+01

**Figure A 13: Survival of enterococci (8 t/ha) from pot trial replicates**

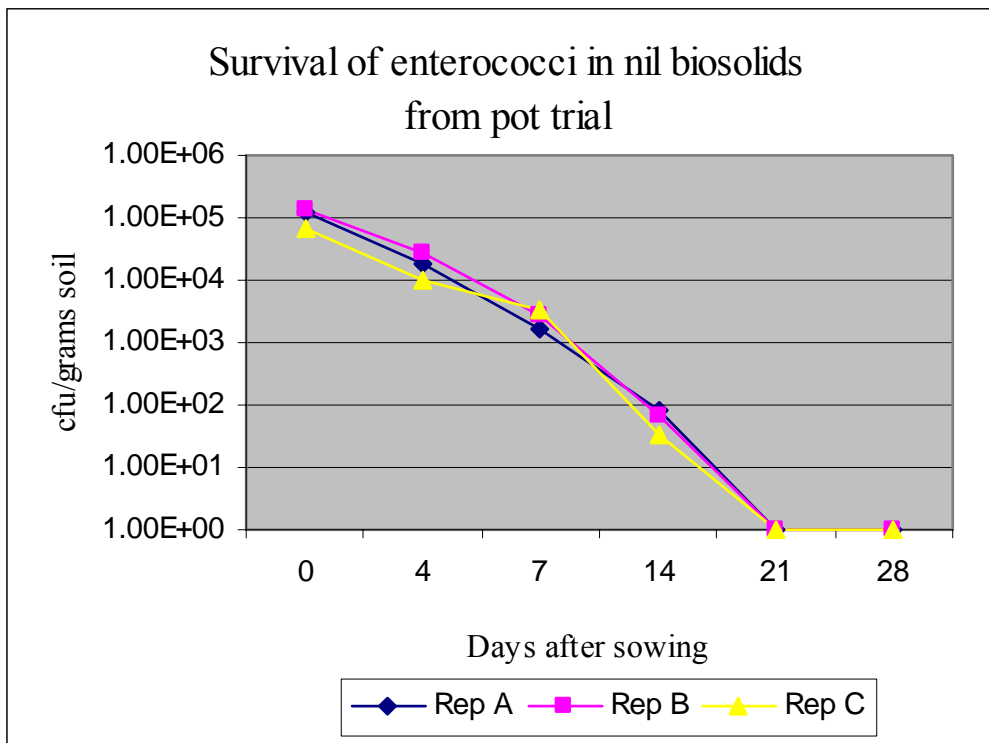


**Table A 19: Enterococci survival from the pot trial (nil biosolids)**

**POT TRIAL DATA SUMMARY**  
**Enterococci (cfu/grams) at nil biosolids**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	1.21E+05	1.29E+05	6.46E+04	1.05E+05	5.25E+04	3.03E+04
4	1.84E+04	2.77E+04	9.88E+03	1.86E+04	1.05E+04	6.06E+03
7	1.58E+03	2.64E+03	3.29E+03	2.50E+03	1.44E+03	8.34E+02
14	8.36E+01	6.78E+01	3.46E+01	6.20E+01	3.75E+01	2.16E+01
21	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
28	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 14: Survival of enterococci (nil biosolids) from pot trial replicates**



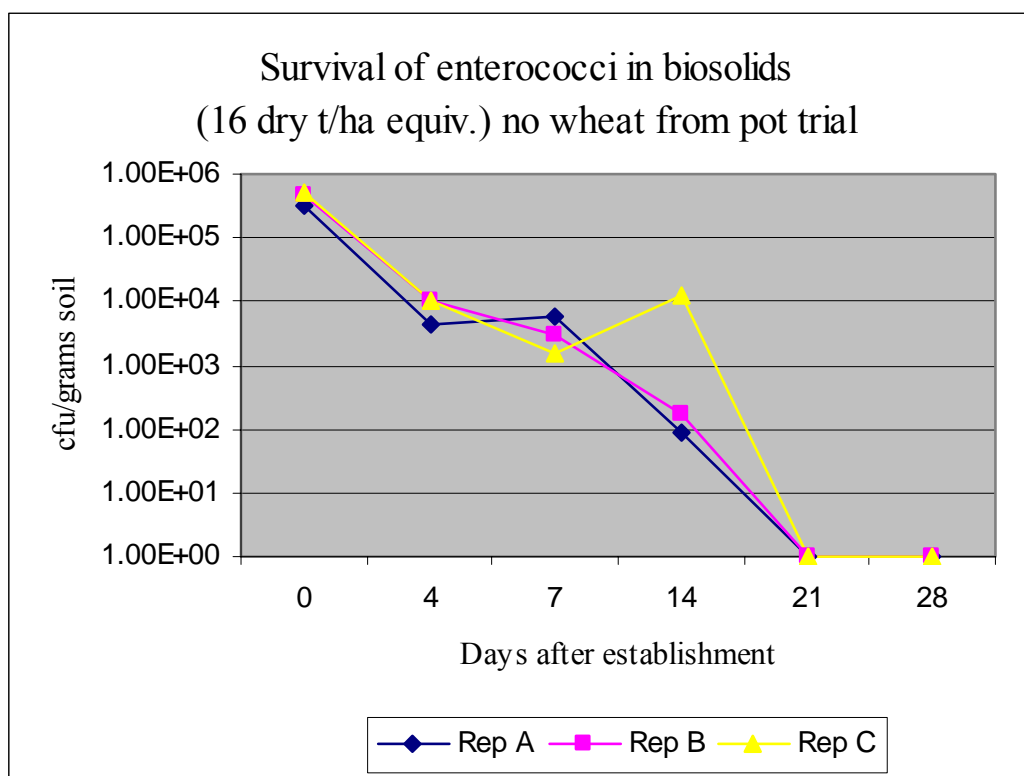
**Table A 20: Enterococci survival from the pot trial (no wheat)**

**POT TRIAL DATA SUMMARY**

**Enterococci (cfu/grams) at no wheat (16 dry t/ha equiv.)**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	3.17E+05	4.89E+05	4.90E+05	4.32E+05	2.41E+05	1.39E+05
4	4.45E+03	1.01E+04	1.00E+04	8.19E+03	3.58E+03	2.07E+03
7	6.05E+03	2.96E+03	1.57E+03	3.53E+03	4.69E+03	2.71E+03
14	8.75E+01	1.66E+02	1.23E+04	4.18E+03	5.00E+03	2.89E+03
21	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
28	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

**Figure A 15: Survival of enterococci (16 t/ha) no wheat from pot trial replicates**

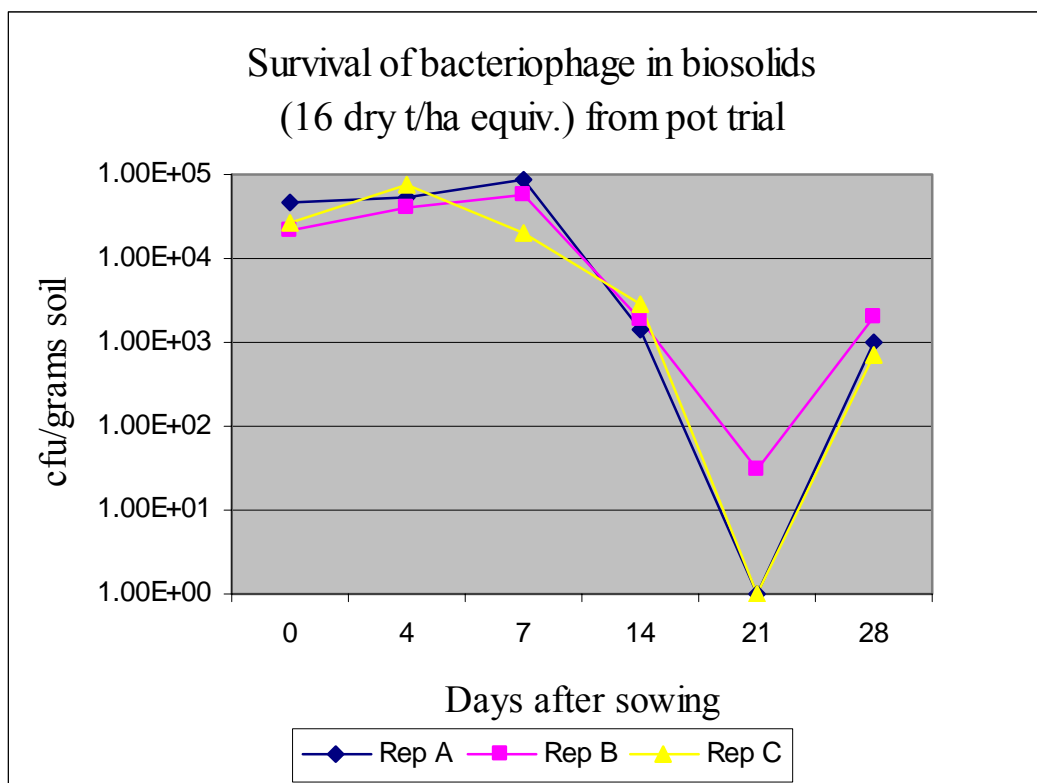


**Table A 21: Phage survival from the pot trial (16 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
**Phage plaques at 16 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	4.62E+04	2.15E+04	2.73E+04	3.17E+04	2.02E+04	1.16E+04
4	5.31E+04	4.01E+04	7.78E+04	5.70E+04	2.47E+04	1.43E+04
7	8.76E+04	5.62E+04	1.96E+04	5.45E+04	3.59E+04	2.07E+04
14	1.37E+03	1.86E+03	2.81E+03	2.01E+03	1.19E+03	6.88E+02
21	0.00E+00	3.12E+01	0.00E+00	1.04E+01	8.06E+02	4.66E+02
28	9.90E+02	2.03E+03	7.17E+02	1.25E+03	6.93E+02	4.00E+02

**Figure A 16: Survival of phage (16 t/ha) from pot trial replicates**

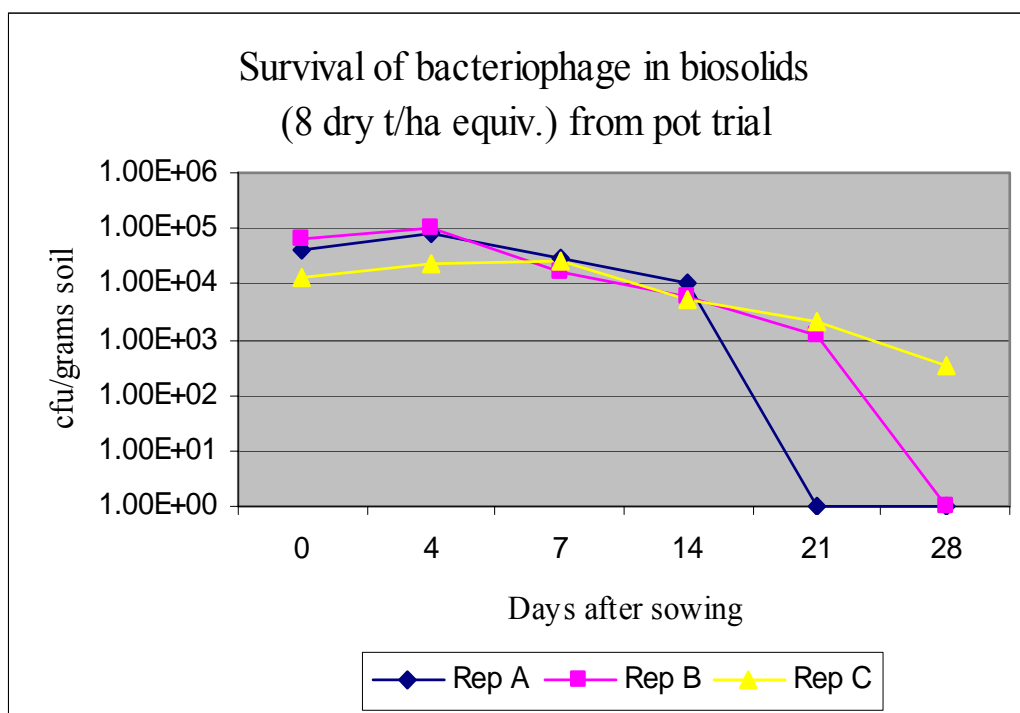


**Table A 22: Phage survival from the pot trial (8 dry t/ha)**

**POT TRIAL DATA SUMMARY**  
**Phage plaques at 8 dry t/ha equiv.**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	4.06E+04	6.67E+04	1.31E+04	4.01E+04	3.49E+04	2.01E+04
4	7.81E+04	1.04E+05	2.20E+04	6.79E+04	3.60E+04	2.08E+04
7	2.96E+04	1.64E+04	2.61E+04	2.40E+04	1.03E+04	5.94E+03
14	1.00E+04	6.17E+03	5.44E+03	7.21E+03	3.74E+03	2.16E+03
21	0.00E+00	1.24E+03	2.19E+03	1.14E+03	9.03E+02	5.22E+02
28	0.00E+00	0.00E+00	3.55E+02	1.18E+02	2.05E+02	1.18E+02

**Figure A 17: Survival of phage (8 t/ha) from pot trial replicates**

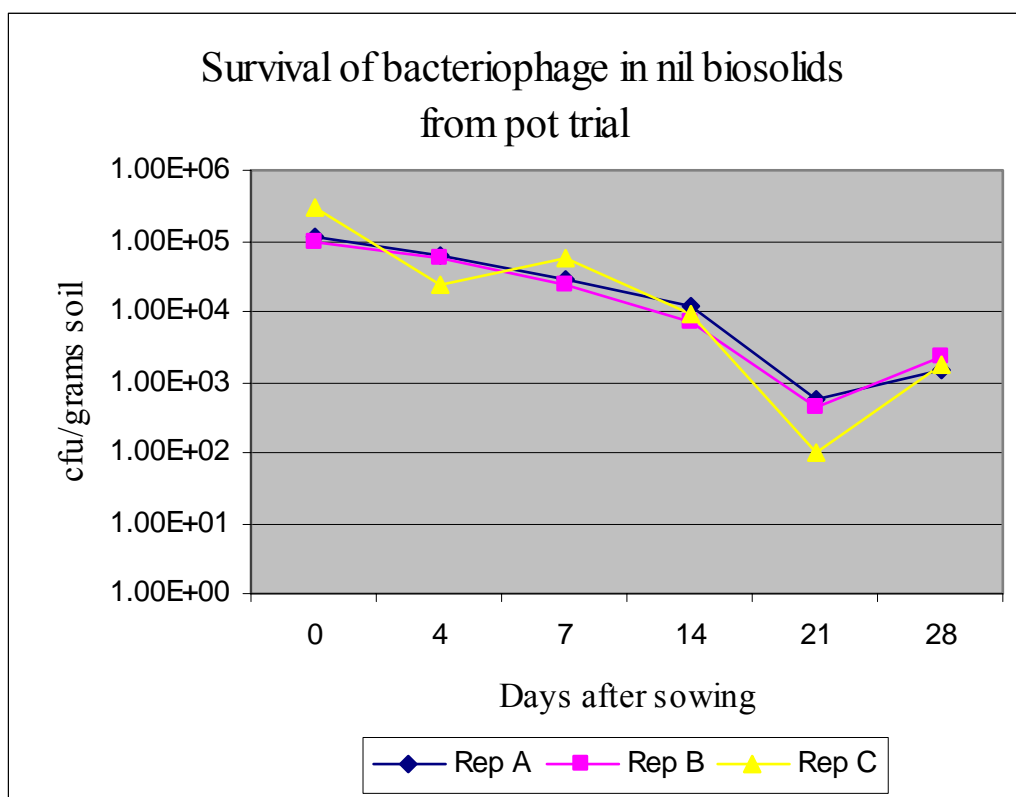


**Table A 23: Phage survival from the pot trial (nil biosolids)**

**POT TRIAL DATA SUMMARY**  
**Phage plaques at nil biosolids**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	1.16E+05	9.83E+04	2.86E+05	1.67E+05	9.32E+04	5.38E+04
4	6.31E+04	5.72E+04	2.40E+04	4.81E+04	1.83E+04	1.06E+04
7	2.83E+04	2.38E+04	5.48E+04	3.56E+04	1.79E+04	1.03E+04
14	1.20E+04	7.30E+03	9.20E+03	9.50E+03	5.21E+03	3.01E+03
21	5.91E+02	4.57E+02	9.65E+01	3.82E+02	8.44E+02	4.87E+02
28	1.48E+03	2.24E+03	1.77E+03	1.83E+03	3.85E+02	2.22E+02

**Figure A 18: Survival of phage (nil biosolids) from pot trial replicates**

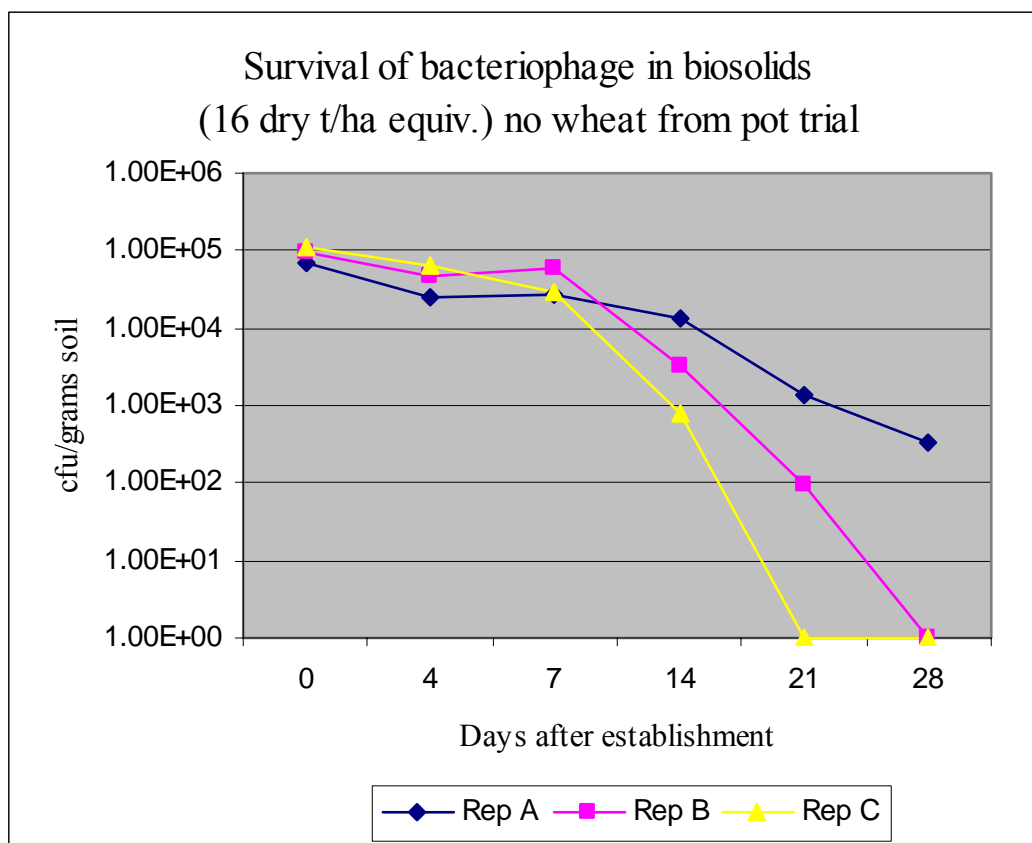


**Table A 24: Phage survival from the pot trial (no wheat)**

**POT TRIAL DATA SUMMARY**  
**Phage plaques at no wheat (16 dry t/ha equiv.)**

Day	Rep A	Rep B	Rep C	Mean	Std Dev	Std Error
0	6.73E+04	9.64E+04	1.14E+05	9.25E+04	3.24E+04	1.87E+04
4	2.44E+04	4.72E+04	6.58E+04	4.58E+04	1.73E+04	9.97E+03
7	2.81E+04	5.71E+04	2.89E+04	3.80E+04	2.09E+04	1.21E+04
14	1.34E+04	3.27E+03	7.93E+02	5.82E+03	5.15E+03	2.97E+03
21	1.42E+03	9.15E+01	0.00E+00	5.04E+02	5.60E+02	3.23E+02
28	3.28E+02	0.00E+00	0.00E+00	1.09E+02	1.89E+02	1.09E+02

**Figure A 19: Survival of phage (16 t/ha) no wheat from pot trial replicates**



**Table A 25: Presence of *E. coli*, enterococci and MS2 from nil (control) pot**

**POT TRIAL DATA SUMMARY**  
***E. coli*, enterococci and phage present in nil (control) pot**

Day	E.coli	Enterococci	Phage
0	1.00E+00	0.00E+00	0.00E+00
4	0.00E+00	0.00E+00	0.00E+00
7	0.00E+00	0.00E+00	0.00E+00
14	0.00E+00	0.00E+00	0.00E+00
21	0.00E+00	0.00E+00	0.00E+00
28	0.00E+00	0.00E+00	0.00E+00

**Table A 26: Conditions at sampling in the glasshouse for pot trial**

**GLASSHOUSE/POT TRIAL DATA**  
**Conditions at sampling**

Day	Soil temperature	G/house temperature	Time	Conditions
0	23.20	20.80	11.30am	Warm, clear skies
4	20.90	23.50	9.30am	Warm, sunny clear skies
7	22.70	27.80	11.10am	Warm, clear skies
14	18.90	20.00	9.00am	Overcast and stormy, rained overnight
21	20.70	20.00	9.30am	Overcast day, 8-13 leaves, 2-3 tillers
28	26.80	31.00	11.30am	Overcast day

**Table A 27: Excel data for enterococci from pot sampling (\*Day 4)**

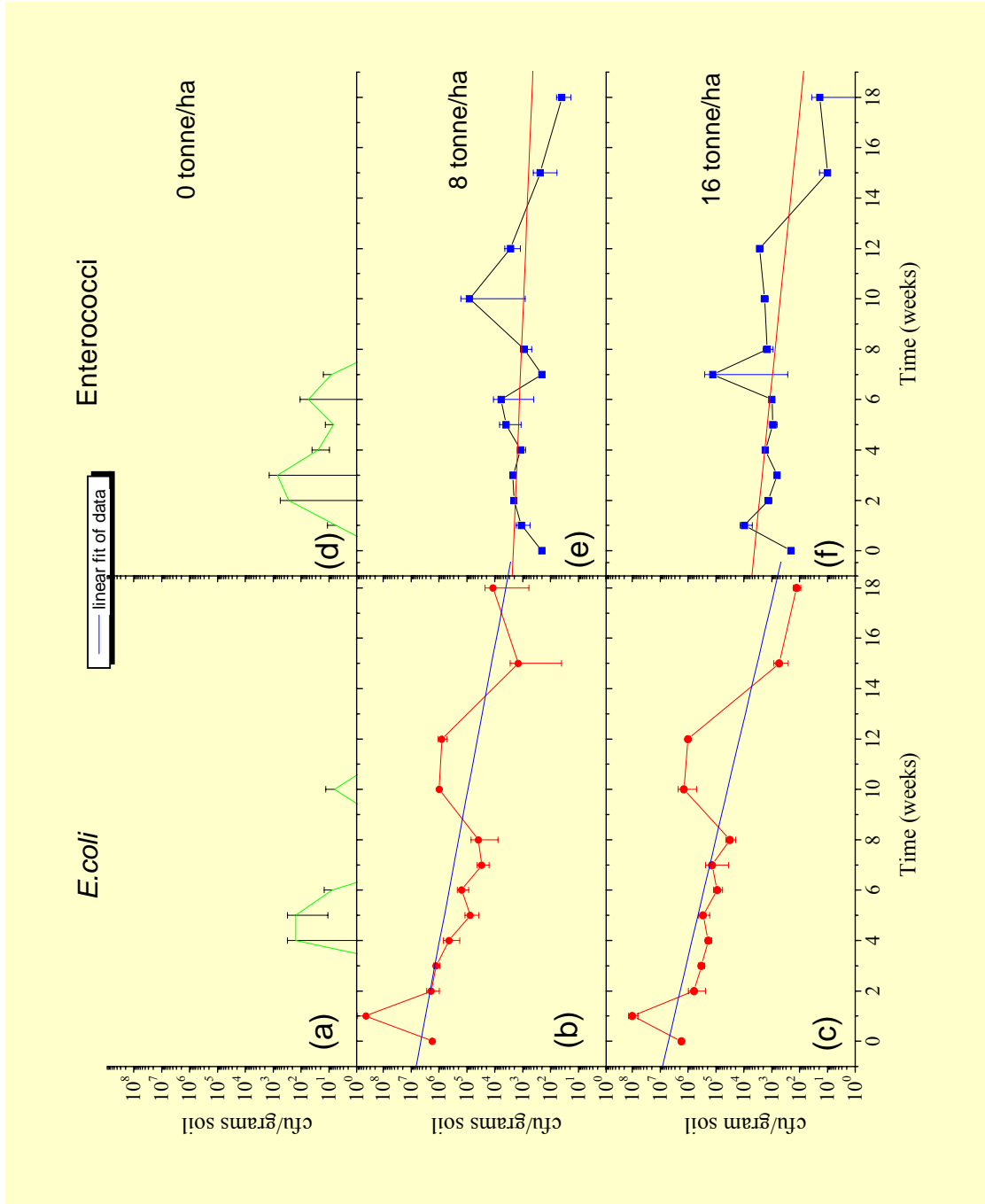
sample id	tube wt	total wt	sample wt	cfu counted	/mL	buffer	cfu / extract	dry wt	multiplication	cfu/g dryweight	average
P16A1	12.89	22.83	9.94	60.00	6.00E+03	15.00	9.00E+04	9.94	0.1006	9.05E+03	
P16A2	12.39	21.52	9.13	80.00	8.00E+03	15.00	1.20E+05	9.13	0.1095	1.31E+04	
P16A3	12.55	23.32	10.77	47.50	4.75E+03	15.00	7.13E+04	10.77	0.0929	6.62E+03	9.60E+03
P16B1	12.91	24.45	11.54	142.50	1.43E+04	15.00	2.14E+05	11.54	0.0867	1.85E+04	
P16B2	12.89	26.08	13.19	20.00	2.00E+03	15.00	3.00E+04	13.19	0.0758	2.27E+03	
P16B3	12.53	23.02	10.49	35.00	3.50E+03	15.00	5.25E+04	10.49	0.0953	5.00E+03	8.60E+03
P16C1	12.42	24.80	12.38	50.00	5.00E+03	15.00	7.50E+04	12.38	0.0808	6.06E+03	
P16C2	12.86	27.08	14.22	40.00	4.00E+03	15.00	6.00E+04	14.22	0.0703	4.22E+03	
P16C3	12.53	24.65	12.12	330.00	3.30E+04	15.00	4.95E+05	12.12	0.0825	4.08E+04	1.70E+04
P8A1	12.91	22.89	9.98	370.00	3.70E+04	15.00	5.55E+05	9.98	0.1002	5.56E+04	
P8A2	12.47	23.76	11.29	40.00	4.00E+03	15.00	6.00E+04	11.29	0.0886	5.31E+03	
P8A3	12.43	22.23	9.80	65.00	6.50E+03	15.00	9.75E+04	9.80	0.1020	9.95E+03	2.36E+04
P8B1	12.46	23.71	11.25	725.00	7.25E+04	15.00	1.09E+06	11.25	0.0889	9.67E+04	
P8B2	12.44	28.64	16.20	107.50	1.08E+04	15.00	1.61E+05	16.20	0.0617	9.95E+03	
P8B3	12.43	28.58	16.15	150.00	1.50E+04	15.00	2.25E+05	16.15	0.0619	1.39E+04	4.02E+04
P8C1	12.50	23.65	11.15	20.00	2.00E+03	15.00	3.00E+04	11.15	0.0897	2.69E+03	
P8C2	12.88	26.13	13.25	0.00	0.00E+00	15.00	0.00E+00	13.25	0.0755	0.00E+00	
P8C3	12.91	24.37	11.46	25.00	2.50E+03	15.00	3.75E+04	11.46	0.0873	3.27E+03	1.99E+03
P0A1	12.44	24.16	11.72	275.00	2.75E+04	15.00	4.13E+05	11.72	0.0853	3.52E+04	
P0A2	12.42	25.51	13.09	13.30	1.33E+03	15.00	2.00E+04	13.09	0.0764	1.52E+03	
P0A3	12.43	21.79	9.36	115.00	1.15E+04	15.00	1.73E+05	9.36	0.1068	1.84E+04	1.84E+04
P0B1	12.51	25.71	13.20	550.00	5.50E+04	15.00	8.25E+05	13.20	0.0758	6.25E+04	
P0B2	12.91	24.23	11.32	0.00	0.00E+00	15.00	0.00E+00	11.32	0.0883	0.00E+00	
P0B3	12.49	23.44	10.95	150.00	1.50E+04	15.00	2.25E+05	10.95	0.0913	2.05E+04	2.77E+04
P0C1	12.51	24.23	11.72	10.00	1.00E+03	15.00	1.50E+04	11.72	0.0853	1.28E+03	
P0C2	12.92	24.65	11.73	0.00	0.00E+00	15.00	0.00E+00	11.73	0.0853	0.00E+00	
P0C3	12.46	20.00	7.54	142.50	1.43E+04	15.00	2.14E+05	7.54	0.1326	2.83E+04	9.88E+03
NWA1	12.53	26.20	13.67	85.00	8.50E+03	15.00	1.28E+05	13.67	0.0732	9.33E+03	
NWA2	12.45	26.93	14.48	13.30	1.33E+03	15.00	2.00E+04	14.48	0.0691	1.38E+03	
NWA3	12.46	26.62	14.16	25.00	2.50E+03	15.00	3.75E+04	14.16	0.0706	2.65E+03	4.45E+03
NWB1	12.43	25.10	12.67	20.00	2.00E+03	15.00	3.00E+04	12.67	0.0789	2.37E+03	
NWB2	12.39	27.08	14.69	147.50	1.48E+04	15.00	2.21E+05	14.69	0.0681	1.51E+04	
NWB3	12.52	26.52	14.00	120.00	1.20E+04	15.00	1.80E+05	14.00	0.0714	1.29E+04	1.01E+04
NWC1	12.54	27.54	15.00	82.50	8.25E+03	15.00	1.24E+05	15.00	0.0667	8.25E+03	
NWC2	12.93	27.72	14.79	57.50	5.75E+03	15.00	8.63E+04	14.79	0.0676	5.83E+03	
NWC3	12.55	25.21	12.66	135.00	1.35E+04	15.00	2.03E+05	12.66	0.0790	1.60E+04	1.00E+04
O1	12.54	25.65	13.11	0.00	0.00E+00	15.00	0.00E+00	13.11	0.0763	0.00E+00	
O2	12.90	25.09	12.19	0.00	0.00E+00	15.00	0.00E+00	12.19	0.0820	0.00E+00	
O3	12.46	24.92	12.46	0.00	0.00E+00	15.00	0.00E+00	12.46	0.0803	0.00E+00	0.00E+00

*\*Excel spreadsheets were produced for sampling days 0, 4, 7, 21 & 28 and are available as Pot Trial.xls data on the CD-ROM Data Files attached.*

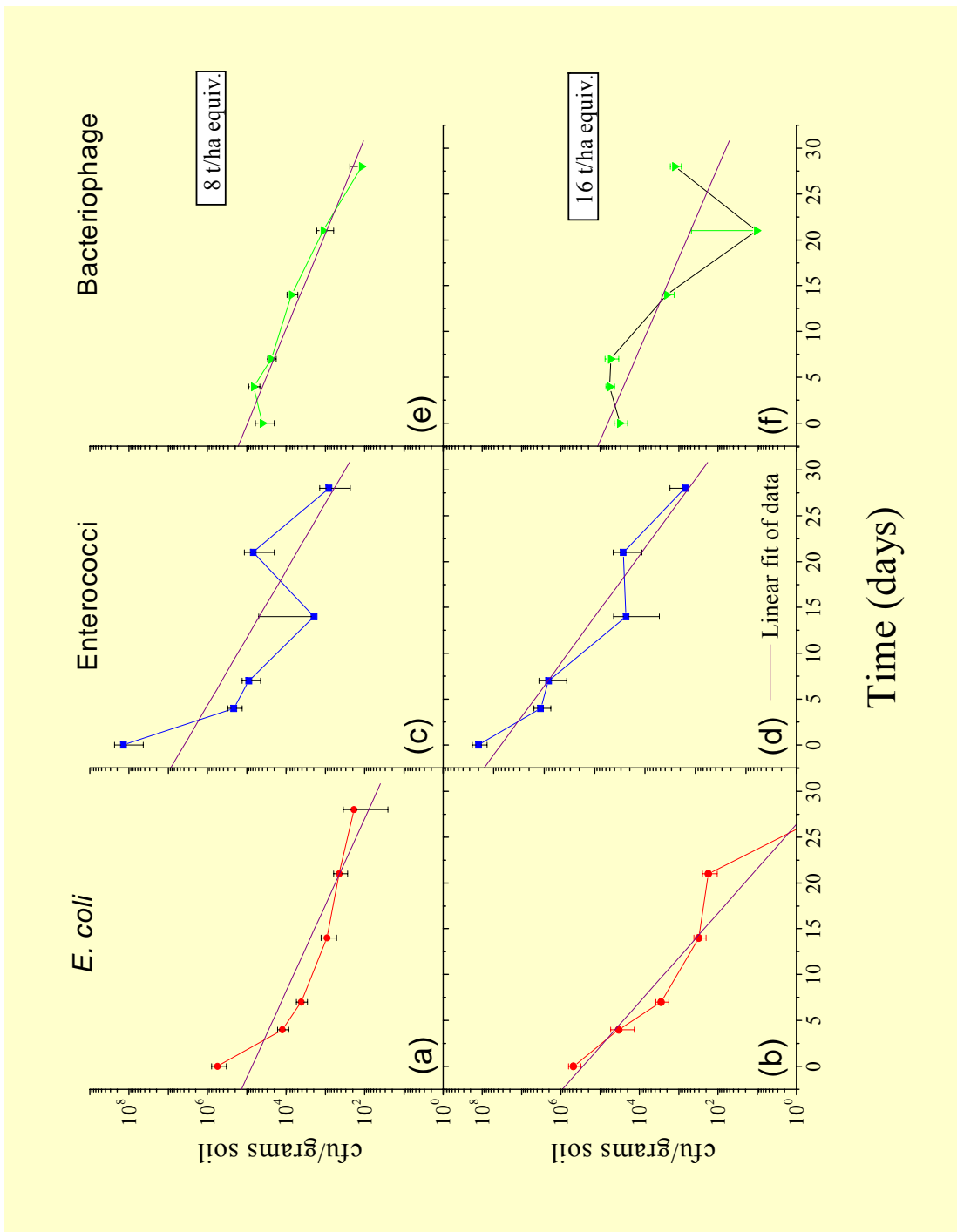
**Table A 28: Significance levels (ANOVA) between treatments in pot trial**

<b>ANOVA between Treatments – Pot Trial</b>						
Day	<i>E. coli</i>		Enterococci		MS2	
	F value	Sig. (P=0.05)	F value	Sig. (P=0.05)	F value	Sig. (P=0.05)
0	1.316	0.335	1.022	0.433	3.803	0.058
4	0.459	0.719	0.992	0.444	0.408	0.752
7	1.003	0.440	0.608	0.629	1.073	0.413
14	1.450	0.299	0.980	0.449	2.083	0.181
21	0.740	0.557	0.979	0.450	1.402	0.311
28	0.862	0.499	1.639	0.256	-	-

Figure A 20: Decay rates of indicator micro-organisms in the field trial



**Figure A 21: Decay rates of indicator micro-organisms in the pot trial (8 and 16 t/ha)**



**Figure A 22: Decay rates of indicator micro-organisms in the pot trial for nil pots and no wheat (16 t/ha)**

