PATHOGEN REGROWTH IN COMPOSTED BIOSOLIDS

A report on research project AS 55078 carried out by Murdoch University for the Water Corporation.

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EXECUTIVE SUMMARY

Biosolids originating from wastewater treatment plants contain many human pathogens, including *Salmonella*, which may present a health hazard to the general public. Composting is commonly used as an effective means of stabilising wastewater biosolids and reducing pathogens to very low levels. However, it has been shown that under certain conditions *Salmonella* can regrow in previously composted biosolids. The main aim of the research was to assess the importance of indigenous microorganisms in the suppression of *Salmonella* regrowth in composted biosolids. In addition, the effect of compost maturity on the pathogen regrowth potential was evaluated.

In this study *Salmonella* inactivation in a full-scale windrow composting process was evaluated. The results of this study suggest that if composting is carried out in accordance with the recommended guidelines (ARMCANZ) then *Salmonella* should be reduced below the detection limit within five weeks of composting. However, *Salmonella* regrowth in stored biosolids coinciding with rainfall after a dry spell, suggested that *Salmonella* can survive the composting process in low numbers and can regrow in the presence of favourable growth conditions. Under the current ARMCANZ guidelines (ARMCANZ et al. 1997) monitoring for thermotolerant coliforms or *Salmonella* in finished compost is recommended. It is suggested that monitoring for thermotolerant coliforms alone would not be sufficient to ensure the absence for *Salmonella*. It is recommended that monitoring of *Salmonella* in all the composted biosolids prior to release for public use should be included in guidelines.
A pathogen regrowth potential test using antibiotic-resistant \textit{S. typhimurium} was developed to evaluate the pathogen regrowth potential and the bio-safety of composted biosolids products. Pathogen regrowth potential is a very valuable tool for laboratory investigation. However, it is not recommended that a requirement to carry out a regrowth potential test should be included in guidelines.

It was found that sufficient bio-available nutrients to support \textit{Salmonella} growth were present in all the composted biosolids samples tested. This includes freshly mixed compost and composted biosolids stored for two years. Contrary to the general belief that storage of biosolids after composting reduces the pathogen regrowth potential, it was found that under composted biosolids had one of the lowest regrowth potential. The inactivation rate of \textit{Salmonella} was seven times higher in biosolids that had been composted for two weeks as compared to composted biosolids stored for two years.

Indigenous microorganisms were the single most important factor which controlled regrowth of \textit{Salmonella} in composted biosolids. The regrowth suppression activity was found to decline with the storage of composted biosolids. Consequently, composting and storage practices should be designed so that they result in a healthy build up and maintenance of the indigenous microbial population. Lengthy storage of composted biosolids is not recommended. However, to reduce the risk associated with \textit{Giardia}, a storage time of approximately 30 weeks following composting is recommended.
Screening of the isolated microorganisms showed that none of them produced secondary metabolites active against *Salmonella*. Limited suppression of growth was observed in the presence of some of the isolated microorganisms. Somatic *Salmonella* (SS) bacteriophages were found to survive in composted biosolids for up to two years. SS phages played a role in the suppression of *Salmonella* regrowth. It is postulated that *Salmonella* growth suppression in compost is due to intense competition for limited nutrients in the presence of biologically active indigenous microorganisms, with some anti-*Salmonella* activity from SS phages. Additional research work should be carried out to improve understanding of the nature of microbial antagonism towards *Salmonella*.

Further research should be carried out to identify the groups of indigenous microorganisms that suppress *Salmonella* regrowth. Resolving this issue could provide a better understanding of the antagonistic effect of indigenous microflora towards pathogenic bacteria in composted biosolids. Additional research is also needed for an improved understanding of optimum composting practices and storage conditions, which result in production and maintenance of a biologically active indigenous microflora. The effect of limiting moisture content and covering of compost piles on the development and maintenance of a taxonomically diverse indigenous microbial population should be investigated.

In order to minimise the health hazard associated with the use of composted biosolids hygienic gardening practices should be emphasised. Livestock wastes are known to contain many pathogenic microorganisms. It is recommended that research work should be carried out to evaluate the health hazard associated with the use of animal manure.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .......................................................................................................... i  
EXECUTIVE SUMMARY .................................................................................................... ii  
TABLE OF CONTENTS ....................................................................................................... v  
LIST OF FIGURES .............................................................................................................. xii  
LIST OF TABLES ................................................................................................................ xv  
LIST OF ABBREVIATIONS .............................................................................................. xvii  

CHAPTER 1: INTRODUCTION ...................................................................................... 1  
  1.1. Biosolids production .................................................................................................. 1  
  1.1.1. Biosolids disposal options .................................................................................... 1  
  1.1.2. Composting and use of composted biosolids ....................................................... 2  
  1.1.3. Risks associated with the use of composted biosolids ....................................... 3  
  1.2. Objectives and scope of research ............................................................................ 4  

CHAPTER 2: LITERATURE REVIEW ........................................................................... 6  
  2.1. Wastewater biosolids ............................................................................................... 6  
  2.1.1. Pathogens in wastewater biosolids ...................................................................... 7  
  2.1.2. Survival of pathogens in biosolids ...................................................................... 9  
  2.1.3. Pathogens of concern and extent of problem ................................................... 12  
  2.2. Finished product standards and regulation ............................................................. 15  
  2.3. Composting process ............................................................................................... 17  
  2.3.1. Definition and basic concept ............................................................................. 17
2.3.2. Wastewater biosolids composting .................................................................20
2.3.3. Microbial ecology and community dynamics ..................................................21
  2.3.3.1. Bacteria ...................................................................................................25
  2.3.3.2. Actinomycetes .........................................................................................27
  2.3.3.3. Fungi .......................................................................................................28
2.4. Pathogens in composted biosolids .................................................................29
2.5. Factors affecting pathogen die-off during composting ......................................31
  2.5.1. Temperature ...............................................................................................32
  2.5.2. Competition for limited nutrients .................................................................34
  2.5.3. Dehydration ...............................................................................................35
  2.5.4. Ammonia toxicity .......................................................................................36
  2.5.5. Antibiotic compounds ................................................................................36
2.6. Correlation between faecal indicators and pathogen die-off ................................38
2.7. Survival of pathogenic bacteria in composted biosolids ....................................41
2.8. Pathogen regrowth in composted biosolids ......................................................43
  2.8.1. Factors affecting Salmonella regrowth in composted biosolids ..................45
    2.8.1.1. Moisture content ..................................................................................46
    2.8.1.2. Bio-available nutrients .........................................................................47
    2.8.1.3. Microbial competition and antagonism ................................................49
    2.8.1.4. Somatic Salmonella bacteriophage ......................................................51
    2.8.1.5. Temperature .........................................................................................54
    2.8.1.6. pH ........................................................................................................54
  2.8.2. Relationship between regrowth of pathogens and indicator bacteria ..........55
2.8.3. Pathogen regrowth potential of composted biosolids ........................................56

2.9. Conclusions ...........................................................................................................58

CHAPTER 3: GENERAL MATERIALS AND METHODS ..............................................60

3.1. Introduction ..........................................................................................................60

3.2. Composting facility ...............................................................................................60

3.3. Materials and Methods ..........................................................................................61

3.3.1. Sampling and storage method .........................................................................61

3.3.2. Sterilisation of samples ..................................................................................62

3.4. Chemical preparations ..........................................................................................63

3.4.1. Ringer's solution ............................................................................................63

3.4.2. Buffered peptone Water .................................................................................63

3.4.3. Rappaport Vassiliadis medium .......................................................................63

3.4.4. Xylose Deoxycholate agar ..............................................................................64

3.4.5. Water extract of compost ...............................................................................64

3.5. Analytical Methods ...............................................................................................65

3.5.1. Solid analysis .................................................................................................65

3.5.2. Total Organic Carbon ..................................................................................65

3.5.3. Total Organic Nitrogen ..................................................................................66

CHAPTER 4: ENUMERATION OF SALMONELLA IN COMPOSTED BIOSOLIDS

AND DEVELOPMENT OF A PATHOGEN REGROWTH POTENTIAL TEST ..........67

4.1. Introduction ..........................................................................................................67

4.2. Experimental approach .........................................................................................69

4.3. Materials and Methods ........................................................................................70
4.3.1. Media preparation ................................................................. 70
4.3.2. Solids analysis ............................................................... 70
4.3.3. Bacterial strains ............................................................... 70
4.3.4. Selection of antibiotic-resistant bacteria ......................... 71
4.3.5. Preparation of inoculum .................................................. 71
4.3.6. Regrowth monitoring ......................................................... 71
4.3.7. Regrowth potential test ..................................................... 72
4.3.8. Enumeration of *Salmonella* .............................................. 73
4.3.9. Enumeration of *E. coli* .................................................... 74
4.3.10. Statistical analysis .......................................................... 74
4.4. Result ..................................................................................... 74
4.4.1. *Salmonella* concentration in composted biosolids .......... 74
4.4.2. Re-producibility of growth curves .................................... 78
4.4.3. Growth of *Salmonella* serovars ..................................... 79
4.4.4. Growth of *E. coli* strains ................................................ 81
4.5. Discussion .............................................................................. 82
4.5.1. *Salmonella* concentration in composted biosolids ........... 82
4.5.2. Selection of a suitable indicator for a pathogen regrowth potential test ... 83

CHAPTER 5: *SALMONELLA* GROWTH IN STERILISED AND NON-STERILISED COMPOST ............................................. 86

5.1. Introduction ......................................................................... 86
5.2. Experimental design .......................................................... 88
5.3. Materials and Methods ....................................................... 89
5.3.1. Sample collection and storage ................................................................. 89
5.3.2. Bacterial strains used in the study........................................................ 89
5.3.3. Sterilisation of biosolids ................................................................. 89
5.3.4. Preparation of inoculum ...................................................................... 90
5.3.5. Regrowth potential test .................................................................. 90
5.3.6. Enumeration of *Salmonella* ............................................................. 90
5.3.7. TON and TOC analysis ................................................................... 91
5.3.8. Statistical analysis ......................................................................... 91
5.4. Results ................................................................................................. 92
5.4.1. Effect of autoclaving on regrowth potential test .............................. 92
5.4.2. *S. typhimurium* growth in sterilised biosolids ............................. 95
5.4.3. *S. typhimurium* growth in non-sterilised biosolids ..................... 97
5.5. Discussion ............................................................................................ 102
5.5.1. Role of bio-available nutrients in pathogen regrowth .................... 102
5.5.2. Role of indigenous microorganisms in suppression of *Salmonella* regrowth 103
5.5.3. Effect of storage on *Salmonella* regrowth potential .................... 104

CHAPTER 6: ROLE OF INDIGENOUS MICROORGANISMS IN SUPPRESSION OF *SALMONELLA* REGROWTH .......................................................... 107
6.1. Introduction ...................................................................................... 107
6.2. Experimental approach .................................................................. 109
6.3. Materials and Methods .................................................................. 110
6.3.1. Sample collection and storage .................................................... 110
6.3.2. Enumeration of indigenous microorganisms ............................... 110
6.3.3. Isolation of indigenous microorganisms from compost ................................ 111
6.3.4. Screening for antagonistic effect .................................................................. 112
6.3.5. Screening for antibiosis ................................................................................ 113
6.3.6. Bacterial host ............................................................................................... 114
6.3.7. Enumeration of somatic Salmonella phage in composted biosolids.......... 115
6.3.8. Plaque purification and host range specificity................................................ 116
6.3.9. Electron microscopy .................................................................................... 117
6.3.10. Preparation of SS phage cultures for seeding ............................................. 117
6.3.11. Regrowth potential test ............................................................................. 118
6.3.12. Statistical analysis ...................................................................................... 119
6.4. Result ................................................................................................................. 119
6.4.1. Enumeration of indigenous microorganisms in composted biosolids........ 119
6.4.2. Colony morphology and microscopic examination of isolated bacteria and
actinomycetes ......................................................................................................... 121
6.4.3. Agar assay for antagonists........................................................................... 123
6.4.4. Compost extract assay with individual bacteria ............................................ 124
6.4.5. Somatic Salmonella phage concentration in composted biosolids .......... 131
6.4.6. Phage morphology and plaque characteristics .............................................. 133
6.4.7. Host range specificity ................................................................................... 137
6.4.8. Effect of SS phage on Salmonella growth .................................................... 138
6.5. Discussion .......................................................................................................... 141
6.5.1. Enumeration of indigenous microorganisms ................................................. 142
6.5.2. Agar assay for antagonists............................................................................ 142
6.5.3. Compost extract assays with isolated bacteria .............................................. 143
6.5.4. SS phage concentration in composted biosolids ........................................... 145
6.5.5. Morphology and host specificity of SS phage ............................................... 147
6.5.6. Effect of SS phage on *Salmonella* growth in composted biosolids .......... 148

CHAPTER 7: GENERAL DISCUSSION ........................................................................ 150
7.1. Major factors affecting pathogen regrowth in composted biosolids ............... 150
7.2. *Salmonella* concentrations and regrowth in composted biosolids ............... 152
7.3. Current method of compost mixing and recommendations for compost handling 153
7.4. Pathogen regrowth in composted biosolids ..................................................... 155
7.5. Risk assessment of pathogen regrowth in composted biosolids .................. 156

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS .................................... 159
8.1. Conclusions ..................................................................................................... 159
8.2. Recommendations ......................................................................................... 160

REFERENCES .......................................................................................................... 162
LIST OF FIGURES

Figure 4.1. *Salmonella* concentrations in composted biosolids of different maturities. ....75

Figure 4.2. Average weekly rainfall recorded at Murdoch University during the storage of composted biosolids. .................................................................77

Figure 4.3. Replicate growth curves of *E. coli* and *S. typhimurium*. 78

Figure 4.4. Growth curves for seed *Salmonella* serotypes in composted biosolids ........79

Figure 4.5. Growth curves for seeded *E. coli* in sterile biosolids. .................................81

Figure 5.1. Growth of seeded *S. typhimurium* in irradiated and autoclaved composted biosolids of different maturity .......................................................93

Figure 5.2. Growth of seeded *S. typhimurium* in sterilised composted biosolids of different maturity .................................................................96

Figure 5.3. Growth of seeded *S. typhimurium* in non-sterilised composted biosolids of different maturity .................................................................98

Figure 5.4. Inactivation rate (k) of seeded *S. typhimurium* in composted biosolids of different maturity .................................................................101

Figure 6.1. Indigenous microbial concentrations in composted biosolids of different maturity .................................................................120

Figure 6.2. Growth curve of seeded *S. typhimurium* in sterilised water extract of composted biosolids .................................................................124

Figure 6.3. Growth curves of seeded *Salmonella* and OC bacteria in sterilised water extract of composted biosolids .................................................................125
Figure 6.4. Growth curve of seeded *Salmonella* in established population of OC bacteria................................................................................................................... 126

Figure 6.5. Growth curve of seeded *Salmonella* in established population of PC bacteria. 126

Figure 6.6. Growth curves of seeded *Salmonella* in established population of ES and BTCR bacteria................................................................................................................... 127

Figure 6.7. Growth curves of seeded *Salmonella* in established population of GYC and SRC bacteria........................................................................................................ 127

Figure 6.8. Growth curves of seeded *Salmonella* in established population of J1, J2 and J3 bacteria. .............................................................................................................. 128

Figure 6.9. Growth curves of seeded *Salmonella* in established population of J4, J5 and J6 bacteria. .............................................................................................................. 128

Figure 6.10. Growth curves of seeded *Salmonella* in established population of J7, J8 and J9 bacteria. .............................................................................................................. 129

Figure 6.11. Growth curves of seeded *Salmonella* in established population of J11, P1 and P4 bacteria. .............................................................................................................. 129

Figure 6.12. Growth response of *Salmonella* to added sucrose in the presence of OC bacteria...................................................................................................................130

Figure 6.13. Growth response of *Salmonella* to added sucrose in the presence of PC bacteria...................................................................................................................131

Figure 6.14. Somatic *Salmonella* phage concentrations in composted biosolids. .......... 132

Figure 6.15. TEM micrograph of Micro 1 isolated from composted biosolids. .......... 134

Figure 6.16. TEM micrograph of Myo 1 isolated from composted biosolids .......... 134

Figure 6.17. TEM micrograph of Myo 2 isolated from composted biosolids. .......... 135
Figure 6.18. TEM micrograph of Podo 1 isolated from composted biosolids..............135
Figure 6.19. TEM micrograph of Stylo 1 isolated from composted biosolids .............136
Figure 6.20. Growth curves of S. typhimurium in the presence of SS phage in composted biosolids of different maturity.................................................................139
LIST OF TABLES

Table 2.1. Pathogenic microorganisms found in wastewater sludge and composted biosolids.................................................................................................................... 8

Table 2.2. Average survival time of some human pathogens in wastewater and biosolids11

Table 2.3. Microbiological standards for biosolids .......................................................... 16

Table 2.4. Microorganisms isolated from composites ......................................................... 23

Table 2.5. Density of various microorganisms during aerobic composting ...................... 27

Table 2.6. Level of temperature and duration required to destroy some pathogens during composting ........................................................................................................... 33

Table 2.7. Regrowth of Salmonella/ E. coli in composted biosolids and biosolids amended soils ................................................................................................................. 44

Table 4.1. Regrowth of indigenous Salmonella in composted biosolids of different maturity after incubation at 37 °C for 48 h ...................................................................................... 76

Table 4.2. Solids analysis of composted biosolids of different maturity ........................... 76

Table 4.3. Bacterial growth kinetics ................................................................................ 80

Table 5.1. Growth rate equations of S. typhimurium seeded in sterilised composted biosolids of different maturity ............................................................................................ 94

Table 5.2. Total organic carbon and nitrogen in the composted biosolids of different maturity ................................................................................................................................. 95

Table 5.3. Inactivation rate equations of seeded S. typhimurium in sterilised composted biosolids of different maturity ......................................................................................... 97
**Table 5.4.** Growth rate equations of *S. typhimurium* seeded in composted biosolids of different maturity..................................................................................................................99

**Table 5.5.** Inactivation rate equations of *S. typhimurium* seeded in composted biosolids of different maturity..................................................................................................................100

**Table 6.1.** Colony morphologies and microscopic appearance of the bacteria isolated from the composted biosolids of different maturity..................................................................................122

**Table 6.2.** Colony morphologies and microscopic appearance of the actinomycetes isolated from the composted biosolids of different maturity..................................................................................123

**Table 6.3.** Particle and plaque descriptions of bacteriophage isolated from composted biosolids.................................................................................................................................137

**Table 6.4.** Host specificity of the five isolated somatic *Salmonella* bacteriophage.................................................................................................................................138

**Table 6.5.** Growth rate equations of *S. typhimurium* in sterilised composted biosolids of different maturity..................................................................................................................140

**Table 6.6.** Growth curves of *S. typhimurium* in the presence of SS phage in composted biosolids of different maturity..................................................................................................................140
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of compost</td>
<td>Maturity in weeks, counted from the beginning of composting process including storage time</td>
</tr>
<tr>
<td>AIA</td>
<td>Actinomycetes Isolation Agar</td>
</tr>
<tr>
<td>ARMCANZ</td>
<td>Agriculture and Resource Management Council of Australia and New Zealand</td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered Peptone Water</td>
</tr>
<tr>
<td>C/N</td>
<td>Carbon to Nitrogen ratio</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DLA</td>
<td>Double Layer Agar</td>
</tr>
<tr>
<td>DS</td>
<td>Dried Solids</td>
</tr>
<tr>
<td>FC</td>
<td>Faecal Coliforms</td>
</tr>
<tr>
<td>FS</td>
<td>Faecal Streptococci</td>
</tr>
<tr>
<td>g⁻¹</td>
<td>Per gram</td>
</tr>
<tr>
<td>h⁻¹</td>
<td>Per hour</td>
</tr>
<tr>
<td>KV</td>
<td>Kilo Volt</td>
</tr>
<tr>
<td>Log₁₀</td>
<td>Log ten</td>
</tr>
<tr>
<td>mL⁻¹</td>
<td>Per millilitre</td>
</tr>
<tr>
<td>MPN</td>
<td>Most Probable Number</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
<tr>
<td>PFRP</td>
<td>Process to Further Reduce Pathogens</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PSRP</td>
<td>Process to Significantly Reduce Pathogens</td>
</tr>
<tr>
<td>RV</td>
<td>Rapport Vassiliadis medium</td>
</tr>
<tr>
<td>SS phage</td>
<td>Somatic <em>Salmonella</em> phage</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TON</td>
<td>Total Organic Nitrogen</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic Soy Agar</td>
</tr>
<tr>
<td>TYGA</td>
<td>Tryptone Yeast Extract Glucose Agar</td>
</tr>
<tr>
<td>TYGB</td>
<td>Tryptone Yeast Extract Glucose Broth</td>
</tr>
<tr>
<td>US EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile Solids</td>
</tr>
<tr>
<td>XLD</td>
<td>Xylose Deoxycholate agar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µ mL</td>
<td>Micro millilitre</td>
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1.1. Biosolids production

Organic waste solids are produced by wastewater treatment processes. When the solids are stabilised and can be used beneficially, they are referred to as biosolids. The disposal of biosolids generated during wastewater treatment is a growing problem in Australia (Varjavandi 1991) and worldwide (Yanko 1988). Biosolids production in Australia during 1996-97 was estimated to be 203,000 dry tones (Carpenter 1998) and is expected to increase with time. The proper management of biosolids is essential to protect public health and prevent contamination of the environment.

1.1.1. Biosolids disposal options

Biosolids disposal options include ocean disposal, landfilling, mine-site rehabilitation and use as a soil amendment. Due to the potential for public health hazard and environmental contamination, ocean disposal has been stopped in many part of the world. Ocean disposal of biosolids is illegal in the United States and Europe (MacKenzie 1998). Landfilling is difficult in Australia, due to the scarcity of urban landfilling sites (ARMCANZ et al. 1997). Incineration is very expensive and its use is restricted by stringent air pollution regulations (Soares 1996).
Use of biosolids, as a soil amendment is another disposal option. The primary objective of an effective wastewater sludge management option is to encourage the beneficial use of sludge and resource recovery (ARMCANZ et al. 1997). Recycling of wastewater sludge as biosolids is a beneficial and environmentally sustainable sludge disposal option. Biosolids contain organic matter and plant nutrients, and hence can be used as a soil conditioner and medium grade fertiliser on agricultural land (Soares et al. 1992). However, biosolids may contain high levels of metals, harmful chemicals and pathogenic microorganisms. As a consequence, practices that reduce public health risks associated with the use of biosolids and ensure resource recovery are gaining popularity. Composting is one of the options used to reduce the public health hazard associated with biosolids.

1.1.2. Composting and use of composted biosolids

The objective of wastewater sludge composting is to biologically convert putrescible organic matter into a stable form and inactivate human pathogens. Composting can also destroy plant pathogens, weed seeds and insect eggs. A number of composting processes such as open windrow, static pile and in-vessel composting can be used to produce good quality product, in a cost-effective manner. Due to technological advances in the composting process, the use of composting to produce marketable products is increasing. The finished product should be aesthetically acceptable, free of pathogens and easy to handle. The current ARMCANZ (ARMCANZ et al. 1997) and USEPA guidelines (US
EPA 1989) address quality aspects such as human pathogen and heavy metal concentrations in biosolids.

When applied to agricultural land, composted biosolids can improve the physical, chemical and biological properties of the soil. Compost is also a source of plant nutrients such as nitrogen, phosphorus and trace elements, which are essential for plant growth. Compost has numerous uses such as in agriculture, horticulture, forestry and mine-site reclamation. Compost based soil amendments are also be used in green houses, gardening and landscaping.

1.1.3. Risks associated with the use of composted biosolids

Although recycling of biosolids as soil amendments has its advantages, there are certain impediments to its wide spread use. Biosolids may contain a wide range of human pathogens including bacteria, viruses, protozoa and parasites (Fradkin et al. 1985; Gibbs et al. 1993) and chemical contaminants. Re-use of improperly treated biosolids can potentially threaten public and environmental health.

Composting can effectively stabilise wastewater sludge and reduce pathogens to low levels (Millner et al. 1987; Deportes et al. 1998). However regrowth of Salmonella in soil amendments and stored biosolids under certain conditions (Burge et al. 1987; Skanvis and Yanko 1994; Gibbs et al. 1997) is of concern. Salmonella and Giardia appear to be the pathogens of major concern in the utilisation of wastewater biosolids.
Composted biosolids were found to contain approximately 1000 times higher Giardia cysts than a level considered to be safe (McInnes et al. 1997). Consequently, it is important to examine the practice of biosolids application to land from a public health standpoint. The conditions, which lead to the survival and regrowth of pathogens, need further investigation.

1.2. Objectives and scope of research

Regrowth of bacterial pathogens such as Salmonella, toxigenic E. coli and Shigella in composted biosolids is a major concern in the utilisation of composted biosolids. Pathogen regrowth in composted biosolids is influenced by inherited properties of the compost and environmental factors, which are highly interdependent. To date, limited information is available on the role of indigenous microorganisms in the suppression of pathogen regrowth. Moreover, there is no satisfactory way of predicting the pathogen regrowth potential of biosolids and the extent of public health hazard linked to it.

Although a large number of enteric bacteria are found in composted biosolids, the scope of this research was restricted to Salmonella regrowth in compost. The investigation of the role of indigenous microbes in inhibition of pathogen regrowth was also limited to the culturable bacteria and actinomycetes. The main objectives of this project are listed below:
1. To determine which are likely to be the factors of major importance when considering pathogen regrowth in anaerobically digested and composted sludge.

2. To assess the occurrence of pathogen regrowth in WA sludge based soil amendment products. *Salmonella* is of particular interest.

3. To assess current methods of soil mixing, and to make recommendations for future practices, based on an understanding of the factors affecting pathogen regrowth.

4. To develop a reliable approach to be used for assessing the regrowth potential of sludge based products.

5. To carry out a risk assessment of pathogen regrowth in sludge based soil amendment products, based on observation concerning existing pathogen levels in marketed products.
CHAPTER 2: LITERATURE REVIEW

In this Chapter the pathogens, of concern the extent of the problem and the role of composting in inactivation of pathogens in wastewater biosolids is reviewed. Factors affecting survival and regrowth of enteric bacteria are also reviewed.

2.1. Wastewater biosolids

In addition to human pathogens, municipal wastewater biosolids also contains chemicals and metal salts from industries. However, the public health risk associated with the chemical toxicants in biosolids is generally much lower than for the risk from microbial infections (ARMCANZ et al. 1997). If wastewater biosolids are left untreated, the organic matter of the wastewater sludge can be putrefied in nature by indigenous microorganisms and this creates odour problem and attracts many vectors.

Uncontrolled disposal of wastewater sludge can create a potential public health hazard. Consequently, proper treatment of wastewater sludge to stabilise organic matter and reduce pathogen concentrations is required prior to disposal. Moreover to encourage the beneficial use of biosolids, product quality is also crucial. A number of technologies such as lime stabilisation, anaerobic digestion, heat-treatment and composting are used to reduce pathogens in wastewater sludge.
2.1.1. Pathogens in wastewater biosolids

As municipal wastewater contains human waste, numerous pathogens can find their way into sludge. Microorganisms of public health concern present in the wastewater can be classified into four major groups: viruses, bacteria, protozoa and helminths (Epstein 1987). The concentration of pathogens in wastewater is highly variable and influenced by the prevailing infection rate in a community (Gibbs et al. 1994) and seasonal change (Soares et al. 1992). The pathogen concentration in sewage sludge depends on the nature of incoming wastewater and also on the ability of sewage treatment process to reduce pathogens (Nell et al. 1983). Another factor is the excretion ration for different pathogens. Infected individuals excrete large number of viral \((10^6)\) and bacterial \((10^8)\) pathogens as compared to protozoan and parasite eggs (Feachem et al. 1983). It is highly likely that the pathogens become concentrated in the raw sludge produced by separation of solids from liquid waste (Fradkin et al. 1989).

Due to high concentration of pathogens in untreated wastewater, a number of processes are used to sanitise biosolids. The degree of sanitation depends upon the intended use of biosolids. These processes used for reducing pathogens in wastewater sludge are divided into two main categories (US EPA 1992). Firstly, Processes to Significantly Reduce Pathogens (PSRP) and secondly, Processes to Further Reduce Pathogens (PFRP). The significance of these processes in wastewater biosolids treatment is discussed in section 2.2. Complete sterilisation of biosolids is difficult to achieve and is undesirable due to the limited potential of health hazard posed by surviving pathogens.
Some of the significant pathogens found in wastewater biosolids and composted biosolids are listed in Table 2.1. Not all of the pathogens listed on Table 2.1 are found in Australian sludges.

**Table 2.1.** Pathogenic microorganisms found in wastewater biosolids and composted biosolids (adapted from Nell *et al.* 1983; De Bertoldi *et al.* 1988; Deportes *et al.* 1995; Deporters *et al.* 1998).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Bacteria</th>
<th>Helminths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrovirus</td>
<td>Arizona hinshawii</td>
<td>Ankyoma duodenale</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>Bacillus cereus</td>
<td>Ascaris lumbricoides</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Bacillus anthracis</td>
<td>Enterobium vermicularis</td>
</tr>
<tr>
<td>Reovirus</td>
<td>Citrobacter spp.</td>
<td>Echinococcus granulosus</td>
</tr>
<tr>
<td>Parovirus</td>
<td>Clostridium botulinum</td>
<td>Echinococcus multieocularis</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Clostridium perfringens</td>
<td>Necator americanus</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Escherichia coli</td>
<td>Taenia saginata</td>
</tr>
<tr>
<td>Elicovirus</td>
<td>Mycobacterium tuberculosis</td>
<td>Taenia solium</td>
</tr>
<tr>
<td>Coxsacivirus</td>
<td>Proteus spp.</td>
<td>Toxocara spp.</td>
</tr>
<tr>
<td>Norwalk virus</td>
<td>Pseudomonas aeruginosa</td>
<td>Trichurus trichura</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Salmonella spp.</td>
<td></td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>Shigellae spp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
<td></td>
</tr>
<tr>
<td>Protozoa</td>
<td>Streptococcus spp.</td>
<td></td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>Vibrio cholera</td>
<td></td>
</tr>
<tr>
<td>Dientamoeba fragilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isospora belli</td>
<td>Vibrio parahaemolyticus</td>
<td></td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td>Yersinia enterocolitica</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In developing countries, parasitic infections are more prevalent (Shuval *et al.* 1986) and therefore a higher number of parasites are expected in wastewater sludge. Conversely, in
developed countries viral and bacterial pathogens are more frequent (Gibbs et al. 1994). The most commonly detected pathogens are enteric pathogens, which result in gastrointestinal illness.

2.1.2. Survival of pathogens in biosolids

The concentration of enteric pathogens in wastewater can be estimated from the number of infected individuals and the average number of pathogens they excrete. In most wastewater treatment processes reduction in pathogen concentration in wastewater mean that pathogens are separated into a sludge product from the treatment process. It is highly likely that the pathogens become concentrated in the raw sludge produced by separation of solids from liquid waste (Fradkin et al. 1989). The concentration of pathogen is sewage sludge is reported to be 50 times more than the sewage (Nell et al. 1983). However, the concentration of surviving pathogens in the biosolids cannot be estimated easily due to the difficulties in methods used for detection (Gibbs et al. 1994). It has been estimated that 90% of the bacterial pathogens die-off very quickly after excretion (Shuval et al. 1986). However, a significant numbers of pathogens may survive for longer periods.

Conventional sludge stabilisation processes such as dewatering, aerobic digestion, anaerobic digestion, air-drying and lime stabilisation are basically more directed toward reduction of volatile solids in sludge which indirectly reduce pathogens. These treatments provide variable reduction in pathogen concentration.
The main factor, which controls the fate of pathogens during sludge treatment, is temperature (Shuval et al. 1986; Straub et al. 1993). Temperature in excess of 55 °C is required for the inactivation of parasite eggs which are more resistant to heat than the bacteria (Carrington, et al. 1991). Since thermal inactivation is a function of time and temperature, better sanitation can be achieved at higher temperatures. Most of the enteric bacteria are inactivated at 70 °C in 30 minutes (Pike et al. 1988). However, in above mentioned processes temperatures as high as 70 °C can not be achieved. Consequently, significant number of pathogens can survive in treated biosolids.

The extent of disinfection differs from method to method, plant to plant and climatic conditions also influence the inactivation of pathogens (Nell et al. 1983). Pederson (1981) concluded that aerobic mesophilic digestion results in less than one log reduction, mesophilic anaerobic digestion between one to two logs and lime stabilisation results in more than two log reduction. Russ and Yanko (1981) reported that dewatered, anaerobically digested sludges contain an average of $10^5$ Salmonella per gram of dried solids. Similarly Reimers et al. (1989) concluded that after anaerobic digestion faecal coliforms and faecal streptococci concentration remain in the range of $10^5$ to $10^8$ organisms per gram dry weight.

The survival of human pathogens in biosolids depends on their ability to survive in the environment. Viruses and parasites cannot multiply outside the host so their population is expected to decline with time. Whereas, under favourable conditions enteric bacteria can multiply in the biosolids (Brandon et al. 1977; Skanavis and Yanko 1994). The
survival time of enteric bacteria outside the host is influenced by temperature, moisture, nutrients, pH and competition from other microflora (Jones and Winkler 1991). Consequently, pathogenic bacterial population in biosolids may fluctuate with time depending upon the environmental conditions. Average survival times for some of the pathogens found in wastewater sludge is presented in Table 2.2.

**Table 2.2.** Average survival time of some human pathogens in wastewater and biosolids at 20-30 °C (Shuval et al. 1986).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pathogens</th>
<th>Average survival time (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses</td>
<td>Enteroviruses</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Hepatitis A virus</td>
<td>3</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Pathogenic <em>E. coli</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>Campylobacter</em> spp.</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em> spp.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> spp.</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> typhi.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio cholerae</em></td>
<td>2</td>
</tr>
<tr>
<td>Protozoa</td>
<td><em>Entamoeba histolytica</em></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Giardia lamblia</em></td>
<td>1</td>
</tr>
<tr>
<td>Helminths</td>
<td><em>Ascaris lumbricoides</em></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Taenia saginata</em></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>Trichuris trichiura</em></td>
<td>10</td>
</tr>
</tbody>
</table>

Generally, viral and bacterial populations die-off within 1-3 months whereas, parasites can survive for a longer period. *Salmonella* has been reported to survive in cattle slurry for long periods of time (Jones et al. 1977 cited by Madsley et al. 1995) possibly due to the availability of nutrients and high moisture content. Sober and Moore (1987) carried
out a literature review on the survival of pathogens in sludge amended soils and found that on average parasites can survive up to 257 days, *Salmonella* 61 days and viruses for 30 days. Therefore, to reduce public health hazard and avoid environmental contamination, further reduction of pathogenic bacteria is required prior to the land application of biosolids.

2.1.3. Pathogens of concern and extent of problem

Many pathogens are found in wastewater sludge (Table 2.1). Some pathogens are of limited concern whereas, others such as *Salmonella*, *Giardia* and *Cryptosporidium*, can survive saprophytically in the environment over a long period (Hinton and Bale 1991). Following the application of contaminated biosolids to land, the potential for environmental contamination exists. Due to the regular use of biosolids on agricultural land, pathogens may accumulate in soil. As a result of indiscriminate use of biosolids, plants, soil and waterways used as drinking water catchment may all become contaminated. The most important pathogens of concern linked to exposure to biosolids are perhaps those responsible for gastrointestinal illness.

Among the bacterial pathogens of concern, *Salmonella* is one of the main causes of gastrointestinal illness (Moe 1996). During the last 10-15 years, *Shigella* spp. and *Campylobacter* spp. were also leading causes of drinking water outbreaks in the United States (Moe 1996). The genus *Salmonella* contains more than 2000 serotypes and the majority of them are pathogenic to humans and animals (Mawdsely *et al.* 1995).
Salmonella is frequently found in wastewater biosolids and is a pathogen of major concern in utilisation of biosolids (Carrington et al. 1991; Jones and Winkler 1991). Salmonella and Campylobacter cells can persist in soil in viable but non-culturable form for long periods of time (Turpin et al. 1993; Jones et al. 1990). However, the public health significance of bacteria in this stage is not known. Some pathogenic strains of E. coli are also responsible for diarrhoeal outbreaks in humans and animals (Mawdsley et al. 1995). The capability of bacterial pathogens to reproduce outside their host is also a major concern. Bacterial pathogens can regrow to hazardous levels in biosolids, which are otherwise considered safe for use.

Enteric protozoa have become one of the main causes of waterborne disease outbreaks (Moe 1996). Four species of protozoa found in sewage are considered to be significant in transmission of human diseases: Entamoeba histolytica, Giardia lamblia, Balantidium coli and Cryptosporidium spp. (Straub et al. 1993). The protozoan cysts are more resistant to heat treatment as compared to the bacterial and viral pathogens and survive in the environment for a long time (Soares et al. 1992). Giardia cysts were detected in biosolids stored up to 6 months (McInnes et al. 1997). Consequently, there is a potential for movement of viable Giardia cysts from soil to water bodies. Cryptosporidium is recognized as an important pathogen of both humans and animals (Mawdsely et al. 1995). Although there is no published literature on the survival of Cryptosporidium in biosolids oocysts are known to survive better than the Giardia cysts. Contamination of water bodies with Giardia and Cryptosporidium through the agricultural use of biosolids is a potential problem because present water treatment methods are not effective to
remove cysts and oocysts. Moreover, infection can occur with the ingestion of as few as ten cysts or oocysts.

The enteric viruses are relatively recently isolated waterborne pathogens and detection of these agents in clinical and environmental samples is limited by inadequate diagnostic technology (Moe 1996). Human enteric viruses such as rotaviruses, adenovirus types 40 and 41, hepatitis A and astrovirus are linked to numerous outbreaks of waterborne diseases. Norwalk-like viruses are the leading cause of epidemic viral gastroenteritis in the United States (Moe 1996). Infected individual can excrete up to \(10^9\) virions per gram of faeces (Wekerle 1986) and infectious doses for these agents can be as low as 1 to 10 infectious units (Moe 1996). Enteric viruses tend to be more persistent in aquatic environments (Moe 1996) and wastewater contaminated soils (Hurst et al. 1980). Temperature and adsorption on to the soil particles appear to be the most important factors for virus survival (Hust et al. 1980). Despite mesophilic digestion, virus concentrations can remain as high as 4000 virions per litre of treated sludge. Considering the low infectious dose of virus and 10-15 tone per hectare of application rate of biosolids on agricultural land (Salt et al. 1996), viruses can be potential risk to public health.

It is clear that there is a potential health hazard associated with the use of wastewater biosolids. This study was aimed at the health hazard posed by the pathogenic bacteria and in particular \textit{Salmonella} regrowth in composted biosolids.
2.2. Finished product standards and regulations

Present US EPA regulations (40 CFR Part 503) specify two classes of pathogen reduction requirements for sludge based products for land application (US EPA 1992). Class A pathogen reduction requires a product to have less than 1000 faecal coliforms g\(^{-1}\) of total solids or less than 3 *Salmonella* 4 g\(^{-1}\) of solids. Additionally, Class A product should also have been treated by a Process to Further Reduce Pathogens (PFRP). Products falling under this category have no restrictions on use. Class B pathogen reduction requires a product to have less than 2 x 10\(^6\) faecal coliforms per gram of total solids or to have been treated by a Process to Significantly Reduce Pathogens (PSRP). Restrictions are imposed on the use of Class B products. The Agriculture and Resource Management Council of Australian and New Zealand (ARMCANZ) regulations are more stringent than the US EPA regulations (Table 2.3). According to the ARMCANZ guidelines (ARMCANZ *et al*. 1997) grade 1A biosolids should have less than 1 *Salmonella* 50 g\(^{-1}\) of dried solids and possess a minimum pathogen regrowth potential, whereas biosolids with 1B grading are expected to possess some pathogen regrowth potential. Only grade 1A products are allowed for unrestricted use, whereas grade 2 and 3 products are used for forestry and landfilling.

The ARMCANZ guidelines for the PSRP and PFRP processes are similar to US EPA regulations. The PSRP are those processes which reduce pathogenic viruses by one log (90%) and indicator bacteria (faecal and total coliforms) by two logs (99%). The examples of PSRP processes are aerobic digestion, air drying, lime stabilisation and
composting. Whereas, the PFRP are those processes which are capable of reducing pathogen concentrations to below detectable limits (Reimers et al. 1990). The examples of PFRP processes are heat drying, composting pasteurisation and gamma irradiation.

Table 2.3. Microbiological Standards for biosolids (ARMCANZ et al. 1997).

<table>
<thead>
<tr>
<th>Pathogen Standard</th>
<th>Microbiological Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grade 1A</strong></td>
<td></td>
</tr>
<tr>
<td>Very low pathogen levels with minimum regrowth potential</td>
<td>1 $&lt; 1$ <em>Salmonella</em> spp. per 50 grams of final product. or 2 $&lt;100$ MPN Thermotolerant coliforms per gram of final product 3 Analysis to be carried out in accordance with AS 1766 with a minimum sample of 50 grams.</td>
</tr>
<tr>
<td><strong>Grade 1B</strong></td>
<td></td>
</tr>
<tr>
<td>Low pathogen levels but some pathogen regrowth potential</td>
<td>1 $&lt; 1$ <em>Salmonella</em> spp. per 50 grams of final product. 2 $&lt;1000$ MPN Thermotolerant coliforms per gram of final product. 3 Analysis to be carried out in accordance with requirements of AS 1766, with a minimum sample of 50 grams.</td>
</tr>
<tr>
<td><strong>Grade 2</strong></td>
<td></td>
</tr>
<tr>
<td>Established process which achieve significant pathogen reduction</td>
<td>No pathogen limit set.</td>
</tr>
<tr>
<td><strong>Grade 3</strong></td>
<td></td>
</tr>
<tr>
<td>Minimum pathogen reduction</td>
<td>No pathogen limit set.</td>
</tr>
</tbody>
</table>

Composting has been listed under both categories depending upon how the process is carried out (ARMCANZ et al. 1997). A number of composting methods such as within vessel, static aerated pile or windrow composting are recommended as PFRP. The temperature of the biosolids should rise to 40 °C or higher and remain at 40 °C or higher for 5 days. For 4 hours during the 5 days, the temperature in the compost pile should exceed 55 °C.
Composting methods are also recommended as PFRP. In this case, for within vessel method or static aerated pile the compost temperature should be maintained at 55 °C for three days. Whereas, for windrow composting method, the compost pile temperature should be maintained at 55 °C or higher for 15 days or longer. During the period when the compost is maintained at 55 °C or higher, there should be a minimum of 5 turnings of the windrow.

The composting conditions outlined above are designed for achieving desired pathogen destruction, but they do not guarantee a bio-hazard free product. To address this problem both the US EPA guidelines and ARMCANZ guidelines include monitoring for Salmonella concentrations in finished products. However, monitoring of Salmonella concentration in finished products remains optional. Under the US EPA guidelines it is believed that when the faecal coliform density is below 1000 MPN g⁻¹ or Salmonella concentration is below 3 MPN 4 g⁻¹, the Salmonella regrowth potential is mitigated.

2.3. Composting Process

2.3.1. Definition and basic concept

Haug (1993) defined the composting process as “the biological decomposition and stabilisation of organic substrate under controlled conditions, which allow the development of thermophilic temperatures as a result of biologically produced heat, to
produce pathogen and plant seed free stable material, which can be applied to land”. The controlled conditions are temperature, moisture and aeration. Consequently, composting is an aerobic, biological decomposition of organic matter at thermophilic temperatures under a controlled environment. The indigenous populations of microorganisms carry out the biological decomposition.

The composting process can be divided into four stages: mesophilic, thermophilic, cooling and maturation. When the moisture content of the compost pile is brought to the appropriate level and the pile is aerated, microbial activity increases. The biological oxidation of organic matter by the microorganisms results in the production of energy, which is used for maintaining metabolic activity. The excess is released in the form of heat. This process continues until only resistant organic matter is left in the system. Further degradation occurs very slowly during the maturation phase of composting.

At the beginning of the composting process, microorganisms capable of thriving at a mesophilic temperature range (25-45 °C) start degrading readily available carbon compounds. This results in an increase in temperature and drop in pH due to the production of organic acids. The mesophilic stage generally lasts for only 24 h.

The thermophilic stage commences with an increase in temperature beyond 45 °C. Biodegradable compounds such as sugars, fats, proteins and starch are degraded rapidly by the thermophilic microflora. The temperature inside the compost piles can rise to more than 80 °C within a few days of composting (McKinley and Vestal 1985).
However, the bio-oxidation rate declines considerably after 60 °C as the optimum temperature for rapid degradation of organic matter is 40 to 50 °C (McKinley and Vestal 1985). The pH of the system starts declining due to the production of ammonia as a result of decomposition of proteins. This stage may last for a couple of weeks depending upon the substrate composition and composting conditions.

Once readily available nutrients are consumed, then bacteria and fungi capable of producing exoenzymes to degrade resistant polysaccharides such as cellulose (which is a major constituent of wood and sewage sludge) commence their activity (Mathur 1998). This results in a slower release of energy and subsequent decline in the temperature, which marks the beginning of the cooling phase. During the cooling phase there is a further decline in pH. The cellulose degrading microbes utilise the earlier liberated ammonia and volatilisation of excess ammonia also takes place. When the temperature begins to drop below 50 °C the mesophilic bacteria and fungi resume their biological activity. The rate of cellulose and pectin degradation is also at its peak during the cooling down phase (De Bertoldi et al. 1980).

In the maturation stage there is slow degradation of the more resistant compounds, which results in humification and subsequent stabilisation of the remaining organic matter. Bacteria, actinomycetes and fungi are active during this stage. However, the fungal population is greater and more diverse during this stage (De Bertoldi et al. 1980). The pH becomes stable and become slightly alkaline in the maturation stage.
2.3.2. Wastewater biosolids composting

Wastewater biosolids are a valuable source of nutrients and also possess soil-conditioning properties for agricultural land. The use of untreated wastewater sludge on agricultural land can cause a number of problems such as inhibition of seed germination, accumulation of heavy metals and pathogens in soil (Epstein 1997) and also may create pollution problem in rivers (De Bertoldi et al. 1980). Composting of wastewater biosolids prior to their application to agricultural land has the capacity to alleviate these problems and therefore is considered an acceptable method for recycling of wastewater biosolids (De Bertoldi et al. 1983).

All types of wastewater biosolids can be composted after mixing with a bulking material to create proper conditions such as C/N balance, aeration and moisture content for composting (De Bertoldi et al. 1980). A variety of bulking materials, such as agricultural waste, grass clippings, wood chips, sawdust, and tree bark, can be used in wastewater biosolids composting (Epstein et al. 1983). After a suitable composting and maturation period the composting mass is converted into stabilised mass, which can be used as soil a conditioner without any ill effect on the environment.

The available compost systems can be divided into two categories, open systems and in–vessel systems (Haug 1993). In-vessel systems use reactors in which the solids flow vertically or horizontally through the reactor. The turning and aeration of composting
material is achieved through mechanical mixing and forced aeration (Goluke and Diaz 1991).

The open systems such as windrow and static pile are more popular due to their low operational and maintenance costs (De Bertoldi et al. 1982). Windrow composting systems consist of a composting mixture placed in open rows. These are turned periodically to maintain aerobic conditions during composting. Turning of compost piles also provides heat dissipation and re-mixing of material. The frequency of turning depends upon many factors such as oxygen demand, nature and condition of the substrate and type and capacity of the turning equipment (Goluke and Diaz 1991). Aeration of windrows can also be achieved by forced aeration or ventilation (De Bertoldi et al. 1982). For forced aeration a network of air ducts consisting of plastic or metal pipes is constructed and laid in the compost pad. The pile is aerated by forcing air through the air ducts by a blower. In the forced aeration system air is pumped into the piles under pressure, whereas in the induced aeration system air is sucked through the pile.

2.3.3. Microbial ecology and community dynamics

Bio-oxidation of organic matter during composting is brought about by a variety of indigenous microorganisms and wide ranges of microorganisms have been isolated from different composting systems (Table 2.4). In the following discussion actinomycetes are referred to as a group of microorganisms distinct from bacteria. Due to ecological and physiological reasons actinomycetes are commonly considered separate from bacteria
(Miller 1992). However, actinomycetes are considered as a group of bacteria by some taxonomists due to the presence of a bacterial cell wall and nuclear material (Haug 1993).

The composting process is very dynamic in terms of the predominance of specific groups of microorganisms during the specific stages of composting (Miller 1992). The overall population dynamics of indigenous microbial communities during the composting process is influenced by a number of factors such as available nutrients, moisture, temperature, oxygen and pH (Epstein 1997). Temperature is very important as it affects the concentration and types of microorganisms during composting (Epstein 1997). Ideally composting temperature range should be between 30 to 40 °C for maximum microbial diversity (Stentiford 1996). In the beginning of the composting process mesophilic/ thermotolerent bacteria and fungi are active. Actinomycetes develop slowly and do not play a role in the early degradation of organic matter.

The population of mesophilic microbes declines during the thermophilic stage, whereas the population of thermophilic bacteria, actinomycetes and fungi increases rapidly (Finstein and Morris 1975). Thermophilic actinomycetes are more resistant to high temperature than fungi and their diversity also increases at 50-55 °C. Thermophilic bacteria are the chief decomposers above 60 °C (Strom 1985a).

The community dynamics during composting is also affected by the availability of substrate. Carbon (C) and Nitrogen (N) are the main sources of energy and they are assimilated differently by bacteria, actinomycetes and fungi.
Table 2.4. Microorganisms isolated from composts (adapted from Strom 1985b; Sharma 1989; Beffa et al. 1996; Epstein 1997).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Actinomycetes</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenes</td>
<td>Actinoplanes spp.</td>
<td>Absidia</td>
</tr>
<tr>
<td>Bacillus brevis</td>
<td>Micromonospora parva</td>
<td>Absidis orchidis</td>
</tr>
<tr>
<td>B. cereus</td>
<td>Micromonospora vulgaris</td>
<td>Allescheria terrestris</td>
</tr>
<tr>
<td>B. circulans</td>
<td>Nocardia brasiliensis</td>
<td>Aspergillus flavus</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>Pseudonocardia thermophila</td>
<td>Aspergillus tamarii</td>
</tr>
<tr>
<td>B. schlegelii</td>
<td>Streptomyces rectus</td>
<td>Byssochlamys spp.</td>
</tr>
<tr>
<td>Chondrococcus exiguus</td>
<td>Streptomyces thermofuscus</td>
<td>Candida</td>
</tr>
<tr>
<td>Clostridium spp.</td>
<td>Streptomyces thermoviolaceus</td>
<td>Charetomium</td>
</tr>
<tr>
<td>Flavobacterium spp.</td>
<td>Streptomyces thermovulgaris</td>
<td>Cladosporium herbarum</td>
</tr>
<tr>
<td>Heterotrophic Bacilli</td>
<td>Thermomonospora curvata</td>
<td>Coprinus cinereus</td>
</tr>
<tr>
<td>Hydrogenobacter spp.</td>
<td>Thermomonospora fusca</td>
<td>Coprinus lagopus</td>
</tr>
<tr>
<td>Micrococcus spp.</td>
<td>Thermomonospora glauca</td>
<td>Cylinndrocaron spp.</td>
</tr>
<tr>
<td>Mycococcus fulvus</td>
<td>Thermomonospora viridis</td>
<td>Fusarium moniliforme</td>
</tr>
<tr>
<td>Mycococcus virescens</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td>Proteus spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcina spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoactinomyces vulgaris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermomonospora curvata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermomonospora fusca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermomonospora glauca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermomonospora viridis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermopolyspora bispora</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermopolyspora polyspora</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermus spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thibacillus denitrificans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thibacillus thiooxidans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glibotrys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hanisenula spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humicola griseus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humicola insilens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipomyces spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortierella wolfii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucor pusillus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucor racemosus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myceliophthora thermophila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myriococcum albomyces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papulaspora thermophila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillium digitatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pichia spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pululloria spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizopus arrhizus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizopus nigricans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizoctonia spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodotorula rubra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scytalidium thermophilum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stysanus stemonitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synecphalastrum spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Talaromyces dupontii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoascus aurantiacus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermidium sulfureum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torula thermophila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torulopsis spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichoderma koningi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichosporon cutaneum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verticillium spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zygorhynchus vuilleminii</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In a mixed microbial population such as in compost, bacteria assimilate 15-30% of C, actinomycetes 30-40% and the remaining 5-10% of C is assimilated by the fungi (Alexander 1977). However, the presence of N is essential for the degradation of C. To degrade one unit of C bacteria require 1-2% N, actinomycetes require 3-4% and fungi 3-4% (Miller 1992). The earlier bacterial degradation of proteinaceous substrate liberates ammonia for the utilisation of subsequent populations.

The population dynamics of bacteria, actinomycetes and fungi in various self-heating systems such as leaves (Hankin et al. 1976), pine bark (Davis et al. 1992), sewage sludge (De Bertoldi et al. 1980; Chino et al. 1983; Nakasaki et al. 1985a) and refuse (Strom 1985a,b) have been investigated. The results of these studies vary according to substrate, microbial recovery techniques used, investigative interest, process management and means of expression.

The factors such as temperature and pH, which determine the population dynamics during composting, are themselves influenced by the microbial activity. The predominance of microbial communities is determined by a continuous change in the physical and chemical conditions established by the previous microbial activity (Miller 1992). The activity of bacteria, actinomycetes and fungi during the composting process is discussed in the following sections.
2.3.3.1. Bacteria

Bacteria are the most important decomposers in the beginning of the composting process due to the availability of readily degradable organic matter and mesophilic temperatures. During mesophilic composting of sewage sludge up to 40% of volatile solids are degraded within a week (Miller and Finstein 1985) and most of the activity (80 to 90%) is attributed to bacteria (Strom 1985a; Haug 1993). A list of commonly isolated bacteria from various types of composts was provided in Table 2.4.

Due to the activity of the mesophilic bacteria at the beginning of the composting process, the temperature in the compost pile starts increasing. The bacterial population shifts from mainly mesophilic to thermophilic when the temperature is more than 50 °C. Composting temperature in the range of 60-80 °C are commonly achieved during wastewater biosolids composting (Finstein and Morris 1975). This temperature range is selective for bacteria and especially for the genus Bacillus (Fermor et al. 1979). Gordon (1977) studied the effect of temperature on diversity of Bacillus species in composted and concluded that maximum diversity is found between 33 to 45 °C. It was further reported that after 60 °C only B. thermophilus was found in the compost pile.

The maximum degradation of organic matter has been reported at 60 °C (Nakasaki et al. 1985a; Strom 1985a). The microbial activity of thermophilic bacteria is faster at higher temperatures but beyond 70 °C however, bio-diversity in the composting system drastically reduces (Nakasaki et al. 1985a). It was found that with the progress of the
composting process the microbial diversity narrowed down from 5 genera (Pseudomonas, Achromobacteria, Flavobacterium, Micrococcus and Bacillus) to Bacillus becoming dominant (Anon. 1955, cited by Finstein and Morris 1975). Similarly Strom (1985b) found that the predominant bacterial genus above 50 °C was Bacillus and beyond 65 °C only B. stearothermophilus survived. In contrast, Beffa et al. (1996) reported that a taxonomically and metabolically diverse population of thermophilic aerobic bacteria was active between 60-80 °C in compost. The bacteria responsible for bio-oxidation above 60 °C included Hydrogenobacter spp., Thermus spp., Bacillus schlegelii and Heterotrophic Bacilli (Beffa et al. 1996). The difference in the results is most likely due to the improved detection techniques used by Beffa et al. (1996).

As the temperature starts going down during the cooling phase the mesophilic bacterial population starts increasing again. Poincelot (1977) analysed densities of various microorganisms during the composting of garden type material. This is shown in Table 2.5. As can be seen from Table 2.5, bacteria are more active during the mesophilic phase and early thermophilic stage of composting. Beffa et al. (1996) reported that although the overall population of bacteria declined during the cooling phase, as compared to the thermophilic phase, there was a considerable increase in taxonomic diversity. However, available information on the activity of various genera of bacteria during composting is very limited.
Table 2.5. Density of various microorganisms during aerobic composting (adapted from Poincelot 1977).

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Mesophilic initial temperature less than 40 °C</th>
<th>Thermophilic 40 to 70 °C</th>
<th>Mesophilic 70 °C to cooler</th>
<th>Number of species identified</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td><strong>Mesophilic</strong> 10^8</td>
<td>10^6</td>
<td>10^11</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Thermophilic</strong> 10^4</td>
<td>10^9</td>
<td>10^7</td>
<td>1</td>
</tr>
<tr>
<td><strong>Actinomycetes</strong></td>
<td><strong>Thermophilic</strong> 10^4</td>
<td>10^8</td>
<td>10^5</td>
<td>14</td>
</tr>
<tr>
<td><strong>Fungus</strong></td>
<td><strong>Mesophilic</strong> 10^6</td>
<td>10^3</td>
<td>10^5</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td><strong>Thermophilic</strong> 10^3</td>
<td>10^7</td>
<td>10^6</td>
<td>16</td>
</tr>
</tbody>
</table>

*Actual number present is equal to or less than the stated value.

2.3.3.2. Actinomycetes

The growth of actinomycetes is favoured by neutral to slightly alkaline pH in the presence of aerobic conditions (Miller 1992). The initial degradation of easily available compounds results in the production of ammonia and consequently an increase in pH. The actinomycetes degrade recalcitrant molecules and polymers such as chitins, cellulose, proteins and waxes, which are not degraded by other microbes (Haug 1993). Species belonging to 3 actinomycetes genera, *Micromonospora*, *Streptomyces*, and *Actinomyces*, are regularly isolated from composts (Haug 1993). Most of the thermophilic actinomycetes are active between 50 to 60 °C and a small number are active...
at 60 °C or more. The activity of actinomycetes also increases with the progress of the composting process. With the progress of the cooling phase, temperature, pH and moisture content decline, which favours the growth of actinomycetes (De Bertoldi et al. 1980).

2.3.3.3. Fungi

Fungi are eukaryotic organoheterotrophs with the majority being saprophytic (Haug 1993). Mesophilic conditions during the early stages of composting favour fungal growth. However, their activity is suppressed due to the high temperature in the thermophilic stage. The optimum temperature for fugal growth is less than 50 °C and only very few fungi can grow at 60 °C (Miller 1992). A large number of mesophilic and thermophilic species of fungi have been isolated from composts (Table 2.4). Fungi as a group are most active during the cooling down phase, when the temperature is moderate and the remaining substrate is predominantly cellulose and lignin (De Bertoldi et al. 1983). However, a large number of fungal species can be isolated during both mesophilic and thermophilic stages of composting (Table 2.5). The thermophilic fungi population is re-established during the cooling down phase at moderate temperatures (50-55 °C). Fungi are well adapted to low moisture, high osmotic pressure and a broad pH range. They also have lower nitrogen requirements as compared to bacteria (Haug 1993). The low nitrogen requirement is of particular significance because the predominant substrate during the later stage of composting is nitrogen deficient cellulose and lignin (De Bertoldi et al. 1980).
2.4. Pathogens in composted biosolids

Composting can effectively reduce pathogen concentrations in wastewater sludge (Russ and Yanko 1981). However, complete sterilisation is difficult to achieve. Sanitation of wastewater sludge through composting has already been investigated by several authors (Nell et al. 1983; Epstein and Epstein 1985, 1989; Deportes et al. 1998; Shaban 1999). However, most of the investigations carried out on the sanitation of biosolids by composting have primarily focused on the faecal indicators and *Salmonella*. Moreover, most of the studies involved seeding of pathogens and investigations were carried out in laboratory scale composting.

It has been demonstrated that one of the greatest risks associated with the recycling of biosolids is *Salmonella* and *Giardia* infection (Gibbs and Ho 1993; Gibbs *et al.* 1994). Very limited literature is available on *Giardia* inactivation during composting. To date there is no satisfactory technique of identifying the viability of *Giardia* cysts in composted biosolids or wastewater. As mentioned earlier, protozoan pathogens and parasite eggs can survive in the environment for long periods of time. Whereas, enteric bacteria can regrow under certain conditions in composted biosolids. In general, the disinfection efficiency of biosolids depends upon the composting method used (Yanko 1987).

Pederson (1981) concluded that composting could be used to achieve sanitation of biosolids, provided that a uniform temperature was maintained throughout the compost
pile for the required time. Yanko (1988) examined compost based products from 26 different composting facilities over one year for pathogenic bacteria, viruses, fungi and parasite eggs. *Salmonella*, toxigenic *E. coli*, *Yersinia enterocolica* and *Aspergillus fumigatus* were detected more frequently than other pathogens in the composted biosolids. *Salmonella* was found to be the most common (in approximately 40% of samples). The highest reported concentration of *Salmonella* was 85000 cells g\(^{-1}\). Yanko (1988) also reported that the frequency of *Salmonella* isolation depended on the composting process and the point of sampling within the compost pile. Conversely, proper mixing and uniformly high temperature (>50 °C) was required inside the compost pile for proper disinfection. The results also showed a need to check the bacterial quality of compost prior to distribution.

Goldstein *et al.* (1988) also carried out a yearlong research project on the presence of pathogenic microorganisms in compost and compost based products. They reported that the compost-based products were free of viruses and parasites. However, *Salmonella* was most frequently detected followed by *Yersinia* and toxigenic *E. coli*. Considering the capacity of bacterial pathogens to regrow in compost, pathogenic bacteria can pose a health hazard.

Skanavis and Yanko (1994) evaluated compost based soil amendments for the presence of *Salmonella* and FC. They detected relatively high concentrations of FC in most of the soil amendments as compared to the composted biosolids, which had lower concentrations. *Salmonella* concentrations in commercial soil amendments varied
between 0.2 to 400 Most Probable Number (MPN) per gram. *Salmonella* was also isolated from the composted biosolids in low numbers when more efficient techniques were used. The results suggest that *Salmonella* may be present in composted biosolids in relatively small numbers.

The sanitisation capacity of a large-scale municipal solid waste composting facility has also been evaluated (Deportes *et al.* 1998). The composting process was found to be effective in reducing faecal indicators and *Salmonella* below the detection limit after 27 days of composting. However, regrowth of *Salmonella* was observed in stored biosolids.

As shown above limited studies have been carried out to investigate the disinfection capacity of full-scale composting plants. The studies described above suggest that composting reduce most pathogens to concentrations below detection limits. However, *Salmonella* appears to survive in some situations. This may be because it is very difficult to achieve a required time-temperature relationship uniformly through out the compost pile, especially with windrow composting.

### 2.5. Factors affecting pathogen die-off during composting

Composting and sanitation are not necessarily synonymous. To assess the pathogen destruction ability of the composting process it is necessary to understand the various factors responsible for pathogen destruction. The inactivation of pathogenic
microorganisms during composting is brought about by the evolution of heat, antagonistic compounds produced by indigenous microbes, and the competitive ability of the consortium of indigenous microorganisms (Mathur 1998). These factors are expected to have a varying effect on different groups of microorganisms. The effect also varies from microbe to microbe. However, the factors, which lead to the destruction of pathogens during composting also reduce indigenous microbial diversity, which is believed to suppress regrowth of pathogens. Some of these factors are also responsible for inhibition of pathogen regrowth in the finished products. The important factors that are responsible for pathogen inactivation during composting are discussed below.

2.5.1. Temperature

Although several factors work towards the sanitation of biosolids during composting, temperature is the factor that can be quantitatively measured and controlled. High temperature is the most effective and reliable means of pathogen inactivation (Martin et al. 1990; De Bertoldi et al. 1988). The extent of thermal inactivation of pathogens during composting depends on the temperature and duration of exposure to a given temperature. A typical composting temperature range is between 55 to 65 °C (Deportes et al. 1995). The temperature and duration of maximum temperature required for inactivation of some of the pathogens during composting is presented in Table 2.6.
Table 2.6. Level of temperature and duration required to destroy some pathogens during composting (adapted from Golueke 1991).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Lethal temperature and necessary time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp</td>
<td>15 to 20 min at 60 °C, 60 min at 55 °C</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>15 to 20 min at 60 °C, 60 min at 55 °C</td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>60 min at 55 °C</td>
</tr>
<tr>
<td><em>Entamoeba hystolitica</em></td>
<td>68 °C, no time mentioned</td>
</tr>
<tr>
<td><em>Taenia saginata</em></td>
<td>5 min at 71 °C</td>
</tr>
</tbody>
</table>

The time-temperature relationship forms the basis of PFRP requirements in the US EPA guidelines as stated in 40CFR part 503 (US EPA 1992). The time-temperature requirement of 55 °C for 15 days during windrow composting is also specified for Grade 1A product in Australian draft guidelines (ARMCANZ et al. 1997). The time-temperature requirements specified are applicable to every particle of the compost (US EPA 1992). *Salmonella* and other enteric bacteria should be destroyed during composting as the average temperature of the composting process is generally more than 55 °C. However, during large scale composting uniform temperatures in the compost pile are difficult to achieve due to improper mixing and other technical problems.

De Bertoldi *et al.* (1982) evaluated several composting systems for their pathogen inactivation efficiency and concluded that the pathogen destruction depended upon the temperature achieved during composting. Maintenance of a high temperature during composting is not only necessary for fostering thermophilic activity but also for sanitation of biosolids. The threshold level of resistance to temperature varies from
microbe to microbe and it also depends upon the inherited properties of compost. Sporigenous and non-sporigenous thermophilic bacteria can withstand even 100 °C temperature, which means that they cannot be eliminated through composting. However, most of the pathogenic microorganisms do not belong to this group and can be eliminated by lower heat treatments. Most of the microorganisms can be destroyed within 30 min. at 70 °C (Pike et al. 1986).

The experiments demonstrating the minimum temperature requirement for pathogen inactivation have generally been carried out on pure cultures and under laboratory conditions. However, several factors may diminish the effectiveness of high temperatures for pathogen inactivation during full scale composting. The shielding effect of compost particles, microbial adsorption onto particles and protective coating of organic matter may contribute to the survival of pathogens (Passman 1978).

2.5.2. Competition for limiting nutrients

A wide range of indigenous microorganisms competes with bacterial pathogens for a limited nutrient supply at every stage of the composting process. The bacterial pathogens present in biosolids are mostly heterotrophic and can only metabolise easily degradable organic matter such as sugars, starch, fatty acids and lipids (De Bertoldi et al. 1990). The organic matter available in biosolids is extremely heterogenous in nature, ranging from easily degradable short chain organic acids to highly complex and stable organic polymers like lignin. Fungi and actinomycetes degrade the complex organic matters such
as lignin, cellulose and chitin first, and later on simplified sugars are available as nutrients for other microorganisms. It is anticipated that in stabilised biosolids growth of enteric bacteria will be restricted. However, with the slow release of simplified sugars there is always a competition for the limited nutrients between enteric bacteria and indigenous microorganisms. The indigenous microbial consortium is considered to be well adapted to the compost conditions and hence competes with enteric pathogens for the limited nutrients more efficiently.

2.5.3. Dehydration

Microbial cells require a suitable moisture content to carry out energy generating metabolic activities such as glycolysis and respiration. Consequently, limiting moisture conditions are expected to irreversibly damage the microbial cells. However, some microorganisms can tolerate moisture stress better than others. Moisture content reduction or de-watering is commonly used for wastewater treatment (Ward et al. 1981).

The bacterial inactivation rates during de-watering of wastewater biosolids depend upon the moisture content (Ward and Ashley 1977). Moisture content of more than 20% is required for growth of Salmonella in biosolids (Russ and Yanko 1981). Consequently, enteric bacterial numbers will decline in biosolids with less than 20% moisture. During the composting process there is a gradual reduction in moisture content, which is expected to favour inactivation of pathogenic bacteria. During storage of composted biosolids a further decline in moisture takes places.
2.5.4. **Ammonia toxicity**

Free ammonia is very reactive and is effective in inactivation of bacterial cells (Cramer and Burge 1977; Burge et al. 1981), faecal coliforms (Taylor et al. 1978) virus and bacteriophage (Cramer et al. 1983). Conversely, it has been reported that the production of ammonia during composting enhances the thermal inactivation of polio and other enteroviruses (Ward and Ashley 1977).

Ammonia is produced during composting due to the decomposition of nitrogen containing organic acids and proteins. Low pH is reported to favour ammonia production in wastewater sludge (Taylor et al. 1978). Since ammonia is bactericidal therefore it is expected that it also play role in inactivation of pathogens during composting. However, there is little information on the role of ammonia in inactivation of pathogens during composting. Production of ammonia leads to loss of nitrogen, which is considered undesirable in composting as it reduces the nitrogen content of the finished product.

2.5.5. **Antibiotic compounds**

Some species of fungi and actinomycetes produce secondary metabolites that suppress the activity of other microorganisms. Penicillium and other actinomycetes such as streptomycetes have been isolated from composting biosolids (Table 2.4). It has been reported that above 60 °C the composting activity is mainly carried out by actinomycetes such as *Streptomyces spp.* and *Thermoactinomycetes spp.* (Mathur 1998). Microbes
belonging to these two genera are known to produce antibiotics. Filamentous fungi such as *Penicillium* are known to produce antibiotics that suppress the growth of *Salmonella* and other enteric pathogens in cheese and other foods (Faber and Geisen 1994; Larsen and Knochel 1997). The majority of antibiotics produced by the soil borne microorganisms such as *Bacillus* spp., *Streptomyces* spp. and *Penicillium* spp are effective against gram positive bacteria (Cross 1968; Salle 1971). However, some thermophilic actinomycetes are known to produce antibiotics active against *Salmonella* (Bakru and Johri 1980).

During composting the production of antibiotics and secondary metabolites from some microorganisms is expected to suppress the growth of enteric bacteria. Antagonistic organisms are expected to produce secondary metabolites in a localised environment in the presence of favourable conditions (Sale 1971). Due to very low production and localised effect of antibiotics they are very difficult to isolate from the environment. Consequently, it is difficult to differentiate between the antagonistic effect of indigenous microorganisms and competition for limited nutrients. The contribution of secondary metabolites and antibiotics produced by indigenous microflora to the suppression of *Salmonella* growth is not known.
2.6. Correlation between faecal indicators and pathogen die-off

As mentioned in Section 2.7.1, complete sanitisation of biosolids based on the time-temperature specifications is extremely difficult to achieve due to operational difficulties. Consequently, monitoring for an indicator organism is generally used to check the proper sanitisation of compost. Monitoring of all the pathogens in biosolids is impossible due to the range of pathogens that may be present and limitations in the existing methodologies. Usually surrogate organisms, which behave in a similar manner to the pathogen of concern, are monitored. However, no single microorganism meets all the essential criteria for use as an indicator, such as abundance, sensitivity to treatment and growth recovery in the biosolids. Faecal coliforms or thermotolerant coliforms (FC) and faecal streptococci (FS) possess most of the desired characteristics and therefore are commonly used as surrogates for pathogen die-off during composting.

Faecal coliforms are present in wastewater biosolids in large number and are often suggested as good indicator for the presence of pathogens. However, faecal coliforms have been reported to grow in compost and their presence in compost cannot be related to inactivation of pathogens (Burge et al. 1981). On the other hand streptococci are consider to be good indicators of Salmonella inactivation during wastewater treatment as they are unlikely to regrow in composted biosolids (US EPA 1992).

A limited number of studies have been carried out relating indicator and pathogen concentrations in biosolids. Berg and Berman (1980) investigated the effect of anaerobic
digestion on the population of viruses and indicators. They observed a large variation in virus numbers over a narrow range of numbers of FC, TC and FC. Moreover, indicator bacteria were found to be 7-8 times more sensitive to mesophilic digestion and 9-10 times more sensitive to thermophilic anaerobic digestion than viruses. On the basis of the similarity between the destruction rate of FS and viruses they suggested that FS could possibly be used as an indicator. However, Pederson (1981) reported that no single microorganism maintained a constant density level as compared to the pathogenic microorganisms in biosolids. Consequently, it is difficult to use a single universal indicator for faecal pollution.

Sorber and Moore (1987) carried out a comprehensive review of the literature available on pathogen survival in sludge amended soils. They found a higher inactivation rate of *Salmonella* in biosolids amended soil as compared to the faecal indicators. Skanavis and Yanko (1994) found significantly lower concentrations of TC and FC in composted biosolids as compared to the compost-based products. This further supported the idea of FC growth in finished products. However, they also observed a correlation between the concentration of FC and occurrence of *Salmonella*.

Gibbs *et al.* (1994) evaluated the usefulness of FS and FC as indicators of *Salmonella* and virus inactivation during mesophilic anaerobic digestion. The authors found no correlation between the reduction in faecal indicators and *Salmonella*. However, a significant correlation was found between FS and *Salmonella* die-off in stored biosolids. A detailed investigation of pathogen die-off in stored biosolids and soils amended with
sewage sludge was carried out by Eamens et al. (1996). They reported that *Salmonella* die-off during mesophilic digestion and storage of biosolids was not correlated to the decline in *E. coli* or FS concentration. They also reported that *Salmonella* die-off in biosolids amended soils was not linked to the decline in concentration of both indicators.

Similarly, Ahmed et al. (1995) determined the inactivation rate of pathogens in stored biosolids. It was reported that *S. typhimurium* was more resistant to inactivation than bacteriophage f2 but less than poliovirus, the inactivation rate was found to be more at higher temperature. However, inactivation rate of FC, TC and FS were not studied in this study. More recently Shaban (1999) compared disinfection efficiency of different composting systems and concluded that coliphage and FS were more resistant to composting than *Salmonella* and FC and should be used as indicator organisms.

From the available literature it appears that the monitoring of faecal indicator concentrations cannot guarantee a *Salmonella* free product. Very limited literature is available on the correlation between pathogen die-off and the concentration of indicator organisms in composted biosolids. Due to the lack of uniform pathogen assay techniques it is difficult to compare the reported results. Furthermore, the reported results are often conflicting. Yanko’s (1988) data showed that when FC concentrations in composted biosolids were below 1000 MPN per gram then chances of *Salmonella* detection were very low. Further validation of this study has not been carried out. AS regrowth of *Salmonella* is unpredictable monitoring of *Salmonella* concentrations along with faecal indicators may be required to safeguard public health.
2.7. Survival of pathogenic bacteria in composted biosolids

Survival of enteric bacteria in composted biosolids and soil amended with biosolids is influenced by a number of factors such as temperature, pH, soil moisture, organic matter, soil colloidal matter and antagonistic microorganisms (Straub et al. 1993; Epstein 1997). Kibbey et al. (1978) reported that survival of S. typhimurium and Streptococcus faecalis increased with an increase in moisture content. Shielding of microbes by compost particles and adsorption on to particles has been reported to prolong survival of pathogens during composting (Passman 1978). Brinton and Droffner (1994) found that Salmonella and E. coli were able to withstand 60 °C temperature for up to 50 days in a process similar to PFRP. They also demonstrated development of temperature resistant mutant strains of E. coli. The role of antagonistic organisms in reducing survival of bacterial pathogens is not very clear.

Salmonella is known to survive in sludge and soil under favourable conditions of temperature and moisture for many months (Straub et al. 1993). Die-off of FS and FC in lagoon-stored sludge in sub-tropical conditions in the USA was investigated by Reimers et al. (1989). They reported little die-off of faecal indicators over a 25 month period. This suggests prolonged survival of enteric bacteria at mesophilic temperatures and adequate moisture conditions.

Storage of composted biosolids is used as a means to further reduce pathogen concentrations after composting. However, limited information is available on the effect
of compost storage on the concentration of pathogens. In the study carried out by Pereira-Neto et al. (1986) it was found that the decline in pathogen concentrations during storage depended upon the concentration of surviving microorganisms after composting.

*Salmonella* have been reported to survive in soil amended with biosolids up for to 7 months (Wary and Callow 1984; Burge and Marsh 1978). Similarly, Eamens et al. (1996) reported *Salmonella* survival times of between 4 to 5 months in biosolids amended soils. Sorber and Moore (1987) observed longer survival and regrowth of FC in sludge amended pasture during the summer, as compared to winter. However, it was claimed that the lengthy survival (more than 7 weeks) occurred due to the excessively high initial concentration \(10^2\) to \(10^7\) g\(^{-1}\) of *Salmonella* in the biosolids applied to land. Consequently, with the application of biosolids with a high initial concentration of pathogens, lengthy survival times in soil are expected.

The process of *Salmonella* inactivation is not well understood so prediction of pathogen concentrations after composting or biosolids application to land is difficult. This is probably due to the fact that inactivation of pathogens is influenced by a large number of variable factors. There is conflicting information available on the survival of *Salmonella* in soils amended with biosolids. Most of the available literature is from studies where soil was irrigated with effluent. It is difficult to extrapolate this data to determine pathogen survival time in compost or biosolids amended soils.
2.8. Pathogen regrowth in composted biosolids

It appears that *Salmonella* can survive composting in some situations. Also biosolids might be properly disinfected through composting, but there is always a possibility of pathogen re-introduction and regrowth in previously disinfected biosolids. Although a wide range of pathogenic microorganisms are present in biosolids, only some pathogenic bacteria and fungi can actually multiply in biosolids (De Bertoldi *et al.* 1990). Bacterial and fungal pathogens may reproduce by feeding on easily consumable organic matter present in composted biosolids. *Salmonella* have been the most frequently isolated bacteria from treated biosolids, sometimes being detected in relatively high concentrations. Skanavis and Yanko (1994) analysed *Salmonella* concentrations in commercially marketed soil amendment products. The *Salmonella* concentration in the compost based soil amendment products varied between less than 0.2 to more than 300 MPN g\(^{-1}\), which exceeds the current Class A limit of less than 3 *Salmonella* 4 g\(^{-1}\) of dry solids. Similarly, Soares *et al.* (1995) evaluated the microbial quality of composted biosolids. They reported that 5 out of 16 samples contained more than 1000 MPN g\(^{-1}\) of *E. coli*, which is more than the currently accepted level for Class A biosolids (US EPA 1992).

Most authors consider *Salmonella* to be the most important pathogen in terms of its regrowth potential in composted biosolids, followed by *Yersinia* and *E. coli*. Re-population of *Salmonella* in biosolids has been reported by several authors (Russ and Yanko 1981; Hussong *et al.* 1985; Millner *et al.* 1987; Burge *et al.* 1987). Regrowth
studies on *Salmonella* in composted biosolids and biosolids amended soils are summarised in Table 2.7. Most of the studies outlined in the Table 2.7 are limited by the fact that regular monitoring of *Salmonella* was not carried out, and more importantly monitoring was discontinued once *Salmonella* concentrations fell below the detectable limit.

**Table 2.7.** Regrowth of *Salmonella/ E. coli* in composted biosolids and biosolids amended soils.

<table>
<thead>
<tr>
<th>Description of study</th>
<th>Seeding</th>
<th>Duration of study</th>
<th>Regrowth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested, dewatered biosolids applied to top 10 cm soil</td>
<td>no</td>
<td>37 Weeks</td>
<td>Regrowth observed after <em>Salmonella</em> remained undetectable for 28 weeks. E. coli regrowth observed in first seven days and then population was monitored at monthly interval, <em>Salmonella</em> became undetectable after one month</td>
<td>Gibbs <em>et al.</em> 1995</td>
</tr>
<tr>
<td><em>Salmonella</em> seeded into compost</td>
<td>Yes</td>
<td>6 months</td>
<td>Regrowth observed after <em>Salmonella</em> remained undetectable for 28 weeks. E. coli regrowth observed in first seven days and then population was monitored at monthly interval, <em>Salmonella</em> became undetectable after one month</td>
<td>Hussong <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>E. coli</em> seeded into compost</td>
<td>Yes</td>
<td>5 days</td>
<td><em>E. coli</em> regrowth observed in two out of 16 samples</td>
<td>Soares <em>et al</em> 1995</td>
</tr>
<tr>
<td>Lagoon-stored biosolids injected into soil, spread on grass land and pasture</td>
<td>no</td>
<td>not stated</td>
<td><em>Salmonella</em> not isolated from field or pasture, <em>Salmonella</em> regrowth in grass land was observed</td>
<td>Wallis <em>et al.</em> 1984</td>
</tr>
<tr>
<td>Bagged compost was dried for one year and then re-wetted</td>
<td>no</td>
<td>21 days</td>
<td><em>Salmonella</em> was undetectable initially and maximum regrowth was observed after five days, followed by die-off</td>
<td>Russ and Yanko 1981</td>
</tr>
</tbody>
</table>
A study carried out by Gibbs et al. (1995) showed that Salmonella could regrow after being undetectable for 28 weeks. It should be noted that viable but non-culturable Salmonella and Campylobacter have been reported to exist in the environment (Turpin et al. 1993; Jones et al. 1990). Culture dependent methods combined with viable but non-culturable cells may lead to a serious underestimation of the Salmonella regrowth potential in compost.

From these studies, it appears that Salmonella can survive and multiply under certain conditions in composted biosolids. The factors that favour survival and regrowth of Salmonella in composted biosolids are not well understood. Consequently, in order to minimise the pathogen regrowth potential of composted biosolids a detailed knowledge of the factors affecting pathogen regrowth is required.

2.8.1. Factors affecting Salmonella regrowth in composted biosolids

The ability of enteric bacteria, particularly Salmonella, to multiply in biosolids is of great concern. The survival and multiplication of microorganisms in biosolids occurs only under favourable conditions. The factors which favour survival and growth of indigenous microorganisms, are also expected to favour regrowth of pathogens. Two types of factors affect regrowth of Salmonella in composted biosolids. Firstly, environmental factors such as temperature, moisture, and atmospheric radiation. Secondly, inherited properties of compost such as bio-available nutrients, the indigenous microbial population and pH. These factors are highly interdependent and pathogen
regrowth is expected to be influenced by the combined effect of these factors. Each factor has its own contribution towards the creation of ideal growth conditions. The major factors affecting regrowth of *Salmonella* in composted biosolids and compost based soil amendments products are discussed below.

2.8.1.1. Moisture content

Like all other biological processes, microbial growth depends on the availability of moisture. Moisture content in the biosolids affects the microbial activity in two ways. Firstly, inadequate moisture during the early stages of composting may lead to premature dehydration of the biosolids, halting bacterial activity and resulting in the production of biologically unstable product (De Bertoldi and Zucconi 1987). Such unstable product, with a high quantity of bio-available nutrients, is ideal for the regrowth of pathogens in the presence of adequate moisture. Secondly, water acts as medium for oxygen transfer to the bacterial cells. However, an excessively high moisture content may restrict oxygen supply by reducing the free air space.

*Salmonella* is known to survive over a long period at very low moisture contents (Yeager and Ward 1981). However, bacterial cell inactivation is faster at less than 50% moisture in compost (Ward *et al.* 1981). A moisture content of 20% was reported as a critical threshold for *Salmonella* regrowth (Russ and Yanko 1981; Yeager and Ward 1981). Soares (1996) reported that in composted biosolids, more rapid regrowth of *E. coli* takes place at 40% moisture content as compared to 30% moisture. McKinley and Vestal
(1985) observed no correlation between indigenous bacterial activity and moisture content over the range of 31 to 60% during the composting of biosolids. The moisture content in composted biosolids is usually high enough to support pathogen growth and wetting of stored biosolids due to rain can also bring moisture content above the critical threshold. Gibbs et al. (1995) observed regrowth of *Salmonella* in biosolids amended soil after onset of winter rainfall. This was possibly due to an increase in soil moisture with the rainfall. It should be noted that *Salmonella* remained undetected for 28 weeks prior to the onset of the rainy season in this study.

It appears that moisture content in excess of 30% is required for unhindered regrowth of *Salmonella*. In the case of stored biosolids or biosolids amended soils an increase in moisture content can take place with the onset of a rainy season, resulting in conditions favourable for *Salmonella* regrowth. Consequently, keeping moisture content below 30% to check *Salmonella* regrowth would only be feasible in covered or bagged composted biosolids.

### 2.8.1.2. Bio-available nutrients

The growth and multiplication of microorganisms depends upon the availability of bio-available carbon and nitrogen and trace elements in the composted biosolids. Nutrient related regrowth of pathogens often occurs in composted biosolids (Goldstein *et al.* 1988). Composted biosolids can provide essential nutrients for the re-population of surviving *Salmonella* in previously treated biosolids.
Russ and Yanko (1981) observed regrowth of *Salmonella* in composted biosolids containing approximately 23% volatile solids (VS). They further reported that *Salmonella* regrowth could not take place at less than 18% VS content. However, care must be taken to interpret the results, as availability of readily assimilable carbon may vary with the type of filling material. Total carbon content present in the composted biosolids should not be considered as bio-available carbon. Most of the available literature suffers from this defect and most of the authors have considered C/N ratio on a solids basis as truly representative of total available carbon. In similar studies, regrowth of *Salmonella* had been reported to take place at more than a 15:1 carbon to nitrogen ratio (Russ and Yanko 1981; Pereira-Neto *et al.* 1986). On the basis of total VS content it cannot be concluded that at less than a 15:1 carbon to nitrogen ratio pathogen regrowth potential is prevented.

A water-soluble nutrient extract of compost has been reported to provide all the essential nutrients for the regrowth of *Salmonella typhimurium* (Burge *et al.* 1987). They found a positive correlation between growth rate, total growth and amount of water extractable carbon in compost. However, no literature was found in which the C/N ratio on a water-soluble basis has been used to predict the pathogen regrowth potential. Additional research is required to establish critical threshold levels of C/N on a water extract basis.

Hussong *et al.* (1985) tested composted biosolids for *Salmonella* regrowth from 30 compost facilities and found that 93% of the collected samples provided nutrients for regrowth. Moreover, composted biosolids are frequently mixed with other ingredients to
make soil amendments, which could provide additional sources of nutrients. As discussed in Section 2.4.1, with slow degradation of complex polymers, simple sugars are continuously released for bacterial consumption.

Following composting, biosolids are generally stockpiled for a period of time to further stabilise organic matter (Epstein 1978). Stabilisation of compost is also believed to reduce the bio-available nutrients (Inbar et al. 1990). However, complete stabilisation through storage is not desirable, as the nutrient quality of compost declines with storage (Haug 1993). Consequently, due to the slow release of nutrients, bio-available nutrients are expected to be present in finished compost.

2.8.1.3. Microbial competition and antagonism

Microbial competition and antagonism have been reported to play a crucial role in the inactivation of pathogens during composting (De Bertoldi et al. 1990). An enormous number of native saprophytes bring about the composting of biosolids. Survival of pathogens depends upon the microbial competition and antagonistic effect of native microflora. The antagonistic effect and competition for nutrients increase at high population densities, which results in the elimination of weak, rival microorganisms such as pathogens. Competition for nutrients can be an important factor in regrowth of Salmonella, especially when nitrogen or other essential elements are limiting.
Yeager and Ward (1981) observed suppression of regrowth and rapid die-off of seeded *S. typhimurium* in the presence of faecal coliforms in dewatered biosolids. Similarly, Hussong *et al.* (1985) monitored regrowth of seeded *Salmonella* in sterile and non-sterile compost. They concluded that competition from indigenous microorganisms suppressed *Salmonella* growth even in the presence of available nutrients.

There is a paucity of information on the role of indigenous microbes in suppression of pathogen regrowth. There appears to be only one study carried out by Millner *et al.* (1987) to understand the antagonistic effect of indigenous microbes towards *Salmonella*. They observed inhibition of seeded *Salmonella* regrowth in compost collected from the mesophilic (20 to 40 °C) zone of a compost pile. The suppression of *Salmonella* regrowth (75%) by faecal coliforms was found to take place only when coliform bacteria were allowed to colonise sterilised compost prior to *Salmonella* inoculation. This shows that an established population of indigenous bacteria could possibly suppress regrowth of *Salmonella* in the event of secondary contamination. Bacteria and actinomycetes were reported to be better competitors as compared to fungi in composted biosolids (Millner *et al.* 1987). This observation is supported by the fact that fungi are generally poor competitors as compared to bacteria for bio-available nutrients (Section 2.5.3).

The suppression of pathogen growth is also influenced by the type and relative number of different indigenous microorganisms present (Millner *et al.* 1987). The role of higher concentrations of indigenous microbes in suppression of *E. coli* regrowth even in the presence of available carbon was highlighted by Soares *et al.* (1995). Soares *et al.* (1995)
reported that growth of indigenous microbes in compost is limited by moisture stress during the composting process, which ultimately results in a higher pathogen regrowth potential. It appears that a healthy population of indigenous microbes, both in number and diversity, is very important for suppression of pathogen regrowth.

From this literature review, it is clear that there is very limited information available on the role of indigenous microorganisms in suppression of pathogen regrowth in composted biosolids. To date it is not known which group or groups of microorganisms are more effective in suppression of *Salmonella* regrowth. One important reason for the scarcity of information is the complex nature of different communities of microorganisms, which are active at different stages of the composting process (Herrmann and Shann, 1997). Furthermore, very little research has been carried out on characterisation of indigenous microorganisms from composted biosolids. To understand the mechanism of *Salmonella* regrowth inhibition, understanding the nature of indigenous microorganisms and their activity is required.

2.8.1.4. Somatic *Salmonella* bacteriophage

The use of somatic coliphage (Cornax *et al.* 1991), F-specific RNA bacteriophage (Havelaar and Hogenboom 1984; Handzel *et al.* 1993) and *Bacteroides fragilis* bacteriophage (Tartera and Jofre 1987) as indicators of enteric virus contamination of drinking and environmental water is well documented. Coliphage (Shaban 1999) and bacteriophage f2 (Burge *et al.* 1981) have also been suggested as indicators for pathogen
inactivation during wastewater biosolids composting. Wastewater sludge is an abundant source of a variety of bacteriophage. The majority of these are coliphages, followed by F-specific phages (Havelaar and Hogenboom 1984). F-specific RNA bacteriophages are considered a useful tool for predicting the behaviour of human enteric viruses during wastewater treatment processes (Havelaar et al. 1986), as they are structurally similar to enteroviruses and norwalk like viruses (Handzel et al. 1993). Most of the information available on the presence and behaviour of bacteriophages in wastewater sludge samples is on coliphages and F-specific RNA bacteriophages.

The somatic coliphage concentrations in wastewater can be as high as 15,900 plaque-forming units (pfu) mL\(^{-1}\) (Dhillon et al. 1970). Some of the coliphages are specific to \(E.\ coli\). Whereas, some of the coliphage, such as \(\phi X174\) and T-phages, show broad hosts range and are known as enterobacter phages (Douglas 1975). Bacteriophage \(\phi X174\) is known to infect \(Salmonella, E.\ coli,\) and \(Shigella\) (Lindberg 1973).

Coliphages in wastewater are mostly absorbed onto solids (Ketratanakul and Ohgaki 1988) and are known to concentrate in wastewater biosolids (Straub et al. 1993). Inactivation of viruses during secondary sludge treatments may differ considerably (Irving and Smith 1981). Consequently, coliphage and other bacteriophages of enterobacters are expected to be present in wastewater biosolids. Due to the relative abundance of coliphages in the environment they are more extensively studied as compared to the other bacteriophage. There is a paucity of information about the concentration of SS phage in wastewater biosolids and composted biosolids.
S. typhimurium WG49 is a specifically engineered bacteria to track FRNA coliphages. However, this strain is also attacked by Salmonella (SS) phage found in environmental waters (Handzel et al. 1993; Stetler and Williams 1996). More recently, somatic S. typhimurium phages were also found in ground water samples (Williams and Stetler 1994). The concentration of SS phage in environmental waters is highly variable (Handzel et al. 1993). These authors reported that up to 70% of the phage detected on WG49 were SS phages. Rhodes and Kator (1991) examined 300 phages from water subjected to faecal pollution for host specificity, and reported that more than 94% were SS phages. It has been reported that due to the presence of high concentrations ($10^{-2}$ mL$^{-1}$) of host bacteria, bacteriophage can multiply in wastewater sludge (IAWPRC et al. 1991).

There is no available information on the behaviour of bacteriophage during composting. Available literature on virus survival in soil may serve as a useful model for the behaviour of enterobacter phage in compost. Bacteriophages and viruses are generally found in most soils (Ostle and Holt 1979) and are known to survive in stored sludge soils treated with effluent up to 6 months (Sorber and Moore 1987; Tierney et al. 1977). The survival of viruses in soils is known to be influenced by soil texture, temperature, moisture (Straub et al. 1993), organic matter and pH (Sobers and Moore 1987).

The presence and survival of SS phages in composted biosolids has not been investigated so far. Due to the repeated phage multiplication cycles in the presence of lysogenic strains of bacteria it is believed that the concentration of host bacteria is influenced by
bacteriophages (Ogata et al. 1980). However, the role of somatic Salmonella phages in suppression of Salmonella regrowth in composted biosolids is not known.

2.8.1.5. Temperature

Temperature is one of the major factors affecting survival and regrowth of bacteria in composted biosolids. Russ and Yanko (1981) observed rapid growth of seeded Salmonella at a mesophilic temperature range (20 to 40 °C) in composted biosolids. Similarly, Yeager and Ward (1981) concluded that the ideal temperature for Salmonella regrowth is 37 °C. These findings suggest that Salmonella growth can take place during the warmer months of the year. However, an ideal regrowth temperature is required in conjunction with other favourable factors such as moisture content, available nutrients and absence of competing microbes, for regrowth to occur.

2.8.1.6. pH

It is well established that extremely high (more than 10) or low (less than 5.0) pH inhibits growth of enteric bacteria. Hussong et al. (1985) reported that the ideal pH for Salmonella regrowth is between 5.0 to 7.7. During the initial stages of composting pH may drop to 4.5 (Goluke 1991) due to the degradation of polysaccharides and cellulose to organic acids. However this stage is transitory only as microorganisms capable of utilising organic acids flourish under these conditions and as a result pH increase to slightly alkaline. During maturation stage pH of compost stays between 7.0 to 8.0.
Consequently, under normal composting conditions the pH of composted biosolids remains close to neutral. Therefore, pH is not a limiting factor for *Salmonella* regrowth under normal circumstances. However, it is possible that when compost is mixed with other ingredients the pH might change.

### 2.8.2. Relationship between regrowth of pathogens and indicator bacteria

Pathogen regrowth in composted biosolids is a relatively new field of research and limited work has been carried out to explore the relationship between traditionally used indicators and pathogens. Regrowth of FC and FS have been reported in stored biosolids (Gibbs *et al.* 1997), composted biosolids (Soares *et al.* 1995), compost based amendments (Skanvis and Yanko 1994) and biosolids amended soils (Wallis *et al.* 1984). However, limited information is available on the relationship between faecal indicator and *Salmonella* regrowth in composted biosolids.

Skanavis and Yanko (1994) observed a high density of coliforms in compost based soil amendments containing a high density of *Salmonella*. Additionally, *Salmonella* was not detected by routine analysis from samples having coliform densities less than 1000 MPN g\(^{-1}\). Similarly Yanko (1980) also reported that the chances of *Salmonella* detection are rare when FC concentration is less than 1000 MPN g\(^{-1}\). However, this criterion might not be applicable in the case of *Salmonella* regrowth in composted biosolids.
Gibbs et al. (1994) observed repopulation of *Salmonella*, FS and FC in stored biosolids and biosolids amended soil. However, regrowth of *Salmonella* was not correlated to faecal indicators. Soares et al. (1995) investigated the regrowth of *E. coli* and TC in composted biosolids. Simultaneous regrowth of *E. coli* and TC was observed. However, no correlation between the two was reported. From the available literature it is clear that regrowth of faecal indicators and *Salmonella* occur under similar conditions. However, on the basis of the available information no conclusion could be drawn on the correlation between the regrowth of pathogen and indicator microorganisms. Further research is required to establish the existence of any relationship between the regrowth of *Salmonella* and faecal indicators.

### 2.8.3. Pathogen regrowth potential of composted biosolids

Wastewater biosolids are stabilised through composting to reduce pathogens, eliminate offensive odours and reduce the potential for putrefaction. However the reduction of pathogen concentrations through composting does not guarantee the bio-safety of the product. Most of the methods developed to assess compost stability such as carbon to nitrogen ratio, volatile solids and cation exchange capacity are inadequate to address the issue of regrowth. These procedures do not distinguish between bio-available carbon and total residual carbon (Inbar et al. 1990). Typical microbiological assays such as respiration rate, microbial activity and biomass are also not sufficient to ensure the bio-safety of compost. Monitoring faecal indicators is more useful than the other procedures.
However, the relationship between *Salmonella* growth and changes in indicator concentration is not well established.

As explained earlier, regrowth of pathogens in composted biosolids is affected by a number of parameters, which are highly interdependent. In the presence of readily assimilable nutrients the chances of pathogen regrowth are greater, provided that other conditions such as moisture and temperature are adequate. However, limited information is available on the role of bio-available nutrients and the antagonistic effect of indigenous microflora on pathogen regrowth. The relationship between these factors and the maturity of the compost is also not clear.

There can be two possible ways to predict the pathogen regrowth potential. First, to have a detailed knowledge of factors affecting regrowth and their interaction with each other, so that by testing various parameters the possibility of regrowth can be estimated. Second a regrowth potential test where a suitable indicator organism can be used to estimate the pathogen regrowth potential. The first option is difficult because of the complexities and interactions of different compost factors. The second option can be more useful as it can provide a direct estimation in a relatively short time.

According to the US EPA guidelines (US EPA 1992) composted biosolids need to be monitored for indicator organisms or *Salmonella* prior to distribution. However, composted biosolids which meet microbiological criteria can, under certain conditions, support pathogen regrowth (Soares 1996). Under those circumstances a regrowth
potential test could be a very useful tool to ensure the bio-safety of the product. It could provide an estimate of the potential regrowth of pathogens in a given lot of compost. The importance of regrowth potential test as a parameter to measure compost stability was previously highlighted by Soares (1996). However, further verification of the usefulness of a pathogen regrowth potential test is required.

2.9. Conclusions

It appears that *Salmonella* regrowth in composted biosolids can lead to a potential public health hazard. However, to date there has been no satisfactory way of predicting the pathogen regrowth potential of composted biosolids. Estimation of potential pathogen regrowth could be very useful to assess the risks associated with the use of composted biosolids.

Regrowth of pathogenic bacteria is controlled by a number of factors, some of which are inherited properties, and others are environmental factors. Limited information is available on the factors affecting pathogen regrowth. It is believed that storage of biosolids is effective in reducing the pathogen regrowth potential by reducing the bioavailable nutrients. However, it has not been established how much storage is sufficient. To be able to control pathogen regrowth in finished products further information on the factors affecting *Salmonella* repopulation is required, especially the role of indigenous microorganisms in suppression of regrowth.
The main aim of this project was to investigate the pathogen regrowth potential of composted biosolids, and to improve understanding of the role of bio-available nutrients and the indigenous microflora in suppression of *Salmonella* regrowth.
CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1. Introduction

This chapter describes general materials and methods that were used in the study. Details of the general procedures adopted for all the experimental work are provided in this section. Details of the specific materials and methods related to each chapter are described at the beginning of each chapter.

3.2. Composting facility

Composted biosolids samples used in this study were collected from a commercial composting plant in Western Australia. This facility received anaerobically digested and de-watered biosolids from a wastewater treatment plant. The composting of biosolids at this composting facility was carried out in open windrows placed longitudinally side by side. The windrows were turned at weekly intervals to maintain aerobic conditions. The windrows were a mixture of 28% biosolids, 16% peat and the remaining 56% was made up of sawdust, finely ground wood chips and green garden waste. The windrows were approximately 20 m long, 4 m wide and 3.5 m high. A section of windrow that had been composting at the composting facility for 13 weeks was stored at Murdoch University. The stored pile comprised roughly 15 cubic meters of compost. The stored compost was used to collect samples at different maturity.
The temperature of biosolids composting for up to 60 days was measured previously at this facility by McInnes et al. (1997). The compost temperature measured at a depth of 0.3 m was found to be more than 53 °C in all the compost piles examined. However, at a depth of 1 m the temperature was found to be 50 °C on several occasions. This composting facility generally achieved the temperature-time requirements specified in ARMCANZ guidelines (ARMCANZ et al. 1997).

The reason for using composted biosolids from one composting facility was to minimise product variability. In this way the effect of the bulking materials and the composting process on the bio-available nutrients and indigenous microbial diversity in the compost samples used for this study was standardised.

### 3.3. Material and Methods

#### 3.3.1. Sampling and storage method

Composted biosolids samples were regularly collected from the composting facility and compost pile stored at Murdoch University. On each sampling occasion the same sampling procedure was adopted. Briefly, 5 replicate samples were collected at 5 randomly selected locations at a depth of 60 cm. Replicate samples were collected to account for the heterogeneity of composted biosolids. Samples were collected in sterile autoclave bags and were transported to the laboratory. Once in the laboratory the
material collected at each replicate sampling point was mixed by hand in sterile conditions to form a composite sample. The sample was stored at 4 °C in the refrigerator until used.

3.3.2. Sterilisation of samples

To sterilise the composted biosolids two different techniques were adopted. The first technique used for sterilisation was autoclaving. For sterilisation by autoclaving approximately 200 g of compost sample was taken in each flask and sealed with stoppers (made from cotton and cheesecloth) covered with aluminium caps. The flasks were sterilised at 121 °C for 20 min. and left for 24 h in order to give time for spore germination. The flasks were then again sterilised at 121 °C for 20 min prior to use.

The second technique used for sterilisation was irradiation of compost. For sterilisation by irradiation 500 g of composted biosolids was packed in sterilised autoclave bags and then placed in a cardboard box. The sample was sterilised by γ- radiation at 3 Mrads from a 60Co source. Both the methods were found to be equally effective. However, for convenience autoclaving was the preferred option.
3.4. Chemical preparations

3.4.1. Ringer’s solution

Quarter strength Ringers solution (Oxoid) was used to adjust the moisture content of the composted biosolids and for making dilutions. The quarter strength Ringers solution was made by dissolving one tablet in 500 mL of distilled water and autoclaving.

3.4.2. Buffered Peptone Water

Buffered Peptone Water (BPW) was used to culture bacteria and for pre-enrichment for enumeration of *Salmonella* throughout this study. The solution was prepared by dissolving 20 g of BPW powder (Oxoid) in 1000 mL of distilled water, followed by immediate autoclaving. The dilution bottles for the MPN (Most Probable Number) procedure were prepared by dispensing 9 mL BPW into 25 mL McCartney bottles and followed by autoclaving.

3.4.3. Rappaport Vassiliadis medium

Enrichment of cultures for enumeration of *Salmonella* was carried out in Rappaport Vassiliadis medium (RV). The RV medium was prepared by dissolving 30 g of RV powder (Oxoid) in 1000 mL of distilled water. Nine mL solution was then dispensed into 25 mL McCartney bottles and immediately autoclaved.
3.4.4. Xylose Deoxycholate agar

Xylose Deoxycholate agar (XLD) was used as a selective medium for the isolation of *Salmonella* and suppression of unwanted bacteria in the MPN procedure. XLD (Life Technologies) was prepared by dissolving 27.5 g powder in 500 mL of distilled water. The media was boiled in a microwave oven for 1 min. and then allowed to cool down. The media was poured into petri plates in a laminar flow cabinet.

3.4.5. Water extract of compost

The water-soluble components of compost are readily assimilated by microorganisms (Chanyasak and Kubota 1981). Consequently, the water-soluble nutrients can give an idea of substrate availability in composted biosolids. The total organic and nitrogen content in composted biosolids was determined from the water extract.

The water extract of composted biosolids was prepared by modifying the method described previously by Chanyasak and Kubota (1981). To prepare a water extract of compost, 20 g of composted biosolids sample was weighed into a flask and mixed with 150 mL of sterilised distilled water at 60 °C. The flask was shaken at room temperature for 30 min. and allowed to stand on a bench for sedimentation. The supernatant was filtered through a Whatman No 4 filter paper using vacuum filtration apparatus. Then the filtrate was passed through a membrane filter with a pore size of 0.45 μm. The filtrate was kept at −20 °C in a freezer until total carbon and nitrogen was determined.
3.5. Analytical Methods

3.5.1. Solids analysis

The solids analysis of composted biosolids samples included the determination of dry solids (DS), volatile solids (VS), moisture content and ash content. Standard gravimetric analysis (APHA et al. 1995) was used for the solids analysis of biosolids samples.

The sample (5 g) was placed in a pre-dried and weighed ceramic crucible. The sample was dried at 105 °C overnight and re-weighed. The remaining sample represented DS and the weight lost was the moisture content of the sample. The dry sample was placed in a furnace and heated to 550 °C for 3 h, and allowed to cool down in a desiccator prior to re-weighing. The remaining solids represented the ash content and weight lost was the VS content of the sample. The moisture content of compost samples was determined prior to the regrowth potential tests and wherever *Salmonella* was seeded in the composted biosolids.

3.5.2. Total Organic Carbon

The total organic carbon content of the composted biosolids samples was measured in the water extract. The procedure used for TOC determination was the combustion infrared method (APHA et al. 1995). A Shimadzu Corporation TOC analyser (Model TOC-5000A) was used for the analysis.
3.5.3. Total Organic Nitrogen

Total organic nitrogen in the composted biosolids samples was also measured in the water extract. The TON was determined by the difference of Total Nitrogen (Kjeldahl method) and the Ammonium Nitrogen (Nesslerization method). In both cases standard methods described in APHA et al. (1995) were used.
CHAPTER 4: ENUMERATION OF SALMONELLA IN COMPOSTED BIOSOLIDS AND DEVELOPMENT OF A PATHOGEN REGROWTH POTENTIAL TEST

4.1. Introduction

Digested wastewater sludge has been found to contain enteric viruses, parasites and enteric bacteria such as *Salmonella, Campylobacter* and *Shigella*. As discussed in Section 2.2.1, among the enteric bacteria, *Salmonella* is a pathogen of major concern. The concentration of *Salmonella* in digested sludge can be as high as 1 cell g\(^{-1}\) wet weight (Jewell *et al.* 1980). Composting of wastewater sludge is considered to be effective in inactivation of pathogens. However, as described in section 2.4 complete sterilisation is difficult to achieve. A number of studies have found that composted biosolids contain *Salmonella* (Yanko 1988; Hussong *et al.* 1985; Skanvis and Yanko 1994).

Composted biosolids are generally monitored for the traditional indicator organisms, such as faecal coliforms, faecal streptococci and *E. coli*, to ensure compost quality. Under the current US EPA guidelines (40 CFR Part 503), at the time of disposal of wastewater biosolids, the FC density should be less than 1000 MPN g\(^{-1}\) of total solids or less than 3 *Salmonella* cells 4 g\(^{-1}\) of total solids. Despite the widespread use of these guidelines, very limited research has been carried out on the enumeration of *Salmonella* from composted biosolids. This information is important since pathogen regrowth takes place in finished composts. As discussed in section 2.6 limited and often conflicting
information is available on the correlation between the concentration of *Salmonella* and indicator organisms in biosolids.

Consequently, testing for an indicator organism alone cannot ensure the bio-safety of composted biosolids. The exact behaviour of *Salmonella* in composted biosolids can only be predicted by monitoring for the pathogen of concern. However, *Salmonella* is often present below detectable limits and later on regrows to hazardous levels when the product is marketed (Skanavis and Yanko 1994). A more practical approach under those circumstances could be testing for the regrowth potential.

This chapter describes experiments that were undertaken to evaluate *Salmonella* inactivation in a full-scale windrow composting process, and to examine the possibility of *Salmonella* regrowth in stored biosolids after concentrations had dropped below the detection limit. To accomplish this goal *Salmonella* concentrations were monitored during the windrow composting process. *Salmonella* concentrations were also monitored in composted biosolids stored for up to 60 weeks.

As described in section 2.8 it has been found that *Salmonella* can regrow in composted biosolids. Consequently, experiments were conducted to select a suitable microorganism for a regrowth potential test. To accomplish this goal the growth kinetics of selected *Salmonella* serovars and *E. coli* strains in composted biosolids were compared.
4.2. Experimental approach

Monitoring for indigenous *Salmonella* was carried out in composted biosolids of different maturity and stored compost. The compost samples up to 13 weeks in age were collected from the composting facility described in section 3.2. Samples stored for more than 13 weeks were collected from the Murdoch University compost pile. The composted biosolids of different maturity were collected as outlined in Section 3.3.1. The presence of indigenous *Salmonella* in composted biosolids of different maturity was monitored twice for some of the compost ages. The collected samples were tested for the presence of *Salmonella* within a week of collection. The samples were refrigerated at 4 °C prior to analysis.

The *Salmonella* serovars used in this study were selected on the basis of their relative abundance in biosolids, and prevalence in the human population (Anon. 1994; Gibbs *et al*. 1994; Gibbs *et al*. 1997). Biosolids composting for two weeks were used in the regrowth experiments to ensure adequate bio-available nutrients for *Salmonella* regrowth. The composted biosolids samples were collected as outlined in Section 3.2.1. Pre-sterilised compost was used in the study to remove the effect of competing indigenous microflora on the growth kinetics of seeded bacteria.
4.3. Materials and Methods

4.3.1. Media preparation

The media preparation procedure is outlined in Section 3.4. BPW and RV media were prepared for pre-enrichment and enrichment of cultures. XLD and McConkey agar plates were used for plating and isolation of presumptive Salmonella.

4.3.2. Solids analysis

The moisture content, ash content, dried solids and volatile solids of compost samples were determined by the procedure outlined in Section 3.5.1.

4.3.3. Bacterial strains

Wild type isolates of S. typhimurium (9451), S. anatum (8414), S. mbandaka (9098), S. chester (8605), S. havana (7685), S. infantis (8527) and E. coli strains (NCTC 8196, ATCC 25922) used in this study were obtained from the PathCentre culture collection in Western Australia. Additional wild type strains of S. typhimurium (Mu-S5-83) and E. coli (MU-E9-83) used in this study were obtained from the Murdoch University culture collection.
4.3.4. Selection of antibiotic resistant *Salmonella*

A rifampicin resistant strain of *S. typhimurium* (9451) was produced by the method adopted by Rhodes *et al.* (1985). Briefly, 10 mL of *S. typhimurium* culture which was grown overnight in buffered peptone water (Oxoid) was added to 90 mL of buffered peptone water containing 50 μg mL⁻¹ of rifampicin. After incubation at 37 °C for 24 h rifampicin resistant colonies were isolated on nutrient agar (Difco) containing 50 μg mL⁻¹ of rifampicin.

4.3.5. Preparation of inoculum

Pure cultures of bacterial strains were grown separately for 24 h at 37 °C in 10 mL of buffered peptone water (Oxoid). Serial ten-fold dilutions were made in quarter strength Ringer’s solution (Oxoid) to a final dilution of 10⁶. The final dilution was used to seed composted biosolids for the regrowth potential tests.

4.3.6. Regrowth monitoring

To monitor the regrowth of indigenous *Salmonella* in compost samples, approximately 200 g of compost sample was placed in a sterile flask sealed with a sterile stopper (made from cotton and cheesecloth) and an aluminium cap was placed on top to prevent contamination. The moisture content in the flasks was found to be more than 45% and
was not adjusted. The flasks were incubated at 37 °C for 48 h prior to enumeration of the indigenous Salmonella concentration.

4.3.7. Regrowth potential test

The composted biosolids were sterilised twice by autoclaving at 121 °C for 20 min. on two consecutive days. Approximately 600 g of sterile biosolids was placed in a sterile beaker and seeded with 30 mL of the $10^6$ dilution of the pure bacterial culture. The sample was blended with a sterile hand held blender. The blended sample was transferred into three sterile one-litre flasks each receiving approximately 150 g of biosolids. The flasks were sealed with sterile stoppers (made from cotton and cheesecloth) and aluminium caps were placed on top to prevent contamination and infiltration of water drops. The moisture content after inoculation was adjusted to between 50 to 55%. The flasks were incubated at 37 °C in a water bath. The bacterial concentration in the samples was determined at regular intervals. Experiments were repeated at least twice for each bacterial strain.

Control experiments were also performed to ensure that sterilisation of compost was effective. For each batch of sterilised compost, approximately 200 g of pre-sterilised compost was placed in a flask and the moisture content was adjusted to 50% prior to incubation in a water bath at 37 °C for 5 days. After every 24 h, the incubated compost was checked for the growth of any indigenous microorganisms. Briefly, 10 g of compost was mixed with 90 mL of sterilised distilled water. Then 0.1 mL of this dilution was
spread plated on nutrient agar plates in duplicate. Plates were incubated at 37 °C for 24 h prior to checking for any bacterial growth.

4.3.8. Enumeration of *Salmonella*

*Salmonella* concentrations in composted biosolids were determined by using modified 5 tube Most Probable Number (MPN) (APHA *et al.* 1995) method for biosolids (Gibbs *et al.* 1994). A master dilution from a composite sample was prepared by blending 60 g of sample with 540 mL of Buffered Peptone Water (Oxoid). From this master dilution, four dilutions containing 0.1, 0.01, 0.001 and 0.0001 g of biosolids in 10 mL of Buffered Peptone Water (BPW) were prepared for pre-enrichment. Pre-enrichment of samples was carried out for 24 h at 37° C. The enrichment of cultures was carried out in RV. Aliquots of 0.1 mL of each pre-enrichment were enriched in 9 mL of RV medium at 42 °C. After 24 and 48 h of incubation the enriched cultures were streaked onto XLD agar plates and incubated at 37° C for 24 h. After incubation plates were observed for typical *Salmonella* colonies with pink borders and black centres. The presumptive *Salmonella* were purified on MacConkey Agar without salt and confirmed by biochemical reactions using API 20E-biochemical-test strip (bioMerieux). The concentration of *Salmonella* in the compost samples was calculated from a standard MPN chart (APHA *et al.* 1995). MPN analysis was always performed in duplicate.
4.3.9. Enumeration of *E. coli*

*E. coli* was enumerated by membrane filtration (APHA *et al.* 1995) using m-FC agar (Difco) as the plating media. Series of decimal dilutions were made in sterile Ringer’s solution. One mL of each dilution was passed through duplicate 0.45 μm membrane filters (Millipore) under vacuum and filters were placed on m-FC agar plates. The colony forming units (cfu) were counted after incubating plates in a sealed container immersed in a water bath at 37 °C for 24 h.

4.3.10. Statistical analysis

StatView software was used to perform a student t-test to assess differences in specific growth rates between *Salmonella* serovars and *E. coli* strains. The critical P-value for the two-tailed t-test was set at 0.05.

4.4. Results

4.4.1. *Salmonella* concentrations in composted biosolids

*Salmonella* concentrations were monitored during the composting process and storage of compost for up to 60 weeks. *Salmonella* enumeration results in composted biosolids of different maturity are presented in Fig. 4.1.
Figure 4.1. Salmonella concentrations in composted biosolids of different maturities.

The lower detection limit of this MPN procedure is 0.02 g⁻¹. Consequently, samples in which Salmonella was not detected were taken as less than 0.02 MPN g⁻¹. The Salmonella concentration declined from 1.1 g⁻¹ initially (2 weeks) to 0.8 g⁻¹ after 5 weeks of composting. Thereafter Salmonella concentrations fell below the detectable limit. However, Salmonella (0.2 g⁻¹) was later detected in the compost pile after it had been stored for 26 weeks.

Table 4.1 shows that limited regrowth of indigenous Salmonella in biosolids composting for two weeks was observed. Other compost samples tested were found to be free of indigenous Salmonella and no regrowth was observed after incubation at 37 °C for 48 h.
Table 4.1. Regrowth of indigenous *Salmonella* in composted biosolids of different maturity after incubation at 37 °C for 48 h.

<table>
<thead>
<tr>
<th>Age</th>
<th>Initial <em>Salmonella</em> con.</th>
<th><em>Salmonella</em> con. after 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>1.00</td>
<td>1.50</td>
</tr>
<tr>
<td>5 weeks</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>7 weeks</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>10 weeks</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>13 weeks</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>5 months</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>8 months</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* *Salmonella* concentration measured in MPN g⁻¹.

The solids analysis results are presented in Table 4.2. The moisture content in the samples used for monitoring *Salmonella* concentrations in composted biosolids was found to be more than 50%. The exception was biosolids stored for 52 weeks or more which had less than 30% moisture.

Table 4.2. Solids analysis of composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Maturity (weeks)</th>
<th>Moisture content (%)</th>
<th>Volatile solids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>60.95</td>
<td>72.37</td>
</tr>
<tr>
<td>5</td>
<td>60.42</td>
<td>65.18</td>
</tr>
<tr>
<td>7</td>
<td>56.09</td>
<td>55.43</td>
</tr>
<tr>
<td>10</td>
<td>49.77</td>
<td>51.76</td>
</tr>
<tr>
<td>13</td>
<td>55.40</td>
<td>47.72</td>
</tr>
<tr>
<td>20</td>
<td>55.23</td>
<td>59.40</td>
</tr>
<tr>
<td>26</td>
<td>55.20</td>
<td>46.64</td>
</tr>
<tr>
<td>52</td>
<td>30.30</td>
<td>36.35</td>
</tr>
<tr>
<td>60</td>
<td>28.43</td>
<td>31.75</td>
</tr>
</tbody>
</table>
The maturation of compost with time resulted in a decline in the volatile solids content and increase in the ash content (Table 4.2).

The average weekly rainfall recorded at Murdoch University during the storage of the compost pile from October 1996 to June 1997 is presented in Figure 4.2.

![Figure 4.2. Average weekly rainfall recorded at Murdoch University during the storage of composted biosolids.](image)

As can be seen from the Fig. 4.2, there was rainfall between week 20 and 26 after a dry spell. The compost pile was sampled during week 26.
4.4.2. Reproducibility of growth curves

Each group of growth experiments was carried out in duplicate. This is illustrated by Fig. 4.3, which shows duplicate growth curves of *S. typhimurium* and *E. coli* in sterilised biosolids.

**Figure 4.3.** Replicate growth curves of *E. coli* (NCTC8196) (○, *E. coli* first replicate; ●, *E. coli* second replicate) and *S. typhimurium* (9451) (▲, *S. typhimurium* first replication; △, *S. typhimurium* second replication).

For each *Salmonella* serovar or *E. coli*, there was good agreement between replicate data.
4.4.3. Growth of *Salmonella* serovars

Bacterial growth curves for *Salmonella* serovars from the first group of experiments are presented in Fig. 4.4. All the *Salmonella* serovars tested showed logistic growth curves (Fig. 4.4). The seeded bacteria grew rapidly in sterile composted biosolids and attained the stationary growth phase after 30 h of incubation.

![Growth curves for seed *Salmonella* serotypes in composted biosolids.](image)

**Figure 4.4.** Growth curves for seed *Salmonella* serotypes in composted biosolids. ○, *S. anatum* (8414); △, *S. chester* (8605); □, *S. mbandaka* (9098); ●, *S. infantis* (8527); ▲, *S. havana* (7685); ■, *S. typhimurium* (9451R); ×, *S. typhimurium* (9451); +, *S. typhimurium* (Mu-S5-83). R = Rifampicin resistant strain.

Results for the growth kinetics of bacterial strains tested are summarised in Table 4.3.
**Table 4.3.** Bacterial growth kinetics

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Specific growth rate (h⁻¹)</th>
<th>Generation time (h)</th>
<th>Maximum observed growth (MPN g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> (Mu-S5-83)ᵃ</td>
<td>0.55</td>
<td>1.26</td>
<td>100 x10⁸</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (Mu-S5-83)ᵇ</td>
<td>0.55</td>
<td>1.27</td>
<td>93 x10⁸</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (9451)ᵃ</td>
<td>0.55</td>
<td>1.26</td>
<td>125 x10⁸</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (9451)ᵇ</td>
<td>0.53</td>
<td>1.30</td>
<td>100 x10⁸</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (9451)Rᵃ</td>
<td>0.55</td>
<td>1.25</td>
<td>130 x10⁸</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (9451)Rᵇ</td>
<td>0.54</td>
<td>1.30</td>
<td>90 x10⁸</td>
</tr>
<tr>
<td><em>S. anatum</em> (8414)ᵃ</td>
<td>0.53</td>
<td>1.31</td>
<td>23 x10⁸</td>
</tr>
<tr>
<td><em>S. anatum</em> (8414)ᵇ</td>
<td>0.50</td>
<td>1.39</td>
<td>24 x10⁸</td>
</tr>
<tr>
<td><em>S. mbandaka</em> (9098)ᵃ</td>
<td>0.50</td>
<td>1.38</td>
<td>13 x10⁸</td>
</tr>
<tr>
<td><em>S. mbandaka</em> (9098)ᵇ</td>
<td>0.50</td>
<td>1.40</td>
<td>11 x10⁸</td>
</tr>
<tr>
<td><em>S. chester</em> (8605)ᵃ</td>
<td>0.51</td>
<td>1.37</td>
<td>9 x 10⁸</td>
</tr>
<tr>
<td><em>S. chester</em> (8605)ᵇ</td>
<td>0.49</td>
<td>1.41</td>
<td>7 x 10⁸</td>
</tr>
<tr>
<td><em>S. havana</em> (7685)ᵃ</td>
<td>0.50</td>
<td>1.38</td>
<td>11 x 10⁸</td>
</tr>
<tr>
<td><em>S. havana</em> (7685)ᵇ</td>
<td>0.50</td>
<td>1.38</td>
<td>11 x 10⁸</td>
</tr>
<tr>
<td><em>S. infantis</em> (8527)ᵃ</td>
<td>0.53</td>
<td>1.32</td>
<td>17 x 10⁸</td>
</tr>
<tr>
<td><em>S. infantis</em> (8527)ᵇ</td>
<td>0.51</td>
<td>1.36</td>
<td>11 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC 8196*ᵃ</td>
<td>0.52</td>
<td>1.33</td>
<td>29 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC 8196*ᵇ</td>
<td>0.52</td>
<td>1.33</td>
<td>28 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922*ᵃ</td>
<td>0.53</td>
<td>1.30</td>
<td>25 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922*ᵇ</td>
<td>0.50</td>
<td>1.40</td>
<td>28 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> (Mu-E9-83)*ᵃ</td>
<td>0.50</td>
<td>1.39</td>
<td>22 x 10⁸</td>
</tr>
<tr>
<td><em>E. coli</em> (Mu-E9-83)*ᵇ</td>
<td>0.50</td>
<td>1.39</td>
<td>19 x 10⁸</td>
</tr>
</tbody>
</table>

R = Rifampicin resistant strain, a = first set of data, b = second set of data, * Growth quantified as cfu.

The maximum population density attained by *S. typhimurium* was significantly higher when compared to the isolates of other *Salmonella* serovars tested (t₀.₀₀₂ (14) = 2.415 which is less than 16.143, calculated value). The isolates of *S. typhimurium* also showed
a significantly higher specific growth rate compared to the isolates of other serovars (t_{0.05/2} (14) =2.145 which is greater than 6.239, calculated value).

4.4.4. Growth of E. coli strains

E. coli strains also grew rapidly in the sterilised composted biosolids and reached the stationary growth phase after 30 h (Fig. 4.5).

![Growth curves for seeded E. coli in sterile biosolids.](image)

**Figure 4.5.** Growth curves for seeded E. coli in sterile biosolids. ○, E. coli (NCTC8196); Δ, E. coli (Mu-E9-83); □, E. coli (ATCC25922).

The specific growth rate and doubling time for all the three strains tested were similar. Maximum observed bacterial concentrations varied from $19 \times 10^8$ to $29 \times 10^8$ g\(^{-1}\) (Table 4.3).
The specific growth rate of the *E. coli* strains in composted biosolids was significantly less than the specific growth rate of *S. typhimurium* ($t_{0.05/2} (10) = 2.228$ is lower than calculated value 5.218). The maximum population density of *E. coli* was also significantly lower as compared to *S. typhimurium* ($t_{0.05/2} (10) = 2.228$ is lower than calculated value 11.436).

4.5. Discussion

4.5.1. *Salmonella* concentrations in composted biosolids

The results of enumeration of *Salmonella* concentrations in composted biosolids on two separate occasions (Fig. 4.1 and Table 4.1), suggest that if composting is carried out in accordance with the recommended guidelines (ARMCANZ *et al.* 1995) then *Salmonella* should be reduced below the detection limit. The present results are in accordance with the earlier reported results by Shaban (1999).

However, the re-population of *Salmonella* in stored biosolids after 26 weeks when *Salmonella* remained undetectable previously, suggests that *Salmonella* can survive the composting process in low numbers and later on re-grow under favourable conditions. It is worth noting that prior to collection of the sample for analysis during week 26 there was rainfall between week 20 to 26. Consequently, it is likely that *Salmonella* regrowth was due to an increase in moisture content and/or increased availability of from leaching.
in the compost pile. Russ and Yanko (1981) have previously reported the survival of low numbers of *Salmonella* during composting. Yanko (1988) also showed that high numbers of *Salmonella* were detected in some stored biosolids samples, suggesting a regrowth phenomenon. Regrowth of *Salmonella* in stored biosolids after rainfall has also been reported earlier by Gibbs *et al.* (1997).

It is possible that *Salmonella* became non-culturable after 5 weeks of composting and reverted back to a culturable form after week 26, with the return of favourable growth conditions. The conversion of culturable *Salmonella* cells into viable but non-culturable form in soil has been reported (Turpin *et al.* 1993). However, from the solids analysis during the period when *Salmonella* were not detected, the moisture and nutrients were not limiting factors for growth.

It is also possible that detection of *Salmonella* during week 26 could be due to contamination of the compost pile. However, since the compost samples were collected from 5 randomly selected sites at a depth of 60 cm, surface contamination by bird droppings or faeces from small animals is not expected to result in an increase in *Salmonella* concentration.

### 4.5.2. Selection of a suitable indicator for a pathogen regrowth potential test

The *Salmonella* serovars and *E. coli* strains that were seeded into composted biosolids grew rapidly reaching more than $10^8$ organisms g$^{-1}$ after 30 h of incubation. This rapid
growth of seeded bacteria in sterilised biosolids suggests that a very limited time was taken by the laboratory grown cultures to adjust to the new environment, which is one of the desired characteristics of an indicator organism. The final bacterial concentrations for all the *Salmonella* serovars in composted biosolids were similar to those reported by other authors (Brandon *et al.* 1977; Hussong *et al.* 1985). The generation time for *S. typhimurium* in this study (0.55 h\(^{-1}\)) was lower than reported by Hussong *et al.* (1985) in a study of irradiated compost (0.65 h\(^{-1}\)). This may have been because the biosolids used in this study had only been composting for two weeks, so may have contained more biodegradable nutrients than the biosolids described by Hussong *et al.* (1985), which were fully composted.

Differences between the growth rates and the maximum concentrations for the *Salmonella* serovars and *E. coli* strains appeared to be small, but were found to be significant. It was found that *S. typhimurium* grew significantly faster and achieved a significantly higher cell count in composted biosolids than *E. coli* and the isolates of the other *Salmonella* serovars tested. These results suggest that *S. typhimurium* could not be used to accurately predict the growth of other *Salmonella* serovars or *E. coli*. However, the results suggest that *S. typhimurium* could serve well as an indicator in a test for the pathogen regrowth potential of composted biosolids, as *S. typhimurium* showed a higher maximum growth rate and cell count as compared to other microorganisms. It therefore should provide a measure of the maximum regrowth potential for other enteric bacteria. *S. typhimurium* was therefore selected as the seed organism for the regrowth potential studies described in chapter 5.
In some situations *S. typhimurium* may not be a suitable indicator of pathogen regrowth potential. If other organisms that were not tested in this study were of particular interest, then these would need to be tested independently. On the basis of present results, *E. coli* is not recommended as the sole indicator in a regrowth potential test as it may provide an underestimation of the pathogen regrowth potential, particularly for *S. typhimurium*.

Sterilised compost was used in these experiments to provide uniformity between experiments and to ensure that indigenous *Salmonella* did not interfere with the results. In sterilised compost, growth was rapid and a high maximum growth was achieved. However, in further experiments non-sterilised compost was used to provide a more realistic prediction of possible regrowth. In non-sterilised compost, the regrowth potential was expected to be lower due to competing indigenous microorganisms (Hussong *et al.* 1985). Also in non-sterilised compost, indigenous *Salmonella* may interfere with the test. This problem was overcome by using antibiotic-resistant *S. typhimurium*. 
CHAPTER 5: SALMONELLA GROWTH IN STERILISED AND NON-STERILIZED COMPOSTED BIOSOLIDS

5.1. Introduction

The regrowth of surviving Salmonella in stored biosolids, compost and compost based amendments has been reported by several authors (Yeager and Ward 1981; Hussong et al. 1985; Skanvis and Yanko 1994; Gibbs et al. 1997). Re-population of Salmonella in finished products under certain conditions can pose a health hazard. As mentioned earlier (Section 2.8.1) re-population of Salmonella is controlled by environmental factors and inherited properties of composted biosolids. It is known that for regrowth to occur, certain conditions such as presence of microorganisms, bio-available nutrients, suitable moisture and temperature and lack of competition from indigenous microorganisms are mandatory. These are highly interdependent and each factor has its own contribution towards creation of favourable growth conditions.

The majority of previous studies carried out on pathogen regrowth have focused on the role of physical factors such as temperature, moisture and nutrients in Salmonella regrowth (Yeager and Ward 1981; Hussong et al. 1995; Soares et al. 1995). These have been discussed previously in Section 2.8.1.
Regrowth and inactivation of *Salmonella* in compost is also influenced by the indigenous microflora (Hussong *et al.* 1985; Millner *et al.* 1987). Previous studies have shown that *Salmonella* can regrow to hazardous levels (more than $10^7$ g$^{-1}$) in sterilised composts (Brandon *et al.* 1977; Hussong *et al.* 1985). This indicates a greater regrowth potential in the absence of indigenous microorganisms. The composting process is very dynamic in terms of the predominance of specific group of microorganisms during the specific stages of composting, including storage. The microbial community structure during the composting process is determined by many factors such as temperature, moisture, and nutrients (Beffa *et al.* 1996). The predominance of microbial communities is determined by a continuous change in the physical and chemical conditions during composting (Miller 1992). The factors which favour regrowth of indigenous microorganisms are expected to support pathogen regrowth. The role of indigenous microorganisms in suppression of *Salmonella* regrowth in the presence of favourable growth conditions is not clear. Moreover, very little is known about the effect of compost maturity on antagonistic microorganisms.

Following composting, biosolids are generally stockpiled for a period of time to further stabilise organic matter (Epstein 1978). Stabilisation of compost is also believed to reduce the bio-available nutrients (Inbar *et al.* 1990) for growth and consequently, limit the regrowth potential. However, complete stabilisation through storage is not desirable, as the nutrient quality of compost declines with storage (Haug 1993). Pathogen regrowth often takes place in previously composted biosolids and compost based soil amendments (Russ and Yanko 1981; Skanvis and Yanko 1994). Methods developed to assess compost
stability are mostly based on bioassays and compost extracts. Most of the procedures adopted to evaluate compost stability do not distinguish between residual carbon and bio-available carbon (Inbar et al. 1990). Consequently, parameters commonly used to ensure stability of compost such as C/N ratio, oxygen demand and respiration rate, may not be sufficient to ensure the bio-safety of composted biosolids.

The main objective of this chapter was to investigate the capacity of indigenous microorganisms to inhibit Salmonella regrowth in the presence of bio-available nutrients and favourable growth conditions. An additional objective was to examine the effect of compost maturation on the potential for Salmonella regrowth.

5.2. Experimental design

Composted biosolids of different maturity were used to evaluate the influence of indigenous microflora on the pathogen regrowth potential of composted biosolids. Regrowth of seeded S. typhimurium in composted biosolids of different maturity was monitored in sterilised and non-sterilised biosolids under laboratory conditions. To evaluate the relative importance of bio-available nutrients and indigenous microflora in suppression of Salmonella regrowth, regrowth potential results of composted biosolids of different maturity were compared.
5.3. Materials and Methods

5.3.1. Sample collection and storage

The composted biosolids samples of different maturity were collected from a commercial composting company and from a compost pile stored at Murdoch University as described in Section 3.2.1. Composite samples for regrowth potential tests were prepared as described in Section 3.2.1. Composite samples were then divided into two halves. One half was sterilised and the other was used un-sterilised for regrowth potential experiments.

5.3.2. Bacterial strain used in the study

A rifampicin resistant strain of wild type isolate of *S. typhimurium* was produced by the method adopted by Rhodes *et al.* (1985). The procedure used for the isolation was outlined previously in Section 4.3.2. The rifampicin resistant bacterial was maintained on XLD agar plates containing 50 µg mL⁻¹ of rifampicin.

5.3.3. Sterilisation of biosolids

Two different techniques were used for sterilisation of composted biosolids samples. In the first technique composted biosolids were sterilised twice by autoclaving at 121 °C for 20 min. on 2 consecutive days. In the second technique γ-radiation at 3 Mrads from a
$^{60}$Co source was used to sterilise composted biosolids samples. The detailed procedure was previously outlined in Section 3.2.2.

5.1.4. Preparation of inoculum

Pure cultures of rifampicin resistant *S. typhimurium* were grown in 10 mL of buffered peptone water (Oxoid) for 24 h at 37 °C. Serial ten-fold dilutions were made in quarter strength Ringer’s solution (Oxoid) to a final dilution of $10^6$. The final dilution was used to seed composted biosolids for the regrowth potential tests.

5.1.5. Regrowth potential test

The procedure adopted for regrowth potential tests for sterilised and non-sterilised samples of the same compost was outlined previously in Section 4.3.4. The moisture content of the compost sample was determined prior to the regrowth potential test as described in section 3.4.1. The moisture content in the samples containing less than 45% moisture was adjusted.

5.1.6. Enumeration of *Salmonella*

*Salmonella* concentrations in the composted biosolids were determined by using a standard 5 tube Most Probable Number (MPN) method as outlined in Section 4.3.3. The XLD agar plates used for isolation of seeded *S. typhimurium* contained 50 μg mL$^{-1}$ of
rifampicin to avoid interference from indigenous *Salmonella*. The MPN procedure was always performed in duplicate.

### 5.1.7. TOC and TON analysis

Total organic carbon and nitrogen in the water extract prepared from the composted biosolids samples was determined by the procedure outlined in Sections 3.4.2 and 3.4.3.

### 5.1.8. Statistical analysis

To compare the growth rate and inactivation rate of seeded *S. typhimurium* in composted biosolids of different maturity, the number of bacterial cells per gram of wet weight of compost were transformed into log$_{10}$ g$^{-1}$ of compost. Linear regression was used to determine the $k$ value (log$_{10}$ growth or reduction per hour) of *Salmonella* by the following equation: log$_{10}$ $N_t/N_0 = -mx + b$, where log$_{10}$ $N_t/N_0$ is the ratio of the log$_{10}$ value at time $t$ (measured in h) to the initial log$_{10}$ value (log $N_0$), $m$ is the slope of the curve, $x$ is the time in hours and $b$ is the intercept value. Microsoft Excel software was used to perform all statistical analysis. Regression analysis was used to determine the regression equations from the curves and the $k$ value was defined as $-m$. A Student $t$-test was performed to compare the maximum growth and inactivation rate of seeded *Salmonella* in composted biosolids. The critical $P$-value for the $t$-test was set at 0.05. To check the influence of indigenous microorganisms and age of compost on the specific
growth rate of *Salmonella*, two-way analysis of variance was performed and the critical
*P*-value was set at 0.05.

5.4. Results

5.4.1. Effect of autoclaving on growth potential

To evaluate the effect of autoclaving on the growth potential, the growth kinetics of *S.
*typhimurium* was compared in autoclaved and irradiated biosolids. Two different sample
types were selected for this study. These were a composted biosolids sample composting
for 2 weeks and a compost sample stored for 33 weeks. The regrowth curves and
regression analysis of *Salmonella* growth are presented in Fig. 5.1 and Table 5.1.
As can be seen from the growth curves in Fig. 5.1, there was good agreements between results of autoclaved and irradiated samples.
Table 5.1. Growth rate equations of *S. typhimurium* seeded in sterilised composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Treatment</th>
<th>Regression equation</th>
<th>$r^2$ (%)</th>
<th>Max. observed growth (MPN g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>autoclaved</td>
<td>$Y = 0.249x + 2.318$</td>
<td>99.2</td>
<td>$40 \times 10^8$</td>
</tr>
<tr>
<td>2 B</td>
<td>irradiated</td>
<td>$Y = 0.238x + 2.418$</td>
<td>98.4</td>
<td>$16 \times 10^8$</td>
</tr>
<tr>
<td>21</td>
<td>autoclaved</td>
<td>$Y = 0.186x + 2.422$</td>
<td>96.0</td>
<td>$4 \times 10^7$</td>
</tr>
<tr>
<td>33</td>
<td>autoclaved</td>
<td>$Y = 0.191x + 2.868$</td>
<td>92.2</td>
<td>$12 \times 10^7$</td>
</tr>
<tr>
<td>33 B</td>
<td>irradiated</td>
<td>$Y = 0.192x + 2.487$</td>
<td>97.8</td>
<td>$16 \times 10^7$</td>
</tr>
<tr>
<td>45</td>
<td>autoclaved</td>
<td>$Y = 0.163x + 2.439$</td>
<td>96.9</td>
<td>$8 \times 10^6$</td>
</tr>
<tr>
<td>65</td>
<td>autoclaved</td>
<td>$Y = 0.159x + 3.412$</td>
<td>92.3</td>
<td>$64 \times 10^6$</td>
</tr>
<tr>
<td>117</td>
<td>autoclaved</td>
<td>$Y = 0.151x + 2.612$</td>
<td>96.9</td>
<td>$8 \times 10^6$</td>
</tr>
<tr>
<td>117 B</td>
<td>autoclaved</td>
<td>$Y = 0.148x + 2.591$</td>
<td>98.0</td>
<td>$6 \times 10^6$</td>
</tr>
</tbody>
</table>

Regression equation is $y = mx + b$, where $(m)$ is the $k$ value, $x$ is the time in hours, and $\log_{10} N_t/N_0$.

There was good agreement between the regression analysis data from the irradiated and autoclaved composted biosolids.

TOC and TON concentration results are shown in Table 5.2. Autoclaving resulted in an increase in TOC and reduction in TON. However, changes in TOC and TON concentrations after autoclaving did not appear to increase the growth rate or maximum observed growth. Autoclaving was therefore used to sterilise biosolids in further experiments.
Table 5.2. Total organic carbon and nitrogen in the composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Treatment</th>
<th>TOC</th>
<th>TON</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Non-sterilised</td>
<td>2.236</td>
<td>1.663</td>
</tr>
<tr>
<td>2</td>
<td>Autoclaved</td>
<td>3.513</td>
<td>0.807</td>
</tr>
<tr>
<td>2</td>
<td>Irradiated</td>
<td>2.496</td>
<td>1.333</td>
</tr>
<tr>
<td>5</td>
<td>Non-sterilised</td>
<td>1.194</td>
<td>0.124</td>
</tr>
<tr>
<td>7</td>
<td>Non-sterilised</td>
<td>1.175</td>
<td>0.117</td>
</tr>
<tr>
<td>13</td>
<td>Non-sterilised</td>
<td>0.973</td>
<td>0.105</td>
</tr>
<tr>
<td>21</td>
<td>Non-sterilised</td>
<td>1.131</td>
<td>0.157</td>
</tr>
<tr>
<td>33</td>
<td>Non-sterilised</td>
<td>2.056</td>
<td>1.474</td>
</tr>
<tr>
<td>33</td>
<td>Autoclaved</td>
<td>3.145</td>
<td>0.647</td>
</tr>
<tr>
<td>33</td>
<td>Irradiated</td>
<td>2.295</td>
<td>1.118</td>
</tr>
<tr>
<td>65</td>
<td>Non-sterilised</td>
<td>1.596</td>
<td>0.176</td>
</tr>
<tr>
<td>117</td>
<td>Non-sterilised</td>
<td>0.056</td>
<td>0.082</td>
</tr>
</tbody>
</table>

*TOC and TON in g\(^{-1}\) Kg of biosolids

5.4.2. *S. typhimurium* growth in sterilised biosolids

Growth curves for seeded *S. typhimurium* in sterilised compost are presented in Figs. 5.1 and 5.2. Regression analysis of the collected data is presented in Tables 5.2 and 5.3. Seeded *Salmonella* grew rapidly in sterile compost and attained the stationary growth phase after 30 h of incubation.

As shown in Table 5.1, all composted biosolids samples could support *Salmonella* growth in excess of 10\(^6\) g\(^{-1}\). For all of the samples seeded *Salmonella* maintained a population density of more than 10\(^6\) g\(^{-1}\) after 100 h of incubation in sterilised compost. The maximum growth of more than 10\(^8\) g\(^{-1}\) was observed in biosolids that have been
composting for 2 weeks. The maximum growth declined with the maturity of the compost. The storage of composted biosolids for 117 weeks (2 years) resulted in a 2 log reduction in the maximum growth potential in sterilised biosolids.

![Diagram](image)

* W = weeks, # B = additional sample of same age

**Figure 5.2.** Growth of seeded *S. typhimurium* in sterilised composted biosolids of different maturity.

The specific growth rate of seeded *Salmonella* in biosolids that had been composting for two weeks was roughly double (0.249 h\(^{-1}\)) that for the compost stored for 2 years (0.148...
h⁻¹). There was a significant negative correlation (-0.850, greater than 95% significant at 
n = 9) between the specific growth rate and the maturity of compost.

Regression analysis of inactivation of seeded *S. typhimurium* in sterilised compost is 
presented in Table 5.3.

**Table 5.3.** Inactivation rate equations of seeded *S. typhimurium* in sterilised composted 
biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Regression equation</th>
<th>$r^2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$Y = -0.004x + 9.317$</td>
<td>63.0</td>
</tr>
<tr>
<td>13</td>
<td>$Y = -0.010x + 7.881$</td>
<td>91.5</td>
</tr>
<tr>
<td>33</td>
<td>$Y = -0.003x + 8.114$</td>
<td>82.1</td>
</tr>
<tr>
<td>45</td>
<td>$Y = -0.004x + 6.995$</td>
<td>91.3</td>
</tr>
<tr>
<td>77</td>
<td>$Y = -0.009x + 7.959$</td>
<td>71.1</td>
</tr>
<tr>
<td>117</td>
<td>$Y = -0.011x + 7.146$</td>
<td>74.0</td>
</tr>
<tr>
<td>117</td>
<td>$Y = -0.008x + 6.973$</td>
<td>75.0</td>
</tr>
</tbody>
</table>

Regression equation is $y = mx + b$, where *m* is the *k* value, *x* is the time in hours, and $\log_{10} N_t/N_0$.

In the absence of competition for nutrients from indigenous microorganisms inactivation 
rate of seeded *Salmonella* in general was low. However, inactivation rate of *Salmonella* 
was lower in freshly composted biosolids as compared to the stored biosolids.

**5.4.3. *S. typhimurium* growth in non-sterilised biosolids**

Growth curves for seeded *S. typhimurium* in non-sterilised compost are presented in Fig. 
5.3. Regression analysis of growth, and maximum observed growth is shown in Table
5.4 and Fig. 5.3. Inactivation data for seeded *Salmonella* in non-sterilised composted biosolids is shown in Table 5.5. The $\log_{10}$ reduction h$^{-1}$ (k value) of *Salmonella* in composted biosolids of different maturity is shown in Fig. 5.4.

![Graph showing inactivation data for seeded *Salmonella* in composted biosolids of different maturity.](image)

$\log_{10}$ *Salmonella* per gram

* W = weeks, # B = additional sample of same age

**Figure 5.3.** Growth of seeded *S. typhimurium* in non-sterilised composted biosolids of different maturity.

Limited growth was observed in non-sterilised biosolids as compared to sterilised samples of the same compost. The growth rate of *S. typhimurium* in the non-sterilised biosolids was significantly lower than the sterilised samples ($t_{0.025}(10) = 2.228$ is lower than calculated value 11.071). The growth of *Salmonella* was severely inhibited in biosolids that had been composting for 2 weeks (Fig. 5.3). Even with a higher initial concentration of inoculum, seeded *Salmonella* declined rapidly in biosolids composting.
for 2 weeks. There was no significant correlation (-0.147) between the maturity of the compost and the specific growth rate of seeded *S. typhimurium* in the non-sterilised comports.

**Table 5.4.** Growth rate equations of *S. typhimurium* seeded in composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Regression equation</th>
<th>$r^2$ (%)</th>
<th>Max. observed growth (MPN g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$Y = -0.012x + 2.78$</td>
<td>8.80</td>
<td>1450</td>
</tr>
<tr>
<td>2B</td>
<td>$Y = -0.035x + 3.896$</td>
<td>77.1</td>
<td>3900</td>
</tr>
<tr>
<td>5</td>
<td>$Y = 0.022x + 2.972$</td>
<td>86.5</td>
<td>2260</td>
</tr>
<tr>
<td>7</td>
<td>$Y = 0.016x + 3.030$</td>
<td>65.8</td>
<td>1700</td>
</tr>
<tr>
<td>21</td>
<td>$Y = 0.024x + 3.350$</td>
<td>98.5</td>
<td>6500</td>
</tr>
<tr>
<td>33</td>
<td>$Y = 0.020x + 2.969$</td>
<td>87.1</td>
<td>4990</td>
</tr>
<tr>
<td>33B</td>
<td>$Y = 0.016x + 2.737$</td>
<td>94.5</td>
<td>3500</td>
</tr>
<tr>
<td>65</td>
<td>$Y = 0.023x + 2.983$</td>
<td>99.5</td>
<td>9800</td>
</tr>
<tr>
<td>65B</td>
<td>$Y = 0.022x + 2.751$</td>
<td>79.4</td>
<td>6080</td>
</tr>
<tr>
<td>117</td>
<td>$Y = 0.006x + 2.353$</td>
<td>58.2</td>
<td>175</td>
</tr>
<tr>
<td>117B</td>
<td>$Y = 0.008x + 2.223$</td>
<td>92.3</td>
<td>90</td>
</tr>
</tbody>
</table>

Regression equation is $y = mx + b$, where (-m) is the $k$ value, $x$ is the time in hours, and $\log_{10} \frac{N_t}{N_0}$.

The inactivation rate of seeded *S. typhimurium* in non-sterilised comports was significantly greater than for the sterilised compost ($t_{0.025} (11) = 2.200$ is lower than calculated value 3.231).
Table 5.5. Inactivation rate equations of *S. typhimurium* seeded in composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Regression equation</th>
<th>( r^2 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>( Y = -0.059x + 3.673 )</td>
<td>99.4</td>
</tr>
<tr>
<td>2B</td>
<td>( Y = -0.057x + 4.628 )</td>
<td>89.1</td>
</tr>
<tr>
<td>5</td>
<td>( Y = -0.051x + 4.779 )</td>
<td>98.8</td>
</tr>
<tr>
<td>7</td>
<td>( Y = -0.042x + 4.165 )</td>
<td>70.0</td>
</tr>
<tr>
<td>21</td>
<td>( Y = -0.029x + 4.700 )</td>
<td>94.1</td>
</tr>
<tr>
<td>33</td>
<td>( Y = -0.020x + 4.306 )</td>
<td>82.1</td>
</tr>
<tr>
<td>33B</td>
<td>( Y = -0.021x + 4.189 )</td>
<td>63.1</td>
</tr>
<tr>
<td>65</td>
<td>( Y = -0.010x + 3.757 )</td>
<td>91.0</td>
</tr>
<tr>
<td>65B</td>
<td>( Y = -0.014x + 3.805 )</td>
<td>98.3</td>
</tr>
<tr>
<td>117</td>
<td>( Y = -0.009x + 2.607 )</td>
<td>85.0</td>
</tr>
<tr>
<td>117B</td>
<td>( Y = -0.008x + 2.545 )</td>
<td>78.0</td>
</tr>
</tbody>
</table>

Regression equation is \( y = mx + b \), where \((-m)\) is the \( k \) value, \( x \) is the time in hours, and \( \log_{10} \frac{N_t}{N_0} \).

The inactivation rate of *Salmonella* in biosolids that had been composting for 2 months was seven times greater than for the composted biosolids stored for 2 years.
Figure 5.4. Inactivation rate (k) of seeded *S. typhimurium* in composted biosolids of different maturity.

The seeded *Salmonella* survived for a longer period in stored biosolids as compared to the freshly composted biosolids (Fig. 6.4). The inactivation rate of *Salmonella* was negatively correlated (-0.855) to the maturity of composted biosolids.
5.5. Discussion

5.5.1. Role of bio-available nutrients in pathogen regrowth

Seeded *Salmonella* grew rapidly in sterilised biosolids reaching a population density of greater than $10^6$ g$^{-1}$ after 30 h of incubation. The rapid growth of *Salmonella* in sterilised biosolids suggests that there was an abundant supply of nutrients in composted biosolids. It was previously reported that the concentration of available nutrients declined with compost maturity (Inbar *et al.* 1990). The storage of composted biosolids in this study also resulted in an overall decline in available organic carbon and nitrogen (Table 5.2). The decline in the specific growth rate and maximum regrowth potential of *Salmonella* with maturity of compost was most likely due to nutrient limitation. The significant negative correlation (-0.850) between the specific growth rate and maturity of compost further proves that the decline in nutrient availability resulted in a drop in *Salmonella* growth.

Although the nutrient limitation with the maturity of compost resulted in a decline in *Salmonella* growth, long term storage did not result in a decline in the bio-available nutrients below a threshold level for growth. The bio-available nutrients were sufficient for *Salmonella* to attain a maximum population density of more than $10^6$ g$^{-1}$ in compost stored for two years. Similarly, Hussong *et al.* (1985) reported that the majority of composted biosolids they tested provided nutrients for *Salmonella* regrowth.
5.5.2. Role of indigenous microflora in suppression of *Salmonella* regrowth

Regardless of the maturity of the composted biosolids, seeded *Salmonella* grew well in sterilised biosolids, whereas growth was suppressed in non-sterilised sample of same compost. The suppression of *Salmonella* regrowth in non-sterilised compost was most likely due to the activity of indigenous microflora. The regrowth inhibitory effect of indigenous microorganisms was also reported by other authors (Hussong *et al.* 1985; Millner *et al.* 1987). The present results suggest that the presence of indigenous microflora suppress *Salmonella* growth even though sufficient bio-available nutrients are present to support high levels of growth.

The inhibitory effect was more pronounced in immature and freshly composted biosolids, and was found to be maximum in biosolids that had been composting for two weeks. This further shows that the indigenous microorganisms are capable of inhibiting *Salmonella* growth even in the immature composts (unstable). The rapid inactivation of seeded *Salmonella* cells in non-sterilised biosolids composting for two weeks could be possibly due to two reasons. Firstly, it may be due to the presence of regrowth inhibitory compounds or secondary metabolites. Secondly, it may be due to the presence of highly active indigenous microorganisms such as somatic *Salmonella* phage. The presence of secondary metabolites appears unlikely, as the growth of *Salmonella* in irradiated biosolids was not inhibited. Consequently, it is likely that somatic *Salmonella* phages were very active during the early stages of the composting process. Wastewater sludge is known to be an abundant source of coliphages such as φ X 174 and T-phages, which
attack *Salmonella* cells (Douglas 1975). Therefore, it is possible that bacteriophage also play a role in the suppression of *Salmonella* growth.

### 5.5.3. Effect of storage on *Salmonella* regrowth potential

The pathogen regrowth potential in composted biosolids is mainly controlled by the activity of indigenous microflora. However, with storage the competition for limited nutrients is expected to intensify which also plays a role in the suppression of *Salmonella* growth. There was no significant correlation between the storage time and the specific growth of *Salmonella*, so it was not concluded that storage resulted in a decline in pathogen regrowth potential. Conversely, the inactivation rate of *Salmonella* declined by seven folds during the storage of composted biosolids for up to two years. This could be due to a decline in the antagonistic effect of indigenous microorganisms with the maturity of compost. Consequently, in the case of reintroduction of *Salmonella*, a longer survival time could be expected in stored biosolids.

Although open windrow composting is very effective in reducing *Salmonella* concentrations, *Giardia* cysts can survive the composting process in relatively high concentrations ranging from 200 to 600 cysts g\(^{-1}\) (Gibbs *et al.* 1998). Storage of composted biosolids for 30 weeks has been found to be effective in reducing *Giardia* cyst concentrations below the detection limit (Gibbs *et al.* 1998). However, the present results suggest that storage of composted biosolids results in a decline in the antagonistic effect of indigenous microflora, which results in an increase in the pathogen regrowth potential.
Additionally, long term storage of compost is also undesirable due to the loss of nutrient quality and requirement for storage space. Consequently, to balance the beneficial effect of storage and loss in antagonistic effect composted biosolids should be stored for the minimum required time after composting. Considering that Giardia can be reduced to below detection limit with 30 weeks of storage, a storage of up to 30 weeks could be a better way to ensure the bio-safety of compost.

The suppression of Salmonella regrowth in non-sterilised biosolids suggests that indigenous microorganisms provide a biological control for pathogen regrowth. The concentration of the indigenous microbial population is also expected to be influenced by environmental factors such as moisture temperature and bio-available nutrients during composting and storage. Different bulking material and composting conditions are expected to change the population dynamics of indigenous microflora, which ultimately affect the regrowth potential of composted biosolids. Consequently, it is possible that the results of this study may not be directly applicable to all composting systems. Care must be taken in extrapolation of regrowth potential of compost produced through different processes.

The results of this study suggest that maturity of compost alone cannot guarantee biosafety of composted biosolids. The concentration and diversity of indigenous microflora appear to be the single main factors which suppress Salmonella regrowth in the composted biosolids. Consequently, the presence of biologically active indigenous microflora is required to reduce the pathogen regrowth potential of compost. Further
research should be carried out to identify indigenous microorganisms, which suppress *Salmonella* regrowth. Resolving this issue could provide a better understanding of the antagonistic effect of indigenous microflora in the environment.
CHAPTER 6: ROLE OF INDIGENOUS MICROORGANISMS IN SUPPRESSION OF SALMONELLA REGROWTH

6.1. Introduction

Results described in Chapter 5 showed the importance of indigenous microorganisms in suppression of Salmonella regrowth. In a previous study indigenous bacteria and actinomycetes were found to suppress Salmonella regrowth in compost assays (Millner et al. 1987). Coliform (Yeager and Ward, 1981) and non-coliform (Millner et al. 1987) bacteria have also been reported to inhibit regrowth of Salmonella in sterilised biosolids. However, limited information is available on the nature of microbial antagonism and the role of different microorganisms in suppression of Salmonella regrowth.

As mentioned earlier in Section 2.8.1.3, up to the present time only one study (Millner et al. 1987) has been conducted to understand the role of indigenous microflora in the suppression of Salmonella regrowth. However, this study did not provide specific information on the mechanism of microbial antagonism and the nature of inhibiting microorganisms. Bacteria and actinomycetes are reported to be better competitors against Salmonella as compared to fungi (Millner et al. 1987). Millner et al (1987) also observed that Salmonella failed to colonise in compost collected from the mesophilic temperature zones (20 to 40 °C) of a composting pile. This was in spite of the fact that
the maximum sustained growth of *Salmonella* also takes place between temperatures of 21 to 37 °C (Ingran and Marr 1996).

It is possible that microorganisms capable of growing under mesophilic temperature conditions are better competitors with *Salmonella* due to identical growth requirements and similar metabolic activity. It is also possible that suppression of *Salmonella* regrowth is not only due to the competition for limited nutrients but also as a result of growth inhibiting compounds secreted by certain microorganisms. Currently, very limited information is available on the antagonistic effect of indigenous microorganisms and the nature of antagonism towards *Salmonella*.

Wastewater biosolids are known to contain a variety of bacteriophage, including phage which attack *Enterobacteriaceae* family members and they are known to show a broad host range (Lindberg 1973). SS phages are known to be present in environmental water and ground water (Handzel *et al.* 1993; William and Stetler 1994). Consequently, it is expected that SS phages are also present in wastewater biosolids. However, the presence of SS phages in biosolids and their rate of survival during composting is not known. SS phages are expected to effect *Salmonella* concentrations due to cell lytic activity (Ogata *et al.* 1980). The influence of SS phages on survival and regrowth of *Salmonella* in composted biosolids is also not known.

The first objective of this chapter was to investigate changes in the concentration of culturable indigenous microorganisms and SS phage during the composting process. The
second objective of this chapter was to isolate indigenous bacteria and actinomycetes from composted biosolids and evaluate their effect on *Salmonella* regrowth, and increase understanding of the mechanism of growth suppression. An additional objective was to examine the role of SS phage in suppression of *Salmonella* growth in composted biosolids.

6.2. Experimental approach

To evaluate the effect of compost maturity on the concentration of indigenous microorganisms, the concentrations of bacteria, fungi and actinomycetes were monitored in compost samples of different maturity. Concentrations of indigenous somatic *Salmonella* phage were also determined in the composted biosolids of different maturity. Monitoring was carried out twice, once during winter and once during summer, to determine seasonal variations.

Indigenous mesophilic bacteria and actinomycetes were isolated from composted biosolids. The main purpose for isolating of mesophilic bacteria and actinomycetes was to select those microorganisms which actually compete with *Salmonella* for limited nutrients in composted biosolids under, moisture and temperature conditions which *Salmonella* prefer.
Isolated indigenous microorganisms were screened for their antagonistic effect towards *Salmonella* in water extracts prepared from composted biosolids that had been composting for two weeks. Sterilised water extracts were used as the preferred media for two reasons. Firstly, it was anticipated that the antagonist effect of secondary metabolites would be more uniform in the water extract as compared to compost. Secondly, it allowed screening of bacteria for their antagonistic effect.

6.3. Materials and Methods

6.3.1. Sample collection and storage

Compost samples were collected from the same compost facility and using the same procedure for sample collection described in Section 3.2.1.

6.3.2. Enumeration of indigenous microorganisms

Indigenous bacteria, fungi and actinomycetes concentrations in composted biosolids of different maturity were determined by a standard spread plate count procedure (APAH *et al.* 1995). To prepare the compost suspension solution, approximately 2 g of compost was suspended in 99 mL of sterile quarter strength Ringer’s solution (Oxoid) and shaken vigorously for 2 to 3 min. Serial ten-fold dilutions were made in quarter strength Ringers solution to a final dilution of $10^{-8}$ of the original suspension. Then 0.1 mL of the series of
dilutions was spread plated on to three different plating media for the isolation of bacteria, actinomycetes and fungi, in triplicate. Prior to spread plating for actinomycetes, dilution bottles were kept in a 55 °C water bath for 5 min. to suppress bacteria (Hatano et al. 1994).

Trypticase soy agar (Life Technologies) plates containing 50 μg mL⁻¹ of cyclohexamide were used for bacterial quantification. Potato dextrose agar (Life Technologies) plates containing 50 μg mL⁻¹ of streptomycin were used for the quantification of fungi. Actinomycete isolation agar (Difco) plates containing 50 μg mL⁻¹ of cyclohexamide were used for actinomycetes. The media were made according to the manufacturer instructions.

Colony forming units (cfu) were counted for bacteria and fungi after incubation at 37 °C for 48 h. Actinomycetes colonies were counted after four days of incubation at 37 °C. The plates containing 20 to 200 cfu were used for the enumeration of microbial concentration. The plates were incubated at 37 °C, to enumerate mesophilic indigenous microorganisms that could grow under conditions favourable for Salmonella growth.

6.3.3. Isolation of indigenous microorganisms from compost

The spread plate procedure was used to isolate mesophilic bacteria and actinomycetes from composted biosolids of different maturity. The procedure adopted for isolation of bacteria and actinomycetes was similar to the procedure outlined in Section 6.3.2. After
the desired incubation the plates with approximately 200 cfu were selected for isolation of bacteria and actinomycetes. Single colonies with distinct morphology were picked up with a sterilised loop and streaked onto fresh TSA medium for bacteria and AlA medium for actinomycetes, to obtain pure cultures. The isolated bacteria, and actinomycetes were further purified 3 or 4 times before colony morphology and microscopic analysis. The purified colonies were examined for colony morphology such as colony appearance, edges, elevation and surface appearance. For bacteria, microscopic examination was also carried out after gram staining.

6.3.4. Screening for antagonistic effect

All isolated microorganisms were analysed for their antagonistic effect towards *S. typhimurium*. A sterilised water extract prepared from biosolids which had been composting for 2 weeks was used as the culture medium in these studies. The water extract of composted biosolids was made by taking 40 g of biosolids in a flask containing 360 mL of distilled water at 60 °C. The flask was stirred at room temperature for 30 minutes and then allowed to stand on a bench for 30 minutes. The supernatant was then passed through a Whatman No 4 filter paper using vacuum filtration apparatus. Fifty mL of water extract was later dispensed into Schott bottles and autoclaved prior to use.

The cultures of *S. typhimurium* and other isolated bacteria were grown overnight in buffered peptone water. Then ten fold dilutions were made in sterile Ringer's solution to the $10^{-6}$ dilution prior to seeding in the water extract of composted biosolids. The
regrowth bottles were incubated at 37 °C for 60 h. Spread plate counts were performed (in duplicate) on XLD agar and TSA to quantify the regrowth of *S. typhimurium* and indigenous bacteria at regular intervals. The results were then expressed as cfu per gram of composted biosolids.

Three types of experiments were conducted. In the first type of experiment the indigenous isolate and *S. typhimurium* were inoculated into the compost extract simultaneously and growth measured over 48 h. In the second type of experiment cultures of indigenous bacteria were seeded into the sterilised water extract of compost and allowed to regrow for 48 h at 37 °C. Then *S. typhimurium* was seeded into the established population of indigenous bacteria and the growth of *S. typhimurium* monitored. The third type of experiment was similar to the second experiment but 0.2 mg of sucrose was added to the compost extract after 8 h.

### 6.3.5. Screening for antibiosis

Two different approaches were adopted to screen bacterial isolates for antibiosis (production of growth inhibitory compounds). In the first approach, pure cultures of isolated indigenous bacteria were grown in buffered peptone water at 37 °C for 72 h on a shaking platform. The host bacteria (*S. typhimurium*) was cultured at 37 °C overnight. To prepare *S. typhimurium* lawns, well dried tryptic soy agar (TSA) plates were flooded with the overnight host culture, and then the excess culture was drained off and then dried at 37 °C for 5 to 10 minutes (Parker 1981). The paper disk method generally used for
measuring antibiotic activity was used for the preliminary screening for the production of bacteriocins (Salle 1971). Sterilised paper disks (7-8 mm) were dipped in the pure culture of indigenous bacteria and were placed aseptically on the *S. typhimurium* lawns grown on TSA agar. The plates were incubated at 37 °C for 48 h prior to checking for clear zones around the filter paper disks, which shows inhibition of host growth.

In the second approach, concentrated cultures of indigenous bacteria were filtered through 0.2 µm membranes and the membranes were aseptically transferred onto TSA plates. The bacterial cells grew in high concentrations on the membrane and the TSA media absorbed secondary metabolites. After incubation for 48 h the filter papers were peeled off with indigenous bacteria on them. *S. typhimurium* was streaked onto the TSA plates. After incubation for 48 h the TSA plates were analysed for suppression of *Salmonella* growth.

Pure cultures of isolated actinomycetes were cultured in actinomycetes isolation medium without agar at 30 °C in flasks on a shaking platform for 72 h. The paper disk method described earlier was used to examine the growth inhibitory effect.

### 6.3.6. Bacterial host

A wild type isolate of *S. typhimurium* (9451) resistant to rifampicin was used as a host bacterium. In order to prevent overgrowth of phage host bacteria by indigenous bacteria rifampicin resistant *Salmonella* was used in this study. The host bacteria was cultured in
Tryptone Yeast extract Glucose Broth (TYGB) supplemented with rifampicin (50 μg mL⁻¹) at 37 °C for 6 to 8 h. Log phase bacterial cultures were stored at -20 °C in 20% glycerol for convenience (Rhodes and Kator 1991).

6.3.7. Enumeration of somatic Salmonella phage in composted biosolids

Somatic *S. typhimurium* (SS) phages in composted biosolids were enumerated by a five tube Most Probable Number (MPN) procedure. Four dilutions containing 10, 1, 0.1 and 0.01 g of biosolids in 10 mL of buffered peptone water were prepared for enrichment. Rifampicin resistant *S. typhimurium* was used as the host bacteria for enrichment. Ten mL of host culture was added to each enrichment tube. The enrichment was carried out for 24 h at 37 °C. The MPN procedure was carried out in duplicate.

A Double Layer Agar (DLA) plate technique was used to prepare the host bacterial lawn. Tryptone Yeast extract Glucose Agar (TYGA) was used as the base agar and soft top agar was prepared with TYGA containing half strength agar. To suppress the activity of indigenous bacteria 50 μg mL⁻¹ of rifampicin was added to the soft agar (Yin *et al.* 1997). The soft agar was melted and cooled to 45 °C and mixed with a 1:10 ratio of host bacteria. The mixture was then poured on to the base agar and allowed to solidify. Then 5 μL of enriched culture was spot inoculated on to DLA plates in triplicate. The plates were then incubated at 37 °C for 24 h and screened for plaque formation.
6.3.8. Plaque purification and host range specificity

Selected plaques with different visible plaque morphology were stabbed with a Pasteur pipette and transferred to fresh host cultures in 10 mL of TYGB for purification. Representative plaques were purified by repeated plaquing on *S. typhimurium*. Purified bacteriophages were examined for lytic activity against other *Salmonella* isolates and *E. coli* to check host specificity.

Host range evaluation of isolated bacteriophage was conducted by plaque assay using wild type isolates *Salmonella* and *E. coli*. The following strains obtained from the PathCentre in WA and the Murdoch University culture collection were used to determine host specificity: *S. typhimurium* (9451), *S. typhimurium* (Mu-S5-83), *S. anatum* (8414), *S. mbandaka* (9098), *S. chester* (8605), *S. havana* (7685), *S. infantis* (8527), *E. coli* (NCTC 8196), *E. coli* (ATCC 25922) and *E. coli* (MU-E9-83).

Host bacteria were cultured in TYGB at 37 °C for 6 to 8 h and the DLA technique was used to prepare a host bacterial lawn. The RNase sensitivity of isolated phage from composted biosolids was determined by adding 100 μg 5 mL⁻¹ of soft agar. The spot inoculation technique described earlier was used to check RNase sensitivity and host range evaluation.
6.3.9. Electron microscopy

Suspensions of bacteriophages and phage adsorbed to host cells were prepared in BPW for electron microscopy. Droplets of phage suspension were applied to carbon coated TEM grids for a 2 minute adsorption period. Then excess material on the grid was removed by filter paper and the grid was negatively stained with 1% phosphotungstic acid, at pH 7 for 2 minutes. Excess stain was removed with a filter paper and the grid was air dried for 5 minutes prior to examination. The grids were examined using a Phillips CM 100 Biotwin electron microscope operating at 80 KV.

6.3.10. Preparation of SS phage cultures for seeding

Pure cultures of *S. typhimurium* were grown overnight in 10 mL of BPW at 37 °C. Serial ten-fold dilutions were made in quarter strength Ringer’s solution to a final dilution of $10^6$. The final dilution was used to seed sterilised biosolids samples.

Different SS phages purified earlier were used to produce a mixture of bacteriophage. *S. typhimurium* was used as host bacterium for bacteriophage culture preparations. The host bacterium cultures in BPW were incubated at 37 °C for 6 h prior to inoculation with a single plaque of each representative phage. Then host and phage were incubated at 37 °C for 4 h before phage harvesting. An aliquot (100 μL) of each phage culture was then inoculated into fresh host bacterial culture (6 h old) to make a mixture of bacteriophage. The culture was incubated for 4 h before preparation of phage titters for seeding. An
aliquot of this eluate was passed twice through a 0.45 μm membrane filter pre-treated with 3% beef extract to remove bacterial cells. The titers were determined in the filtrate at the time of seeding by the double layer agar method using *S. typhimurium* as host bacteria. Then the $10^2$ dilution of the membrane filtrate (containing $10 \times 10^5$ pfu mL$^{-1}$) was used to seed sterilised biosolids.

### 6.3.11. Regrowth potential test

Sterilised biosolids (600 g) samples were placed in a sterile beaker and seeded with 20 mL of the $10^2$ dilution of bacteriophage culture and blended. Then 20 mL of the $10^6$ dilution of *S. typhimurium* culture was seeded into the sample and again blended. The blended sample was then transferred into three flasks each receiving 150 g of sample. An additional 600 g of each composted biosolids samples were seeded with 20 mL of the $10^6$ dilution of *S. typhimurium*. Sterilised distilled water (20 mL) was added to the compost to adjust the moisture content. The inoculated biosolids were then transferred into three flasks receiving 150 g of sample. These served as controls for comparison of *S. typhimurium* regrowth in the presence and absence of bacteriophage. The bacterial concentration in both samples was determined at regular intervals by the MPN procedure described in Section 4.3.8.
6.3.12. Statistical analysis

To compare the growth rate of *S. typhimurium* in sterilised biosolids and in the presence of SS phage, linear regression analysis was used, as discussed in Section 5.1.7.

6.4. Results

6.4.1. Enumeration of indigenous microorganisms in composted biosolids

The concentration of bacteria, actinomycetes and fungi in composted biosolids of different maturity, sampled during winter and summer months, is shown in Fig. 6.1. There was no observable seasonal variation between bacterial and fungal populations. However, actinomycetes populations were one log higher in the composts sampled during the winter period.

The bacterial and actinomycetes concentrations were 5 to 7.3 x 10^8 g^-1 and 3.3 to 18.8 x 10^6 g^-1 respectively in the biosolids which had been composting for 2 weeks. The bacterial concentration declined gradually to 10^6 g^-1 in composted biosolids stored for two years. The population of actinomycetes increased with the progress in composting and was at a maximum (10^7 g^-1) in biosolids composting for 13 weeks. The population declined to 10^5 g^-1 after storage for 2 years.
Fungus 1, Actino 1 and Bacteria 1 are winter samples whereas the others are summer samples.

**Figure 6.1.** Indigenous microbial concentrations in composted biosolids of different maturity.

The fungi population was $10^4 \text{ g}^{-1}$ in biosolids composting for two weeks and gradually increased during composting and storage to more than $10^5 \text{ g}^{-1}$ in composted biosolids stored for 2 years.
6.4.2. Colony morphology and microscopic examination of isolated bacteria and actinomycetes

The identification of bacteria from composted biosolids of different maturity was based on colony morphology and microscopic examination. Most of the bacteria were isolated from biosolids that had been composting for 2 weeks or 13 weeks. Table 6.1 summarises the results of colony morphology and microscopic examination for bacteria isolated from the composted biosolids. All of the bacteria were gram-positive. The majority of isolated bacteria were cocci and others were rods resembling *Bacillus* species. No positive identification of bacteria was made on any of the isolated bacteria. However, genus level identification was carried out on some of the isolates. PC bacterium was catalase positive, strictly aerobic and belonged to genus *Micrococcus*. GYC was a very motile non-spore forming bacteria, which may belong to genus *Microbacterium*. Bacteria OC was non-motile, non-spore forming and catalase positive, which may belong to genus *Microbacterium*. Bacteria BTCS was non-motile, catalase positive and resembled *Bacillus* species. SRC bacterium was catalase positive, non-spore forming and a very slow growing bacteria, which may belong to genus *Micrococcus*. On the basis of visual observations of colony characteristics during the isolation of bacteria, diversity declined considerably in biosolids stored for more than 5 months.
Table 6.1. Colony morphologies and microscopic appearance of the bacteria isolated from the composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Sample Age (weeks)</th>
<th>Sample ID</th>
<th>Colony appearance</th>
<th>Edges</th>
<th>Elevation</th>
<th>Surface appearance</th>
<th>Microscopic appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>PC</td>
<td>circular</td>
<td>entire</td>
<td>pluvinate</td>
<td>yellow</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>2</td>
<td>GYC</td>
<td>circular</td>
<td>entire</td>
<td>low convex</td>
<td>very motile, yellow</td>
<td>gram +, ovoid</td>
</tr>
<tr>
<td>2</td>
<td>OC</td>
<td>circular</td>
<td>entire</td>
<td>low convex</td>
<td>orange</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>2</td>
<td>BTCS</td>
<td>irregular</td>
<td>undulate</td>
<td>pulvinate</td>
<td>blister like, granular</td>
<td>gram +, rods</td>
</tr>
<tr>
<td></td>
<td>SRC</td>
<td>circular</td>
<td>entire</td>
<td>low convex</td>
<td>Opaque, slow growing</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>13</td>
<td>J1</td>
<td>circular</td>
<td>lobate</td>
<td>convex papilate</td>
<td>halo effect</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>13</td>
<td>J2</td>
<td>circular</td>
<td>undulate</td>
<td>low convex</td>
<td>deep orange, slow growing</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>13</td>
<td>J3</td>
<td>irregular</td>
<td>crenate</td>
<td>convex papilate</td>
<td>halo effect</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>13</td>
<td>J4</td>
<td>circular</td>
<td>low convex</td>
<td>small, off-white</td>
<td>white</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>2</td>
<td>J5</td>
<td>irregular</td>
<td>undulate</td>
<td>convex</td>
<td>concentric rings</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>2</td>
<td>J6</td>
<td>irregular</td>
<td>undulate</td>
<td>convex</td>
<td>cream</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>2</td>
<td>J7</td>
<td>circular</td>
<td>entire</td>
<td>low convex</td>
<td>translucent</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>2</td>
<td>J8</td>
<td>irregular</td>
<td>undulate</td>
<td>low convex</td>
<td>translucent</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>2</td>
<td>J9</td>
<td>irregular</td>
<td>undulate</td>
<td>low convex</td>
<td>off white</td>
<td>gram +, rods</td>
</tr>
<tr>
<td>13</td>
<td>J11</td>
<td>circular</td>
<td>entire</td>
<td>low convex</td>
<td>tiny colonies</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>13</td>
<td>P1</td>
<td>circular</td>
<td>entire</td>
<td>convex</td>
<td>cream</td>
<td>gram +, cocci</td>
</tr>
<tr>
<td>13</td>
<td>P4</td>
<td>irregular</td>
<td>undulate</td>
<td>low convex</td>
<td>powdery</td>
<td>gram +, rods</td>
</tr>
</tbody>
</table>

The identification of actinomycetes isolated from the composted biosolids was carried out by the traditional method outlined for identification of *Streptomyces* by Shirling and Gottlieb (1966). The main criteria used for identification were morphology of aerial mycelia, colour of aerial mass and colour of substrate mycelium. All isolated
actinomycetes belonged to genus *Streptomyces* with spiral or loop type aerial mycelia.

The results of actinomycetes identification are presented in the Table 6.2.

**Table 6.2.** Colony morphologies and microscopic appearance of the actinomycetes isolated from the composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Sample age (weeks)</th>
<th>ID</th>
<th>Colony size</th>
<th>Colour of substrate mycelia</th>
<th>Colour of aerial mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>A1</td>
<td>small</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>2</td>
<td>A2</td>
<td>small</td>
<td>gray</td>
<td>gray and brown</td>
</tr>
<tr>
<td>2</td>
<td>A3</td>
<td>small</td>
<td>gray</td>
<td>yellow</td>
</tr>
<tr>
<td>2</td>
<td>A4</td>
<td>medium</td>
<td>gray</td>
<td>yellow</td>
</tr>
<tr>
<td>2</td>
<td>A5</td>
<td>medium</td>
<td>brown</td>
<td>dark brown</td>
</tr>
<tr>
<td>13</td>
<td>A6</td>
<td>small</td>
<td>gray</td>
<td>gray</td>
</tr>
<tr>
<td>13</td>
<td>A7</td>
<td>small</td>
<td>white</td>
<td>yellow</td>
</tr>
<tr>
<td>13</td>
<td>A8</td>
<td>small</td>
<td>gray</td>
<td>gray</td>
</tr>
</tbody>
</table>

**6.4.3. Agar assays for antagonists**

All of the isolated bacteria were screened for antagonistic effect on agar plates and none of them was found to produce secondary metabolites which inhibited growth of *Salmonella*. Similarly, all the isolated actinomycetes were also screened for the production of growth inhibitory metabolites. No growth inhibit effect was recorded on the agar plates.
6.4.4. Compost extract assays with individual bacteria

*S. typhimurium* regrowth in the sterilised water extract of composted biosolids was monitored over a period of 80 h to evaluate the maximum regrowth potential in the absence of competing microorganisms. Seeded *Salmonella* reached a concentration of more than $10^7$ g$^{-1}$ within 48 h of incubation and maintained this population level during 80 h of incubation (Fig. 6.2).

![Graph](image)

**Figure 6.2.** Growth curve of seeded *S. typhimurium* in sterilised water extract of composted biosolids.

The maximum growth potential of *Salmonella* in the absence of competing microorganisms was used as a standard to compare the growth inhibitory effect of indigenous bacteria on seeded *Salmonella*. As can be seen from Fig 6.3., when
Salmonella and OC were seeded together the maximum growth of Salmonella was one log lower ($10^7$ g$^{-1}$) as compared to the standard growth curve (Fig. 6.2).

![Growth curve of Salmonella and OC](image)

**Figure 6.3.** Growth curves of seeded Salmonella and OC bacteria in sterilised water extract of composted biosolids.

In order to evaluate the antagonistic behaviour of isolated bacteria against Salmonella, Salmonella was seeded into an established population of indigenous bacteria. Growth curves of seeded *S. typhimurium* in the presence of established populations of pure cultures of indigenous bacteria are presented in Figures 6.4 to 6.11.

The maximum growth of seeded Salmonella was found to be $10^6$ g$^{-1}$ in the presence of most of the indigenous bacteria tested. The maximum regrowth was approximately 2 logs lower as compared to the standard growth curve for Salmonella. However,
maximum regrowth suppression was observed in the case of SRC bacteria, where maximum regrowth was suppressed more than $10^3$ g$^{-1}$ (Fig. 6.6).

Figure 6.4. Growth curve of seeded *Salmonella* in established population of OC bacteria.

Figure 6.5. Growth curve of seeded *Salmonella* in established population of PC bacteria.
Figure 6.6. Growth curves of seeded *Salmonella* in established population of ES and BTCR bacteria.

Figure 6.7. Growth curves of seeded *Salmonella* in established population of GYC and SRC bacteria.
Figure 6.8. Growth curves of seeded *Salmonella* in established population of J1, J2 and J3 bacteria.

Figure 6.9. Growth curves of seeded *Salmonella* in established population of J4, J5 and J6 bacteria.
Figure 6.10. Growth curves of seeded *Salmonella* in established population of J7, J8 and J9 bacteria.

Figure 6.11. Growth curves of seeded *Salmonella* in established population of J11, P1 and P4 bacteria.
Whereas, growth of *Salmonella* was suppressed by less than or equal to log one by J6, J9, J11 and P4 (Fig. 6.8 to 6.11). However, growth of seeded *Salmonella* remained unaffected in the presence of bacteria J1 to J5, J7, J8 and P1 (Fig. 6.7 to 6.11).

In order to understand the mechanisms of growth suppression, an additional carbon source (sucrose) was supplied to the seeded *Salmonella* in the presence of an established population of indigenous bacteria. The growth response to added sucrose (0.2 mg 50 mL$^{-1}$) in the water extract of composted biosolids was evaluated. Growth curves of *Salmonella* in the presence of bacteria OC and PC are presented in Fig. 6.12 and 6.13.

* Arrow indicates addition of 0.2 mg 50 mL$^{-1}$ of sucrose

**Figure 6.12.** Growth response of *Salmonella* to added sucrose in the presence of OC bacteria.
* Arrow indicates addition of 0.2 mg 50 mL$^{-1}$ of sucrose

**Figure 6.13.** Growth response of *Salmonella* to added sucrose in the presence of PC bacteria.

In both cases addition of extra carbon source resulted in additional growth of *Salmonella* as compared to growth pattern of *Salmonella* in Fig. 6.4 and 6.5.

### 6.4.5. Somatic *Salmonella* phage concentration in composted biosolids

A total of 14 biosolids samples were tested for the presence of SS phage. Results of SS phage concentrations in compost samples collected during the summer and winter are presented in Figure 6.14. The seasonal variation in bacteriophage concentrations was
found to be negligible. The SS phage concentration in anaerobically digested and
dewatered sludge was 1100 pfu 10 g⁻¹. The maximum bacteriophage concentration of
9000 pfu 10 g⁻¹ was found in biosolids composting for 2 weeks. The bacteriophage
population gradually declined to 90 pfu 10g⁻¹ at the end of composting (13 weeks) and
continued to decline further during the storage of compost.

Figure 6.14. Somatic Salmonella phage concentrations in composted biosolids

SS Phages lytic to S. typhimurium were present at a low concentration (4 pfu 10 g⁻¹) in
composted biosolids stored for 2 years. However, in the sample collected during the
winter period no phages were detected in the composted biosolids stored for 2 years. The decline in the bacteriophage concentration was negatively correlated to the compost maturity (-0.758, greater than 95% significant at n = 7).

6.4.6. Phage morphology and plaque characteristics

Bacteriophage isolated from the composted biosolids showed four types of morphologies. Phage particle and plaque measurements of these isolates are presented in Table 6.3. Electron microscopic examination of five SS phage isolates revealed that they represent Microviride, Myoviridae, Podoviridae and Styloviride classes of bacteriophage as described by Francki et al. (1991). Electron microscope micrographs are shown in Fig. 6.15 to 6.19.

The isolated phage particles showed characteristic morphology and produced a characteristic plaque. Micro 1 represents the Microviridae family with a hexagonal head without tail (Fig. 6.15). Myo 1 and Myo 2 represent the Myoviridae, Myo1 has an elongated head and a contractile tail with tail fibbers (Fig. 6.16). Whereas Myo 2 has an isometric head with a contractile tail (Fig. 6.17). Podo 1 belongs to family Podoviridae with an isometric head and a rudimentary tail (Fig. 6.18). Stylo 1 belongs to family Styloviridae and has an isometric head with long non-contractile tail (Fig. 6.19).
Figure 6.15. TEM micrograph of Micro 1 isolated from composted biosolids.

Figure 6.16. TEM micrograph of Myo 1 isolated from composted biosolids.
Figure 6.17. TEM micrograph of Myo 2 isolated from composted biosolids.

Figure 6.18. TEM micrograph of Podo 1 isolated from composted biosolids.
Figure 6.19. TEM micrograph of Stylo 1 isolated from composted biosolids.
Table 6.3. Particle and plaque descriptions of bacteriophage isolated from composted biosolids.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Particle description</th>
<th>Dimensions in (nm)</th>
<th>Plaque description</th>
<th>Plaque diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Head</td>
<td>Tail</td>
<td></td>
</tr>
<tr>
<td>Micro 1</td>
<td>Circular tail absent</td>
<td>29</td>
<td>-</td>
<td>Clear plaque with halo effect</td>
</tr>
<tr>
<td>Myo 1</td>
<td>Elongated head with contractile tail</td>
<td>78 x 64</td>
<td>102</td>
<td>Well defined plaque with halo effect</td>
</tr>
<tr>
<td>Myo 2</td>
<td>Isometric head with contractile tail</td>
<td>74</td>
<td>57</td>
<td>Well defined clear plaque</td>
</tr>
<tr>
<td>Podo 1</td>
<td>Isometric head with rudimentary tail</td>
<td>61</td>
<td>5 ?</td>
<td>Opaque plaque</td>
</tr>
<tr>
<td>Stylo 1</td>
<td>Isometric head with non contractile tail</td>
<td>81</td>
<td>92</td>
<td>Clear plaque</td>
</tr>
</tbody>
</table>

6.4.7. Host range specificity

Results of the host range evaluation of isolated bacteriophage are presented in Table 6.4. Plaque assay of the isolated bacteriophage revealed that none of the isolated bacteriophage formed plaques on the *E. coli* strains tested. All the isolated bacteriophage formed plaques on *S. typhimurium* and *S. chester*, whereas no plaques were observed on the other *Salmonella* serotypes tested.
Table 6.4. Host specificity of the five isolated somatic *Salmonella* bacteriophage.

<table>
<thead>
<tr>
<th>Strain of bacteria</th>
<th>Micro 1</th>
<th>Myo 1</th>
<th>Myo 2</th>
<th>Podo 1</th>
<th>Stylo 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. chester</em></td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. anatum</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. infantis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. mbandaka</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. havana</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> NTCC 8196</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

6.4.8. Effect of SS phage on *S. typhimurium* growth

Growth curves of seeded *S. typhimurium* in the presence of SS phage in composted biosolids of different maturity and regression analysis data are presented in Fig. 6.20 and Table 6.5. In order to compare the effect of SS phage on *S. typhimurium* growth, growth curves of *S. typhimurium* in sterilised biosolids are also presented in Fig. 6.20. and Table 6.6.
#2,13,45 and 117 represent age of compost, * Sal = Salmonella growth in the absence of phage, ** Sal + phage = growth of Salmonella in the presence of SS phage.

**Figure 6.20.** Growth curves of *S. typhimurium* in the presence of SS phage in composted biosolids of different maturity.

Growth kinetics of *Salmonella* in the sterilised biosolids was similar to previously reported results (Section 5.4.2) with a maximum population density of more than $10^6$ g$^{-1}$. As can be seen from Fig 1, growth of *Salmonella* was suppressed in the presence of SS phage and the maximum growth was $10^1$ to $10^2$ g$^{-1}$ lower than growth of *Salmonella* in sterilised biosolids. Maximum growth suppression ($10^2$ g$^{-1}$) was observed in the biosolids that had been composting for 13 weeks.
Table 6.5. Growth rate equations of *S. typhimurium* in sterilised composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Regression equation</th>
<th>$r^2$ (%)</th>
<th>Max growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$Y = 0.200x + 3.649$</td>
<td>84.8</td>
<td>$8 \times 10^8$</td>
</tr>
<tr>
<td>13</td>
<td>$Y = 0.203x + 2.462$</td>
<td>97.5</td>
<td>$8 \times 10^8$</td>
</tr>
<tr>
<td>45</td>
<td>$Y = 0.163x + 2.439$</td>
<td>96.8</td>
<td>$8 \times 10^6$</td>
</tr>
<tr>
<td>117</td>
<td>$Y = 0.124x + 2.456$</td>
<td>96.9</td>
<td>$5 \times 10^6$</td>
</tr>
</tbody>
</table>

*Regression equation is $y = mx + b$, where (-m) is the $k$ value, $x$ is the time in hours, and $\log_{10} N_f/N_0$.*

Table 6.6. Growth curves of *S. typhimurium* in the presence of SS phage in composted biosolids of different maturity.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Regression equation</th>
<th>$r^2$ (%)</th>
<th>Max growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$Y = 0.159x + 2.599$</td>
<td>98.5</td>
<td>$30 \times 10^6$</td>
</tr>
<tr>
<td>13</td>
<td>$Y = 0.075x + 3.368$</td>
<td>67.8</td>
<td>$80 \times 10^6$</td>
</tr>
<tr>
<td>45</td>
<td>$Y = 0.154x + 2.050$</td>
<td>93.9</td>
<td>$23 \times 10^5$</td>
</tr>
<tr>
<td>117</td>
<td>$Y = 0.107x + 2.152$</td>
<td>97.6</td>
<td>$5 \times 10^5$</td>
</tr>
</tbody>
</table>

*Regression equation is $y = mx + b$, where (-m) is the $k$ value, $x$ is the time in hours, and $\log_{10} N_f/N_0$.*

The specific growth rate of *Salmonella* in the presence of bacteriophages was also lower as compared to the specific growth rate in sterilised biosolids (Tables 6.5 and 6.6). Complete inhibition of *Salmonella* growth was not observed even in the presence of $10^6$ pfu mL$^{-1}$ of bacteriophages seeded in the biosolids.
6.5. Discussion

6.5.1. Enumeration of indigenous microorganisms

It is important to note that the dilution-plate method adopted in this study gives an estimate of the culturable cell, spore and fungal mycelium capable of growing on the nutrient agar. Nevertheless, the dilution plate method is useful in providing information on the general trends in the groups of microorganisms during the composting process. The overall changes in microbial community structure during composting and storage were comparable to previously reported results for composting of garden type material by Poincelot (1977).

Bacteria as a group were more active during the early stages of composting and their concentration declined with the maturity of compost (Fig. 6.1). This is most likely due to the availability of readily degradable organic matter as discussed in Section 2.3.2.1. The increase in the concentration of actinomycetes with the maturity of compost is most likely due to conditions becoming more favourable for actinomycetes growth. During the cooling down phase pH is closer to neutral and complex polymers such as chitins and cellulose are the main nutrient source, which favour actinomycetes growth (Section 2.3). The increase in fungal population with the maturity of compost is most likely due to the decline in pH, temperature and moisture (De Bertoldi et al. 1982) which takes place during the storage of composted biosolids. Fungi as a group are known to be active at a
low moisture content in the presence of predominantly lignin and cellulose (De Bertoldi et al. 1983).

The present results suggest that the overall population of bacteria declined with the progress of composting, whereas the population of actinomycetes and fungi remained relatively unaffected. It is possible that in addition to the decline in bacterial concentration, total microbial diversity also declined with the storage of compost, which was not reflected by the overall changes in the microbial concentrations. Beffa et al. (1996) reported that the overall bacterial population declined during the cooling down phase, but taxonomic diversity increased during this period. Consequently, in the absence of information on the changes in the population of different groups of indigenous microorganisms, a decline in antagonistic effect with storage may not only be due to the decline in total bacterial concentration. In addition to the total microbial concentration, a greater diversity is expected to increase the competition for limited nutrients among indigenous microflora, which ultimately results in the elimination of weak rivals such as pathogens. Further investigation is required to understand the role of the total microbial concentration and taxonomic diversity in suppression of Salmonella regrowth.

6.5.2. Agar assays for antagonists

None of the isolated bacteria (17) or actinomycetes (8) were found to produce growth inhibitory compounds effective against Salmonella. Millner et al. (1987) also screened 23 bacteria and 61 actinomycetes isolated from composted biosolids for their growth
inhibitory effect and no growth inhibition was recorded. There was 100% agreement between agar assay results and compost extract assay results. Consequently, it can be concluded that none of the indigenous microorganisms isolated in this study showed antibiosis against *S. typhimurium*.

6.5.3. Compost extract assays with isolated bacteria

The maximum *Salmonella* growth observed in the water extract of biosolids that had been composting for 2 weeks was slightly lower (10^8 g^-1) than the maximum growth of *Salmonella* in sterilised composted biosolids (Section 5.4.2). Nevertheless, the results were similar to the previously reported results (Burge *et al.* 1987). This variation in maximum growth rate is possibly due to less diffusion of substrate from the compost particles into water and subsequently lowered availability of nutrients.

The present results show that growth of *Salmonella* is suppressed in the presence of an established population of gram-positive bacteria. Similarly, Millner *et al.* (1987) also reported suppression of *Salmonella* growth in the presence of an established population of coliforms. Most of the antagonistic indigenous bacteria suppressed growth of *Salmonella* by 1 to 2 logs with the exception of SRC bacteria, which suppressed growth by 3 log. The results show that isolated indigenous bacteria differ in terms of their degree of antagonism towards *Salmonella*. The suppression of growth only takes place when *Salmonella* is seeded in the presence of actively growing indigenous microorganisms. This highlights the importance of available nutrients and the degree of
competition for the nutrients. The ability of *Salmonella* to out compete some of the indigenous bacteria shows that the nature of the competing bacteria influences the growth potential of *Salmonella* in addition to the available nutrients.

Since none of the isolated bacteria showed production of growth inhibitory compounds active against *Salmonella* on TSA agar plates, suppression of *Salmonella* growth when seeded in the presence of an established population is possibly due to nutrition limitation. Availability of substrate is known to influence *Salmonella* growth in a water extract of compost (Burge *et al.* 1987). This observation is also supported by the fact that seeded *Salmonella* achieved a population equal to maximum achievable concentration with the addition of extra carbon source.

Since the research was focused on the culturable bacteria and actinomycetes, it is possible that groups of non-culturable but viable microorganisms also played a role in the suppression of *Salmonella* growth in composted biosolids. However, it is clear that suppression of *Salmonella* growth is not due to a single group of microorganisms. It appears that the mechanism of growth suppression works on the basis of intense competition for limited nutrients. It is possible that inhibition of *Salmonella* growth in non-sterilised compost is due to the collective effect of competing indigenous microorganisms, including SS phage. The effect of the number and kind of bacteria on suppression of *Salmonella* growth was also reported by Millner *et al.* (1987). However, additional studies are needed to identify specific microbes and groups of microorganisms, which are more antagonistic towards *Salmonella*. 
It is expected that the higher the taxonomic diversity and concentration of indigenous microorganisms the greater will be the competition for limited nutrients. Since indigenous microorganisms are more adapted to the compost conditions, introduced *Salmonella* is expected to be at a disadvantage in competition for nutrients. However, it is important to note that with the decline in available nutrients and adverse growth conditions, concentration of indigenous microorganisms are expected to decline. Under those circumstances, with secondary contamination of *Salmonella* and with the return of favourable growth conditions such as moisture and temperature, uncontrolled growth of *Salmonella* may result. Consequently, actively growing and taxonomically diverse indigenous population is essential to lower the chances of pathogen regrowth in composted biosolids.

6.5.4. **SS phage concentration in composted biosolids**

Wastewater sludge is an abundant source of a variety of bacteriophage with the majority being coliphage (Section 2.8.1.4). Coliphages are known to show a broad host range among members of the *Enterobacteriaceae* family (Douglas 1975). Somatic coliphage are a heterozygous group of *E. coli* phage found in high numbers in wastewater (Donnison and Ross 1995) and have been reported to multiply in wastewater (IAWPRC 1991). Most of the bacteriophage are known to be associated with the solids of wastewater they are known to be present in composted biosolids (Shaban 1999). Consequently, the presence of SS phage in composted biosolids was not unexpected.
A nine-fold increase in the concentration of SS phage was observed in biosolids composting for two weeks as compared to dewatered biosolids. The increase in SS phage density in compost was possibly due to the multiplication of phage in the presence of host bacteria. It is likely that viable Salmonella cells present in the dewatered biosolids regrows after mixing of biosolids with the bulking material during composting. The initial increase in Salmonella cell concentration may serve as host for the multiplication of SS phage. Faecal coliform concentrations in composted biosolids can be more than $10^4 \text{g}^{-1}$ (De Bertoldi et al. 1982) and they may also serve as alternate host. As described here for Salmonella phage, Salmonella concentrations were maximum at 2 weeks of composting and bacterial densities declined thereafter (Section 4.4.1). Salmonella was well below the detection limit after 5 weeks of composting. The decline in SS phage concentration also showed a similar trend and was significantly correlated to the maturity of the compost.

The minimum concentration of host bacteria required for the multiplication of bacteriophage in sewage sludge is $10^2 \text{mL}^{-1}$ (IAWPRC et al. 1991). Consequently, the detection of SS phage in the absence of Salmonella (after 5 weeks of composting) suggests that either SS phages have multiple hosts or that they can survive in compost in the absence of host bacteria. Enteric bacteriophages are known to be more resistant to environmental factors than faecal coliforms (Borrego et al. 1990).

Survival of SS phage in composted biosolids stored for 2 years further suggests that bacteriophage can survive in biosolids stored over a long period of time and possibly in
the absence of host. Somatic coliphage such as T1 and T7 are known to be adsorbed on clay minerals in soil (Schiffenbauer and Stotzky 1982) which may prolong their persistence in the absence of host (Stotzky 1980). Prolonged persistence of SS phage in composted biosolids also suggests that bacteriophages may play a role in the suppression of Salmonella regrowth throughout composting and storage. However, due to very low concentration of bacteriophage in biosolids stored for more than 30 weeks the cell lytic activity may not be as higher as in the mesophilic stage of composting.

6.5.5. Morphology and host specificity of SS phage

The isolation of bacteriophage on the basis of single plaque isolation is a highly selective procedure (Ackermann and Nguyen 1983). Moreover, coliphage produce a diversity of plaques and it is difficult to identify phage on the basis of plaque morphology alone (Pedroso and Martins 1995). Consequently, it is unlikely that all the bacteriophage active against S. typhimurium in composted biosolids were isolated in this study. Nevertheless, bacteriophage representing four different morphological families was recovered from the compost. The present results suggest that a diverse population of bacteriophage is active against S. typhimurium in composted biosolids. This is not unexpected as wastewater sludge is known to contain a variety of bacteriophage, and polyvalency among phages of family Enterobacteriaceae is common (Ackermann et al. 1978).

Identification of isolated bacteriophage as somatic Salmonella phage is supported by the host range evaluation results (Table 6.4). The host range specificity of isolated
bacteriophages suggest that they are *S. typhimurium* bacteriophage and none of them infected other bacterial strains tested apart from *S. chester*. Since single host bacteria (*S. typhimurium*) was used in this study, it is likely that bacteriophage which attack other serovars of *Salmonella* were also present in composted biosolids.

The host range specificity of the isolated phage suggests that their natural host in the compost is *S. typhimurium*. Although phage did not produce plaques on tested *E. coli* strains and other *Salmonella* serovars, it is possible that that other bacteria in the compost also serve as host cells in the absence of *Salmonella*. These bacteriophages might not be genus-specific. Since *S. typhimurium* was used as host strain, alternate hosts are expected to be limited to the *Enterobacteriaceae*. However, Rhodes and Kator (1991) screened 82 coliforms isolated from sewage polluted water for alternate host for *S. typhimurium* phage and found that none of the them was susceptible to somatic phages isolated on *S. typhimurium*. Therefore, results suggest that prolonged survival of SS phage in composted biosolids in the absence of *Salmonella* spp. is likely.

**6.5.6. Effect of SS phage on *S. typhimurium* growth in composted biosolids**

Growth of seeded *S. typhimurium* was suppressed by one to two logs in the presence of SS phage. The suppression of growth in the presence of bacteriophages is most likely due to cell lysis as a result of phage activity (Ogata et al. 1980). The growth suppression effect was found to be maximum in biosolids composting for two weeks. It is possible that due to the higher specific growth rate of *Salmonella* in two weeks old compost the
availability of host cells was maximum during this period. This might have resulted in rapid multiplication of phage and subsequently resulted in higher suppression of growth due to high concentration of SS phage.

Growth of *Salmonella* was not completely inhibited even in the presence of high numbers of bacteriophage ($10^6$ pfu mL$^{-1}$ of seed). The results suggest that SS phage particles might have adhered to compost particles, which resulted in a localised effect of phage. Coliphages and other bacteriophages are known to be adsorbed on clay minerals in soils (Schiffenbauer and Stotzky 1982; Yin *et al.* 1997). Under these conditions the effect of bacteriophage is expected to be limited to it's localised environment. This might have resulted in unchecked growth of *Salmonella* in some pockets in the absence of phage activity.

Growth of seeded *Salmonella* was found to be inhibited in biosolids that had been composting for 2 weeks (Section 5.5.2). The present results suggest that suppression of *Salmonella* regrowth is not solely controlled by the activity of bacteriophage. Moreover, none of the isolated bacteria were able to inhibit the growth of *Salmonella* in composted biosolids. Consequently, it is more than likely that the regrowth suppression of *Salmonella* in composted biosolids is due to the collective effect of indigenous microorganisms including bacteriophage, which results in elimination of weak rivals in competition.
CHAPTER 7: GENERAL DISCUSSION

The results of this study suggest that there is always a possibility of pathogen regrowth in composted biosolids. However, in the case of reintroduction of *Salmonella*, a lower survival time is expected in freshly composted biosolids as compared to stored composted biosolids. It was found that the presence of biologically active population of indigenous microflora is the single main factor which controls pathogen regrowth. To ensure the bio-safety of composted biosolids, a healthy build up and maintenance of the indigenous microbial population in compost is very important. The implications of this study for biosolids management are discussed in this chapter.

7.1. Major factors affecting pathogen regrowth in composted biosolids

The regrowth of pathogens in composted biosolids depends upon a number of factors, such as the moisture content, bio-available nutrients, temperature and indigenous microorganisms. The role of physical factors, such as temperature, moisture and nutrients in *Salmonella* regrowth has been investigated by several authors (Yeager and Ward 1981; Hussong *et al.* 1995; Soares *et al.* 1995). The regrowth and inactivation of *Salmonella* in compost is also influenced by the indigenous microflora (Hussong *et al.* 1985; Millner *et al.* 1987).
The role of the indigenous microflora and bio-available nutrients in *Salmonella* regrowth were investigated in this study. It was found that the bio-available nutrients for pathogen regrowth were present in all of the composted biosolids samples tested, including those that had been stored for up to 2 years. As expected (Inbar *et al.* 1990) bio-available nutrients declined with the storage of compost. However, in all the samples tested they were present in sufficient concentrations to support *Salmonella* growth in the absence of competition from indigenous microorganisms. Consequently, bio-available nutrients were not a critical factor in pathogen regrowth in composted biosolids.

The results of this study suggest that the indigenous microbial activity was the single main factor which controlled *Salmonella* regrowth in composted biosolids. The youngest compost, which had been composting for two weeks, appeared to have the highest concentration of bio-available nutrients. However, inhibition of *Salmonella* growth in biosolids that had been composting for 2 weeks suggests that the biologically active indigenous microflora had the capacity to suppress *Salmonella* growth. It was also found that the antagonistic effect of indigenous microbes towards *Salmonella* declined with the storage of compost. Contrary to the general belief that the pathogen regrowth potential of composted biosolids declines with storage, it was found that the survival time of *Salmonella* in stored biosolids was longer as compared to the under composted and freshly composted biosolids. Consequently, to reduce the pathogen regrowth potential of composted biosolids, a taxonomically diverse indigenous microbial population should be maintained.
A number of bacteria and actinomycetes that had been isolated from composted biosolids were assayed for their antagonism towards *Salmonella*. The role of SS phage in suppression of *Salmonella* growth was also evaluated. The growth of *Salmonella* was found to be suppressed by 1 to 3 logs in the presence of established populations of indigenous bacteria and bacteriophage. Since none of the isolated microbes produced any growth inhibitory compounds against *Salmonella* therefore, the mechanism for growth suppression appears to be intense competition for limited nutrients. Competition for limited nutrients is expected to increase in the presence of a taxonomically diverse population due to different substrate utilisation.

### 7.2. *Salmonella* concentrations and regrowth in composted biosolids

In this study, *Salmonella* inactivation during large-scale windrow composting was evaluated. It was found that if composting is carried out in accordance with the recommended guidelines (ARMCANZ *et al.* 1995), *Salmonella* should be reduced to below detection limits within five weeks of composting. After five weeks *Salmonella* concentrations in composted biosolids were less than one MPN 50 g\(^{-1}\), which was the lowest detection limit of the method used for enumeration of *Salmonella*. Under draft Australian guidelines for biosolids re-use an acceptable *Salmonella* concentration in finished products is less than one MPN 50 g\(^{-1}\) (ARMCANZ *et al.* 1997). Thus the composted biosolids produced by windrow composting generally met Australian standards.
However, the re-population of *Salmonella* in stored compost after *Salmonella* were below the detection limit for 26 weeks, suggests that *Salmonella* can survive the composting process in low numbers. The surviving *Salmonella* may grow to hazardous levels in the presence of favourable growth conditions and the absence of competition from indigenous microflora. In this study, it was found that *Salmonella* regrowth was greater in stored biosolids as compared to freshly composted biosolids. Moreover, the inactivation rate for *Salmonella* was significantly lower in stored biosolids. Consequently, it is recommended that lengthy storage (more than 30 weeks) should be avoided to minimise the pathogen regrowth potential.

7.3. **Current method of compost mixing and recommendations for compost handling**

Different bulking materials, composting and storage conditions are expected to influence the population dynamics of indigenous microorganisms and ultimately pathogen regrowth potential. Some of these factors such as moisture content and initial C/N ratio could be managed to ensure a rapid build up of indigenous microflora.

The decline in concentration of microorganisms including *Salmonella* in composted biosolids is partially due to the unfavourable growth conditions. *Salmonella* regrowth can take place in the previously sanitised compost and compost mixed with other amendments with the return of favourable growth conditions. Skanvis and Yanko (1994)
reported the presence of *Salmonella* in 25% of compost based products they analysed. They also found a higher concentration of *Salmonella* and faecal coliforms in compost mixed with other materials as compared to the composted biosolids. Growth of *Salmonella* in the water extract of compost in this study suggests that there is a possibility of additional supply of water-soluble nutrients from wood sap and other materials used for making soil amendments. Consequently, mixing of compost with other bulking material to produce soil amendments needs careful consideration. It is possible that with the availability of additional nutrients and absence of a strong antagonistic effect from indigenous microorganisms, sudden regrowth of *Salmonella* in the presence of adequate moisture and temperature may result. Further research is required to improve understanding of the role of bulking materials in the supply of additional nutrients, and the response of reintroduced *Salmonella* to such conditions.

In this study, regrowth of *Salmonella* was observed in stored biosolids with the onset of rains. Similarly Deportes *et al.* (1998) also observed growth of *Salmonella*, streptococci and faecal coliforms in uncovered compost piles as compared to covered piles. It is possible that a decline in the moisture content in stored compost results in a decline in the concentration of antagonistic microorganisms. The sudden increase in the moisture content in this case could provide an opportunity for surviving *Salmonella* to regrow. Regrowth of *Salmonella* is also expected to be favoured by the previous decline in antagonistic microorganisms. Consequently, one way of ensuring a healthy population of antagonistic microorganisms in compost is to manage moisture content during the storage. It is recommended that the moisture content of compost should be more than the
critical limit of 20% (Russ and Yanko 1981) during composting and storage for maintaining activity of indigenous microorganisms. This could be achieved by monitoring the moisture content during composting and covering composted biosolids after composting. *Giardia* concentrations are known to decline with the storage of compost (Gibbs *et al.* 1998). However, the effect of covering compost on *Giardia* inactivation is not known and it needs further investigation.

### 7.4. Pathogen regrowth of composted biosolids

Limiting and often conflicting information is available on the correlation between *Salmonella* and indicator microorganisms such as FC, FS and *E. coli* during composting and storage of compost. Moreover, *Salmonella* is often present below detectable limits and later on regrows to hazardous levels when the product is marketed (Skanavis and Yanko 1994). Consequently, testing for an indicator organism alone cannot ensure the bio-safety of composted biosolids. The behaviour of *Salmonella* in composted biosolids can only be confidently predicted by monitoring for *Salmonella*.

A regrowth potential test using *S. typhimurium* is a very useful laboratory tool for understanding the role of various factors in *Salmonella* regrowth. It can also provide information on the bio-safety of the product. However, it is not recommended as a routine test for monitoring compost. Regular monitoring of *Salmonella* concentrations especially prior to the distribution of compost seems to be best option. At present under
both ARMCANZ and US EPA guidelines monitoring for *Salmonella* is optional. It is recommended that *Salmonella* should be monitored along with the total coliforms to ensure the bio-safety of compost based products.

### 7.5. Risk assessment of pathogen regrowth in composted biosolids

The results of this study suggest that there is always a risk of pathogen regrowth in composted biosolids. Skanvis and Yanko (1994) reported detectable levels of *Salmonella* in 25% of composted biosolids products. It has been previously demonstrated that *Salmonella* can survive the composting process (Russ and Yanko 1981) and monitoring of biosolids for pathogens is not routinely carried out. Consequently, there is a risk associated with the use of composted biosolids.

The most likely route of exposure to *Salmonella* in composted biosolids and compost based soil amendments is ingestion of compost. The children with pica behaviour are assumed to be at greater risk. Pica children ingest more than 1 g of soil per day (Calabrese and Stanek 1993). It has been also shown that children commonly ingest 5 g or more of soil in one day (Calabrese and Stanek 1995). The risk assessment in this study was carried out on the basis of a child ingesting 5 g of composted biosolids.

The annual reported *Salmonella* infection rate (1991) in pica category (0 to 4 Years) in Australia was 400 per 100 000 (Anura and Hall 1992). Since this is considered to be an acceptable level, this gives a probability of infection equal to 4 x 10^{-3}. It has been
suggested that acceptable level of illness due to biosolids may be from 1 to 10% of background rate of infection from other sources (Gibbs and Ho 1995). If we consider 10% of this background probability then the acceptable annual risk will be $4 \times 10^{-4}$ and $4 \times 10^{-5}$ at 1% of background level. If regrowth occurs, then the lowest concentration of *Salmonella* growth found was (90 g$^{-1}$) (Table 5.4). Pica children may ingest 5 g of compost which would be 400 *Salmonella*. An adult is more likely to ingest 0.1 g which come out to be 9 *Salmonella*.

The beta distributed probability model generated by Rose and Gerba (1991) can be used to calculate the probability of infection with ingestion of different concentration of *Salmonella*. The model used to work out the probability of infection in the case of *Salmonella* regrowth was:

$$p = 1 - \left(1 + \frac{N}{139.9}\right)^{-0.33}$$

Where $p$ = probability of infection and $N$ = number of microorganisms.

It was found that the probability of infection was more than acceptable limit for both adults and pica children being 0.02 and 0.37 respectively. Composted biosolids are also used to make soil amendments and mulches. Assuming that 25% of soil amendments is compost the probability of infection is still more than the acceptable limit for both adults and children. Consequently, it is concluded that regrowth of *Salmonella* in composted biosolids or compost based amendments may lead to a health hazard.
This study has shown that composted biosolids ranging from fresh to compost stored for up to 2 years has the potential to support growth of *Salmonella* to levels which are hazardous. *Salmonella* may survive the composting process at concentrations lower than the detection limit for *Salmonella* or may be reintroduces to compost. The recent study carried out by Simmonds *et al* (1999) also showed that there is a significant risk of contacting respiratory illness through the use of biosolids in gardens. However, other products that may provide similar level of hazard are being used in home gardens as well. Animal manure may be particularly more hazardous then the composted biosolids. Some manures may contain higher initial concentration of *Salmonella* in addition to supporting growth of *Salmonella*.

Consequently, it needs to be stressed a number of soil amendment material used in gardens have some level of microbial hazard associated with them. It is recommended that the hygienic practices with gardening should be emphasised.
8.1. Conclusions

1. All composted biosolids contain sufficient bio-available nutrients to support pathogen regrowth. Consequently, in the presence of favourable growth conditions pathogen regrowth can take place where *Salmonella* is reintroduced.

2. *Salmonella* can survive the composting process in numbers below the detection limit and regrow under favourable growth conditions. Consequently, there is always a risk of pathogen regrowth when compost is used for gardening.

3. The presence of a biologically active and taxonomically diverse population of indigenous microorganisms was the single main factor which controlled pathogen regrowth in composted biosolids.

4. The antagonistic effect of indigenous microorganisms was found to decline with the maturity of compost. Consequently, in the case of reintroduction of *Salmonella*, longer survival times could be expected in stored composted biosolids as compared to freshly composted biosolids.

5. Storage of composted biosolids to achieve compost stability and reduce the pathogen regrowth potential cannot guarantee the bio-safety of composted biosolids.
8.2. Recommendations

1. Under the current ARMCANZ guidelines (ARMCANZ et al. 1997) monitoring for thermotolerant coliforms or Salmonella in finished compost is recommended. It is recommended that in all the cases monitoring for Salmonella concentrations in composted biosolids should be carried out prior to release for public use. The relationship between indicator bacteria and Salmonella concentrations in composted biosolids should be investigated.

2. Composting and storage practices should aim towards a healthy build up and maintenance of the indigenous microbial population.

3. Long term storage of composted biosolids is not recommended as it results in a decline in the antagonistic effect. However, to reduce the risk associated with Giardia, following composting a storage time of approximately 30 weeks is recommended.

4. Livestock wastes are known to contain many pathogenic microorganisms. Research should be carried out to evaluate the health hazard associated with the use of animal manure.

5. To minimise the health hazard associated with the use of composted biosolids hygienic gardening practices should be emphasised.

6. Further research should be carried out to identify which groups of indigenous microorganisms suppress Salmonella regrowth. Resolving this issue could provide a better understanding of the antagonistic effect of indigenous microflora towards pathogenic bacteria in composted biosolids.
7. Additional research work should be carried out to improve understanding of the nature of microbial antagonism towards *Salmonella*.

8. Additional research is also needed for an improved understanding of optimum composting practices and storage conditions, which result in the production and maintenance of a biologically active indigenous microflora. The effect of the following factors on the indigenous microbial population should be considered:

- Effect of limiting moisture content on microbial diversity during composting and storage should be investigated.

- The compost pile could be covered after composting to preserve moisture, which may ensure the presence of a biologically active indigenous microflora. However, the effect of covering on the survival of *Giardia* and other pathogenic bacteria needs further investigation. Also, covering may create anaerobic conditions and may result in increased occupational health and safety risks. These issues need to be investigated.
REFERENCES


