CRANFIELD UNIVERSITY

SCHOOL OF ENERGY, ENVIRONMENT AND AGRIFOOD

DOCTOR OF PHILOSOPHY

ADADEMIC YEAR 2014 – 2015

STEPHANIE ELLIS

INTERACTIONS BETWEEN SEWAGE SLUDGE AND THE SURVIVAL OF PATHOGENIC BACTERIA IN SOIL

SUPERVISED BY KARL RITZ, SEAN TYRREL, KARL RICHARDS AND BRYAN GRIFFITHS

FEBRUARY 2015

© CRANFIELD UNIVERSITY 2015 ALL RIGHTS RESERVED. NO PART OF THIS PUBLICATION MAY BE REPRODUCED WITHOUT THE WRITTEN PERMISSION OF THE COPYRIGHT HOLDER.

THIS THESIS IS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Abstract

Sewage sludge is a potentially valuable resource that can enhance both the structure and fertility of soil. However, it can also harbour enteric pathogens which pose a significant socio-economic risk to society. Therefore it is important to understand the factors that govern the persistence of such pathogens in soil, when co-introduced with sewage sludge, in order to mitigate risk and to further avail of such a valuable resource. This research aimed to clarify how microbial activity and the presence of sewage sludge would influence the persistence of co-introduced enteric pathogens in soil. It was theorised that the addition of sewage sludge to soil would cause the formation of organic matter (OM) and nutrient-rich niches. Such niches, in turn, would encourage the enhanced activity of the local soil microbial community, instigating greater competition for local resources, i.e. a hot spot of microbial activity that would lead to a decline in the introduced enteric pathogens. It was also hypothesised that the interface between the soil and sewage sludge may influence such interactions, as the physicochemical characteristics could affect the extent of exposure and subsequent interactions between enteric pathogens and the soil microbial community. These theories were investigated using four different perspectives that linked closely with each other.

In initial studies, two cohorts of microcosms consisting of different proportions of sewage sludge to soil were inoculated with either *E. coli* or *S.* Dublin and destructively sampled over a 42 day period. *E. coli* prevailed at greater numbers when inoculated directly into soil and sewage sludge, whilst it declined to the greatest extent within mixed microcosms containing 25% sludge. All treatments containing *S.* Dublin appeared to decline at a similar rate, which was more linear than the decline observed within treatments inoculated with *E. coli*. From these findings, it can be concluded that there are no direct relationships between the proportion of sludge to soil and its affect on pathogen survival. A subsequent experiment implemented a similar treatment strategy, whilst using indigenous sewage sludge *E. coli*. The use of this microbe provided data which was more suited to the original premise of this work, as under such scenarios it would be indigenous sewage sludge *E. coli* that would be of concern. Therefore, microcosms consisting of different proportions of sewage sludge, containing indigenous *E. coli*, were destructively sampled over a 56 day period. The indigenous sewage sludge *E. coli* exhibited a more consistent linear decline after the first week.

However, the indigenous *E. coli* were again not significantly affected by different proportions of sewage sludge to soil. It was theorised that this lack of variation in response to varying proportions of sewage sludge to soil may have been associated with a lack of available substrate within the system, or some form of partitioning effect between soil and sewage sludge matrices, which prevented the microbial communities from interacting.

To further develop these concepts, the effect of two contrasting substrate amendments and their location (either sewage sludge, soil or within both matrices) was also investigated in relation to the persistence of sewage sludge-derived *E. coli*. Microcosms consisting of both pure samples and mixtures of sewage sludge or soil were inoculated with sewage sludge-derived *E. coli* and destructively sampled over a 42 day period. Respired CO_2 and microbial carbon were also quantified. The addition of a simple substrate, glucose, instigated a peak in microbial respiration and accelerated the decline of sewage sludge-derived *E. coli* and also marginally increased the microbial biomass. This is similar to the original concept proposing that a hot spot of microbial activity could instigate pathogen die-off. In contrast, amendment with a more complex substrate, yeast extract, had little effect on the decline of sewage sludge-derived *E. coli*. Nor did respiration increase immediately after amendment. There was also no observable partitioning effects between soil or sewage sludge with either amendment. This suggests that a lack of available substrate could influence microbial dynamics and thus the decline of *E. coli*. To further explore this phenomenon the repeated addition of glucose and its effect on the survival of sewage sludge-derived *E. coli* was investigated. It aimed to highlight the impact of sustained competition for resources on persistence, whilst mimicking the recurrent input of carbon that occurs in plant/soil systems. Microcosms consisting of both pure and mixtures of sewage sludge or soil were inoculated with sewage sludge-derived *E. coli* and destructively sampled over a period of 105 days. Respired CO_2 and microbial carbon were again analysed. It was found that the repeated addition of glucose did not cause a significant decline in the survival of sewage sludge-derived *E. coli*. Notably, some small increases in *E. coli* numbers were observed after the second and third amendments of glucose.

Overall, these findings suggest that hot spots of activity can instigate a decline in enteric pathogens, though such interactions are dependent upon the availability and quantity of nutrients and organic carbon within the matrices. These findings could aid in developing the use of amendments in sewage sludge that would minimise the survival of enteric pathogens in soil. They also provide a framework which pinpoints the factors that should be considered when investigating the persistence of enteric pathogens in the soil environment. Such amendments and knowledge pertaining to the key factors in the survival of enteric pathogens could further decrease the social and economic risk which the use of sewage sludge poses when used in agricultural systems.

This work was funded by the EU FP7 Environment theme - Grant no. 265269 Marketable sludge derivatives from a highly integrated wastewater treatment plant (END-O-SLUDG).

Acknowledgements

To my supervisors, Karl Ritz, Sean Tyrrel, Karl Richards and Bryan Griffiths, thank you. Your support and guidance have been invaluable in the few years we have worked together. To Karl Richards, thank you for putting up with me sporadically calling into your office. It helped to keep me focused! To Bryan Griffiths, thank you for introducing me to such a fascinating subject area. I hope I get to continue within this area for as long as this *slight* obsession holds! I would especially like to thank Karl Ritz and Sean Tyrrel for their time and patience. You have both been the cornerstones to the development of this project. It is not just the research that inspired me, but you also.

To my colleagues and peers at Teagasc, Johnstown Castle, I would also like to extend my gratitude. It has been both a pleasure and a privilege to work amongst so many brilliant individuals. I would particularly like to thank Emma Moynihan. You made doing a PhD a little less stressful! Additionally, I would like to thank Denis Brennan, Cathal Somers, Paul Massey and Dote Stone for their advice and support. You are all invaluable. I would also like to thank the END-O-SLUDG project and the Walsh Fellowship Program for funding this work. As well as Andrew Sprigings, Katherine Miller and colleagues at United Utilities, Ellesmere Port who provided the sludge cake with which this research was undertaken.

To my family and friends, you have been far too patient and kind with me. I'm not sure I will be able to repay such a debt, though I plan on trying! To my Dad, your advice helped ground me and our walks helped clear my head. To Jen and Nicki, thank you for the fun nights out and evenings spent talking too much over wine. To Aidan, I don't think I would have made it this far without you by my side. Your support kept me together and kept me going on days I did not think I would get through.

Finally, to all those who have provided me the opportunities and the education to get to this point, thank you. I won't forget it. To anyone else that I may have missed, forgive me for that is not my intent. Feel free to demand retribution in the form of alcoholic beverages at any point in the future!

Table of contents

Title page I

Abstract..II

Acknowledgements V

Table of contents VI

Figures XIII

Tables XVIII

1. Introduction 1

2. Literature review..6

2.1. Introduction 6

2.1.1. Epidemiology 6

2.2. Sewage sludge 10

2.2.1. Treatment..10

2.2.2. Physicochemical properties 12

2.2.3. Biological properties..14

2.2.4. Legislation 16

2.2.5. Management 18

2.3. Persistence of pathogenic bacteria in soil..20

2.3.1. Physicochemical factors 21

2.3.2. Biological factors..23

2.4. A hot spot of activity 25

2.5. Aims and research questions 26

2.5.1. Objectives 27

3. General methods 28

3.1. Introduction 28

3.2. Collection, preparation and analysis of samples 28

3.2.1. Site description: soil 28

3.2.2. Site description: sewage sludge 28

3.2.3. Sample preparation and analysis 29

3.3. Preparation, extraction and enumeration of pathogens 31

3.3.1. Pathogens..31

3.3.2. Inoculum preparation..32

3.3.3. Extraction of pathogens 32

3.3.4. Enumeration of model pathogens 33

3.3.5. Enumeration of indigenous sewage sludge and sewage sludge-derived pathogens 33

3.4. Respiration..35

3.4.1. Background..35

3.4.2. Sample preparation 36

3.4.3. Headspace sampling 36

3.4.4. Gas chromatography procedure..37

3.5. Microbial carbon..38

3.5.1. Background..38

3.5.2. Fumigation procedure 39

3.5.3. Extraction procedure 39

3.5.4. Calculation of microbial carbon 39

3.6. Statistical analysis 40

4. The significance of sewage sludge loading on the persistence of pathogenic bacteria in soil...41

4.1. Introduction 41

4.2. Aims, objectives and hypotheses..45

4.2.1. Aim 45

4.2.2. Objectives 45

4.2.3. Hypothesis 45

4.3. Materials and methods..46

4.3.1. Sample collection and analysis...46

4.3.2. Treatment preparation 46

4.3.3. Survival of model pathogens *E. coli* and *S*. Dublin.......................48

4.3.4. Statistical analysis 48

4.4. Results 49

4.4.1. Background analysis...49

4.4.2. Survival of model pathogen *E. coli* 51

4.4.3. Survival of model pathogen *S*. Dublin..53

4.5. Discussion...55

4.5.1. Conclusion 57

5. The significance of sewage sludge loading on the persistence of indigenous sewage sludge pathogens in soil 58

5.1. Introduction 58

5.2. Aims, objectives and hypotheses 60

5.2.1. Aim 60

5.2.2. Objectives 60

5.2.3. Hypothesis 60

5.3. Materials and methods 61

5.3.1. Sample collection and analysis 61

5.3.2. Treatment preparation 61

5.3.3. Survival of indigenous sewage sludge *E. coli* 62

5.3.4. Statistical analysis 63

5.4. Results 64

5.4.1. Background analysis 64

5.4.2. Survival of indigenous sewage sludge *E. coli* 66

5.5. Discussion 68

5.5.1. Conclusion 69

6. The effect of supplementary nutrients on the survival of pathogens in soil, co-introduced with sewage sludge 71

6.1. Introduction 71

6.2. Aims, objectives and hypotheses 75

6.2.1. Aims 75

6.2.2. Objectives 75

6.2.3. Hypotheses 75

6.3. Materials and methods 77

6.3.1. Sample collection and analysis 77

6.3.2. Treatment preparation 77

6.3.3. Analysis of microbial activity and survival of sewage sludge-derived *E. coli* 78

6.3.4. Statistical analysis 79

6.4. Results 80

6.4.1. Background analysis 80

6.4.2. Survival of sewage sludge-derived *E. coli*; glucose amendment ..83

6.4.3. Survival of sewage sludge-derived *E. coli*; yeast extract amendment 85

6.4.4. Respiration; glucose amendment 87

6.4.5. Respiration; yeast extract amendment 89

6.4.6. Microbial carbon; glucose amendment 91

6.4.7. Microbial carbon; yeast extract amendment 93

6.5. Discussion 95

6.5.1. Conclusion 97

7. The effect of repeated, supplementary nutrients on pathogens survival in soil, co-introduced with sewage sludge 99

7.1. Introduction 99

7.2. Aims, objectives and hypotheses 102

7.2.1. Aim 102

7.2.2. Objectives 102

7.2.3. Hypotheses 102

7.3. Materials and methods..103

7.3.1. Background analysis..103

7.3.2. Treatment preparation 103

7.3.3. Analysis of microbial activity and survival of sewage sludge-derived *E. coli*..104

7.3.4. Statistical analysis 105

7.4. Results 107

7.4.1. Background analysis..107

7.4.2. Survival of sewage sludge-derived *E. coli* 110

7.4.3. Survival of general coliforms 112

7.4.4. Respiration..115

7.4.5. Microbial carbon..117

7.5. Discussion..119

7.5.1. Conclusion 122

8. Discussion..123

8.1. Conclusion..133

8.2. Limitations and future work 135

References 137

Appendix 1 162

Appendix 2 182

Appendix 3 186

Figures

Figure 1.1. (a) Input and decomposition of sewage sludge containing pathogenic bacteria; (b) potential physical interactions that could influence survival; (c) potential competitive and predatory interactions between sewage sludge-derived pathogens and the soil microbial community (partially adapted from Ingham, 2000)............................3

Figure 1.2. Flow chart overview of thesis 5

Figure 2.1. Simplified graph representing the growth kinetics of bacteria (adapted from Buchanan, 1918; Roszak and Colwell, 1987)..7

Figure 2.2. Simplified schematic of wastewater treatment process (simplified adaptation from AWA, 2009)...11

Figure 2.3. Schematic showing (i) sewage sludge tilled into crop land and (ii) sewage sludge spread onto grazing land 20

Figure 3.1. Equipment used for chromogenic membrane filtration protocol (a) Microsart® e.jet pump; (b) Combisart stainless steel manifold apparatus; (c) placement of membrane filter; (d) removable funnels; (e) latch to secure funnels to the main body, with spigot to open/close inner valve to allow liquid through 35

Figure 3.2. Apparatus used to hold and obtain headspace samples; (a) 10 ml syringe with needle attached, inserted through rubber septum; (b) 100ml sterile, plastic, screw-cap container holding sewage sludge and soil samples...37

Figure 4.1. Treatment outline, detailing amendment strategy. Key: ☐ soil; ▨ sludge; ■ inoculated with model pathogen; ▦ inoculated with model pathogen only, i.e. no soil or sewage sludge present within treatment..47

Figure 4.2. Survival of *E. coli* within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE). Key: —●— inoculated control soil; —○— mixed 25% sludge; —▼— mixed 50% sludge; —△— mixed 75% sludge; —■— inoculated control sludge 52

Figure 4.3. Death rates for *E. coli* in microcosms containing mixtures of sewage sludge and soil, calculated using CFU gDs^{-1} (n=3, Mean ± SEM). Letters denote homogeneous means at 5% significance level..52

Figure 4.4. Survival of *S.* Dublin within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE). Key: —●— inoculated control soil; —○— mixed 25% sludge; —▼— mixed 50% sludge; —△— mixed 75% sludge; —■— inoculated control sludge. *errors are smaller than symbols 54

Figure 4.5. Death rates for *S.* Dublin in microcosms containing mixtures of sewage sludge and soil, calculated using CFU gDs^{-1} (n=3, Mean ±SEM). Letters denote homogeneous means at 5% significance level 54

Figure 5.1. Treatment outline, detailing proportion of soil to sewage sludge. Key: □ soil; ▨ sludge; ■ presence of indigenous sewage sludge *E. coli* 62

Figure 5.2. Survival of indigenous sewage sludge *E. coli* within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE; error bars fall within confines of points in most instances). Key: —●— mixed, 15% sludge; —○— mixed, 25% sludge; —▼— mixed, 50% sludge; —△— mixed, 75% sludge; —■— Control sludge. *errors are smaller than symbols 66

Figure 6.1. Treatment outline, detailing substrate amendment strategy. Key: □ soil; ▨ sludge; ■ amended with substrate. Sewage sludge-derived *E. coli* was present within sludge in this instance..78

Figure 6.2. Survival of sewage sludge-derived *E. coli* across all treatments, amended with glucose (n=4, ± pooled SE). Key: —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge.........................84

Figure 6.3. Survival of sewage sludge-derived *E. coli* across all treatments, amended with yeast extract (n=4, ± pooled SE). Key: —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge 86

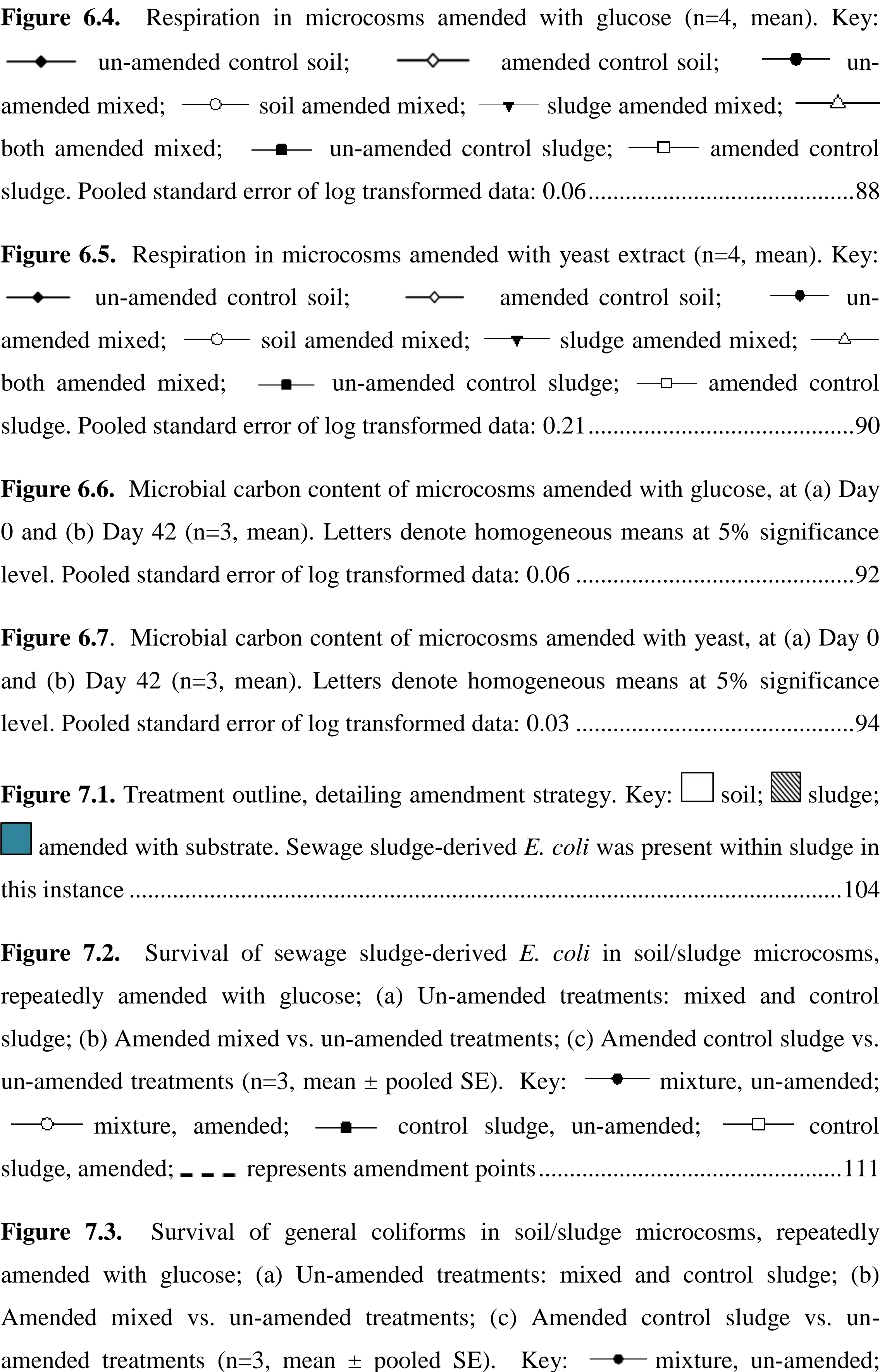

Figure 6.4. Respiration in microcosms amended with glucose (n=4, mean). Key: un-amended control soil; amended control soil; un-amended mixed; soil amended mixed; sludge amended mixed; both amended mixed; un-amended control sludge; amended control sludge. Pooled standard error of log transformed data: 0.06 88

Figure 6.5. Respiration in microcosms amended with yeast extract (n=4, mean). Key: un-amended control soil; amended control soil; un-amended mixed; soil amended mixed; sludge amended mixed; both amended mixed; un-amended control sludge; amended control sludge. Pooled standard error of log transformed data: 0.21 90

Figure 6.6. Microbial carbon content of microcosms amended with glucose, at (a) Day 0 and (b) Day 42 (n=3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.06 92

Figure 6.7. Microbial carbon content of microcosms amended with yeast, at (a) Day 0 and (b) Day 42 (n=3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.03 94

Figure 7.1. Treatment outline, detailing amendment strategy. Key: soil; sludge; amended with substrate. Sewage sludge-derived *E. coli* was present within sludge in this instance 104

Figure 7.2. Survival of sewage sludge-derived *E. coli* in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended treatments: mixed and control sludge; (b) Amended mixed vs. un-amended treatments; (c) Amended control sludge vs. un-amended treatments (n=3, mean ± pooled SE). Key: mixture, un-amended; mixture, amended; control sludge, un-amended; control sludge, amended; represents amendment points 111

Figure 7.3. Survival of general coliforms in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended treatments: mixed and control sludge; (b) Amended mixed vs. un-amended treatments; (c) Amended control sludge vs. un-amended treatments (n=3, mean ± pooled SE). Key: mixture, un-amended;

—○— mixture, amended; —■— control sludge, un-amended; —□— control sludge, amended. Estimate key: ┈◇┈ control sludge, un-amended;┈▽┈ mixture, amended; ┈⬡┈ control sludge, amended; ▬ ▬ ▬ represents amendment points. *Day 15 omitted as there was no data collected 114

Figure 7.4. Respiration in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended control soil vs. amended control soil; (b) Un-amended mixture vs. amended mixture; (c) Un-amended control sludge vs. amended control sludge (n=3, mean). Key: —◆— control soil, un-amended; —◇— control soil, amended; —●— mixture, un-amended; —○— mixture, amended; —■— control sludge, un-amended; —□— control sludge, amended; ↓ represents removed anomalous points; ▬ ▬ ▬ represents amendment points. Pooled standard error of log transformed data: 0.00 116

Figure 7.5. Microbial carbon content of soil/sludge microcosms, repeatedly amended with glucose, at (a) Day 0 and (b) Day 105 (n = 3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.08..118

Figure 1a. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* lys 9; ▬ linear regression equation 164

Figure 1b. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* lys 9; ▬ linear regression equation 168

Figure 1c. Optical density of *E. coli* O157 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* O157; ▬ linear regression equation 168

Figure 1d. Optical density of *L. monocytogenes* in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *L. monocytogenes*; ▬ linear regression equation 169

Figure 1e. Optical density of *S*. Dublin in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *S*. Dublin; ▬ linear regression equation169

Figure 1f. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data ta. Key: ▲ optical density vs. *E. coli* lys 9; ▬ linear regression equation 176

Figure 1g. Survival of indigenous sewage sludge *E. coli* within microcosms comprising of sewage sludge mixtures enumerated using basic plating technique (n = 4, mean ± SEM). Key: —●— *E. coli*. *SEM falls within the width of the observed symbols 179

Figure 1h. Survival of indigenous sewage sludge *E. coli* and general coliform bacteria within microcosms comprising of sewage sludge mixtures enumerated using chromogenic membrane filtration technique (n=4, mean ± SEM). Key: —●— *E. coli*; —▲— general coliforms. *SEM falls within the width of the observed symbols 180

Figure 1i. Survival of indigenous sewage sludge *E. coli* within microcosms comprising of sewage sludge mixtures enumerated using MPN technique (n=4, mean ± SEM). Key: —●— *E. coli*. *SEM falls within the width of the observed symbols 180

Figure 2a. Optical density of *E. coli* in LB broth (tenfold dilution series) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli*; ▬ linear regression equation......................184

Figure 2b. Optical density of *S.* Dublin in LB broth (tenfold dilution series) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *S.* Dublin; ▬ linear regression equation..........184

Figure 2c. Recovery rate of (a) *S.* Dublin and (b) *E. coli* in microcosms containing mixtures of sewage sludge and soil (n=3, Mean ± Pooled SE) 185

Tables

Table 2.1. Chemical composition of sewage sludge (simplified adaptation from Fytili and Zabaniotou, 2008) 14

Table 2.2. Pathogens associated with wastewater and sewage sludge, with associated diseases or symptoms (reproduced from Gerba and Smith, 2005) 15

Table 2.3. Use of sewage sludge in agriculture (reproduced from ADAS, 2001). ✔ All applications must comply with the Sludge (Use in Agriculture) Regulations and Department of Environment Transport and Regions (DETR). ✘ Applications not allowed (except where stated conditions apply) 18

Table 2.4. The re-use or disposal route for sewage sludge within Europe (reproduced from DEFRA, 2012b) 20

Table 3.1. Soil analyses and corresponding references 30

Table 3.2. Sewage sludge analyses and corresponding references 31

Table 4.1. Treatment outline (n = 3) based on volume basis (%), with equivalent weight of sewage sludge and soil (± 0.05 g) 47

Table 4.2. Chemical characteristics of soil (Mean ± SEM, n = 3) 49

Table 4.3. Chemical characteristics of the sewage sludge (Mean ± SEM, n = 3) 50

Table 4.4. Biological characteristics of the sewage sludge and soil, prior to inoculation of model pathogenic bacteria (Mean ± SEM, n=3) (* n=2) 50

Table 5.1. Treatment outline (n = 3), with equivalent weight of sewage sludge and soil (± 0.02g) 62

Table 5.2. Chemical characteristics of soil (Mean ± SEM, n = 3) 64

Table 5.3. Chemical characteristics of the sewage sludge (Mean ± SEM, n = 3) 65

Table 5.4. Biological characteristics of the sewage sludge and soil (Mean ± SEM, n = 3). 65

Table 6.1. Treatment formulations (n = 4), with equivalent dry weight (± 0.02 g) of sewage sludge and soil 78

Table 6.2. Time points and respective analyses....................79

Table 6.3. Physicochemical characteristics of soil (Mean ± SEM, n = 3)....................81

Table 6.4. Physicochemical characteristics of sewage sludge (Mean ± SEM, n = 3) 82

Table 6.5. Biological characteristics of soil and sewage sludge (Mean ± SEM, n = 3) .82

Table 7.1. Treatment formulations (n = 3), with equivalent dry weight (± 0.02 g) of sewage sludge and soil 104

Table 7.2. Time points, amendment regime and respective analyses....................105

Table 7.3. Physicochemical characteristics of soil (mean ± SEM, n = 3) 108

Table 7.4. Physicochemical characteristics of sewage sludge (mean ± SEM, n = 3)...109

Table 7.5. Biological characteristics of both soil and sewage sludge (mean ± SEM, n = 3)....................109

Table 1a. Treatment outline (n = 3) based on fresh weight of soil....................164

Table 1b. Estimated quantity of inoculated *E. coli* lys 9, obtained from the regression equation calculated from the data in Figure 10.1. vs. plate count data. * calculated using regression equation where X equals average absorbency (600 nm) 165

Table 1c. Quantity of *E. coli* lys 9 recovered from each treatment (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment. *Not applicable (NA) as the soil within these treatments was not inoculated prior to extraction 165

Table 1d. Treatment outline (n = 3) based on fresh weight of soil....................167

Table 1e. Estimated quantity of inoculated model pathogens, obtained using the regression equation obtained from the preceding Figures vs. against plate count data. * calculated using regression equation where X equals average absorbency (600 nm) 170

Table 1f. Quantity of each model pathogen recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment..........170

Table 1g. Treatment outline (n = 3) based on the fresh weight of soil and sewage sludge (± 0.05 g) 172

Table 1h. Quantity of each *E. coli* lys 9 recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment.................172

Table 1i. Treatment outline (n = 3) based on fresh weight of soil (± 0.05 g). *contained 50ml 1/4 strength Ringer's 175

Table 1j. Estimated *E. coli* lys 9, obtained from the regression equation calculated from the data in Figure 1.6. against actual plate count data. * calculated using regression equation where X equals average absorbency (600 nm) 176

Table 1k. Quantity of each model pathogen recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment..........177

Table 2a. Estimated quantity of inoculated model pathogens, obtained from the regression equation calculated from the preceding figures vs. plate count data. * calculated using regression equation where X equals average absorbency (600 nm) 183

Table 3a. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 42) on *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 186

Table 3b. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 7) on *S.* Dublin counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 186

Table 3c. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 14 - 42) on *S.* Dublin counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 187

Table 3d. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 14) on indigenous sewage sludge *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 187

Table 3e. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 28- 56) on indigenous sewage sludge *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 188

Table 3f. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0- 6) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 188

Table 3g. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 13- 42) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 189

Table 3h. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0 - 6) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 189

Table 3i. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 13 - 42) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 190

Table 3j. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0 - 3) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05190

Table 3k. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 6 - 13) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05191

Table 3l. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 28 - 42) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05192

Table 3m. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0 - 6) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05193

Table 3n. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 13 - 42) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05194

Table 3o. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0, 42) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05...195

Table 3p. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per treatment (Treatments: control soil, un-amended – mixture, soil amended) on microbial carbon content (mg l^{-1}). Letters denote

homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 195

Table 3q. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per treatment (Treatments: mixture, sludge amended – control sludge, amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 196

Table 3r. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0, 42) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 196

Table 3s. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (Treatments: control soil, un-amended – mixture, soil amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 197

Table 3t. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (Treatments: mixture, sludge amended – control sludge, amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 197

Table 3u. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 0 - 14) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 198

Table 3v. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 15 -

29) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 199

Table 3w. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 15 - 29) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 199

Table 3x. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 0 - 14) on general coliform counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 200

Table 3y. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 17 - 31) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05. [*no data for Day 15] 200

Table 3z. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 35 - 105) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 201

Table 3aa. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0, 105) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 201

Table 3ab. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (all treatments) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05 202

1. Introduction

Sewage sludge can provide organic matter (OM) and nutrients to soil, thus improving both its structure and fertility. However, it can also introduce enteric pathogens into the soil environment, leading to their potential transferral onto crops or into watercourses (Jones, 1999). From here, enteric pathogens can potentially infect individuals through contact with contaminated water, produce, soil particles or from direct contact (Buchholz, *et al*, 2011; Davis, *et al*, 2005; Hrudey, *et al*, 2003). Therefore, their persistence in such environments is of continual concern, as they pose a significant socio-economic threat. This research, then, intends to clarify our understanding of the mechanisms which affect the persistence of enteric pathogens in soil, when co-introduced with sewage sludge. In particular the impact of physicochemical properties of sewage sludge on microbial interactions and how they impact the persistence of such pathogens.

Enteric pathogens are human gut flora which cause a variety of diseases in humans; *Salmonella,* typhoid fever and gastroenteritis; *Shigella*, bacillary dysentery; *Escherichia*, haemorrhagic diarrhoea and haemolytic uremic syndrome (Prescott, *et al.* 2005a; Kolling, *et al.* 2012). These diseases and their associated pathogens are transmitted in faeces, leading to direct host-host contact from inadequate hygiene or indirect contact from contaminated soil, water or food (Karch, *et al.* 1995; Maule, 2000; Solomon, *et al.* 2002; Rangel, *et al.* 2005). Not only do such diseases pose a risk to individuals, especially the more vulnerable (e.g. infants, the elderly and immunocompromised), but they also place a heavy economic burden on society (Käferstein, *et al.* 1997). Currently, the global burden of food-borne disease is unknown, with the World Health Organisation (WHO) expected to provide new estimates in 2015 (WHO, 2015). Previous research, estimated that there were approximately 21 million cases of typhoid fever in 2000 and approximately 1.7 billion cases of diarrhoeal disease annually (Crump, *et al.* 2004; WHO, 2013). Furthermore, Rayner and Scarborough (2005) estimated that food-borne illnesses cost the NHS, UK, £6 billion each year. These examples illustrate the potential socio-economic threat posed by enteric pathogens and that research into their persistence in the environment is both valid and essential to future socio-economic security.

The transmission of enteric pathogens also poses a significant issue for agriculture. The faecal matter, as well as other waste products, accumulated at wastewater treatment facilities (WWTFs) provides a valuable source of fertiliser: sewage sludge (Mantovi, *et al.* 2005). Prior to application to land, the sludge can undergo several treatment processes, to stabilise the OM and to reduce presence of harmful substances, as well as the pathogen load (EC, 2001a). However, some pathogens can survive such processes, with their survival potentially leading to their introduction to land with the sewage sludge (Sahlström, *et al.* 2004; Pourcher, *et al.* 2007). Therefore, outbreaks of disease that are associated with crops or farmland can subsequently incur significant monetary losses within the agricultural sector.

However, the re-use of sewage sludge as an agricultural fertiliser is the most cost effective and sustainable mode of handling this wastewater by-product, as it is both a cheaper alternative to chemical fertilisers and decreases the volume of the material that is incinerated or that enters landfill sites (Grey, 2004). Its re-use is significant in developing countries because of the cost and finite nature of chemical fertilisers. Whilst in developed countries, such as the UK or Germany, European legislation continues to promote sustainable alternatives. For example, the Landfill Directive 99/31/EC sets targets which reduce the amount of biodegradable waste going to landfill and will eventually lead to the phasing out of the disposal of sewage sludge in this manner (RPA, *et al*. 2008). Therefore, it will be important to find alternate routes for the safe disposal or re-use of sewage sludge in the future. Further supporting the importance of studying pathogen survival in sludge applied to land.

There are several biological and physicochemical factors which can affect the survival of pathogens in the soil environment. These include biological factors such as antagonism, competition, mutualism and symbiosis; and physicochemical factors such as temperature, pH, structure, nutrient availability and land management (van Veen, *et al.* 1997; Harper, *et al.* 2006). As the soil microbial community is potentially better adapted to a harsher and more changeable environment than indigenous sewage sludge pathogens, there is the potential for them to out-compete pathogens for available resources. This in combination with the input of organic carbon and nutrients from sewage sludge application to land, leads to the following question: How will the input

of organic carbon and nutrients and, associated with sewage sludge applied to land, affect soil microbial activity and the persistence of pathogenic bacteria found within sewage sludge?

It is theorised that the addition of sewage sludge to soil would cause the formation of a nutrient- and OM- rich niche (Figure 1.1a). This niche, in turn, would encourage the enhanced activity of the local soil microbial community, instigating greater competition for local resources, i.e. a hot spot of microbial activity that would lead to a decline in the introduced enteric pathogens (Figure 1.1c). It was also speculated that the interface between the soil and sewage sludge may influence such interactions; as the physicochemical characteristics could affect the extent of exposure or interaction enteric pathogens and the soil microbial community (Figure 1.1b).

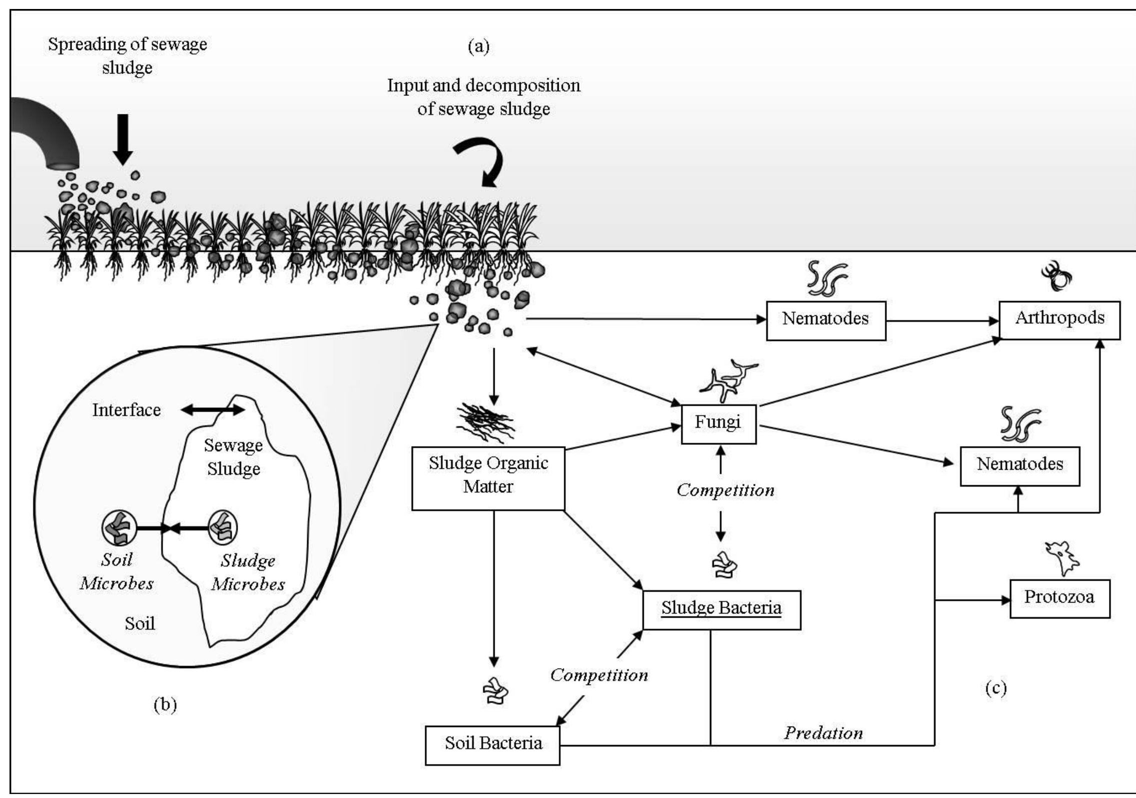

Figure 1.1. (a) Input and decomposition of sewage sludge containing pathogenic bacteria; (b) potential physical interactions that could influence survival; (c) potential competitive and predatory interactions between sewage sludge-derived pathogens and the soil microbial community (partially adapted from Ingham, 2000).

From this initial concept, a further set of questions was developed over the course of the project. Each of the questions was built upon the outcome of the previous question and is further outlined in Figure 1.2, which sets the questions within the context of the entire thesis. These questions were:

1. Does the proportion of sewage sludge to soil affect the persistence of bacterial pathogens? (Chapter 4).

2. Does the proportion of sludge to soil affect the persistence of indigenous sewage sludge *E. coli*? (Chapter 5).

3. How will the addition of supplementary substrate (yeast extract and glucose) to differential phases of sludge and soil mixtures affect the survival of sewage sludge-derived *E.coli*? (Chapter 6).

4. How will the repeated additions of supplementary substrate (glucose), mimicking the recurrent input of carbon that occurs in plant/soil systems, affect the survival of sewage sludge-derived *E. coli* or indigenous sewage sludge general coliforms? (Chapter 7).

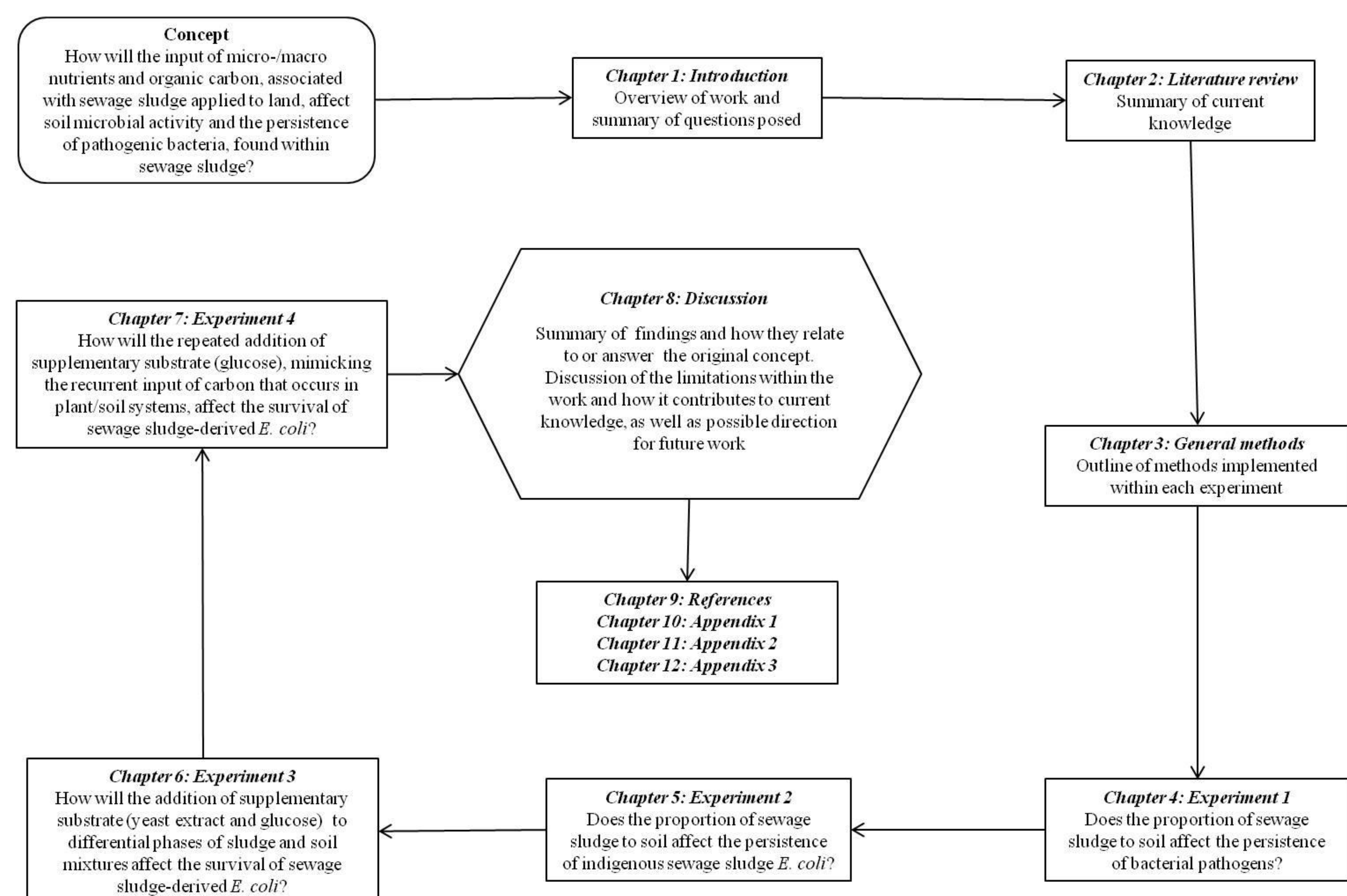

Figure 1.2. Flow chart overview of thesis.

2. Literature review

2.1. Introduction

The fate of enteric pathogens in soils receiving sewage sludge is a complex, multidisciplinary issue. It requires knowledge of pathogen survival, transmission routes, wastewater treatment practices, legislation and associated risks. It also requires understanding between the physicochemical and biological interactions of both soil and sewage sludge. Therefore this Chapter endeavours to provide salient information necessary to develop and support the questions and hypotheses posited throughout this work.

2.1.1. Epidemiology

The primary niche of enteric pathogens is the gastrointestinal tract of warm blooded animals, such as humans or livestock (Troxler, *et al.* 2012). This environment provides a constant temperature and a continual input of nutrients; optimum conditions for survival. After initial contact, infection begins with colonisation, followed by growth and reproductive phases within the gut. They are then shed in the excrement or diarrhoea of infected individuals (Hammer and Hammer 2004a). This waste is accumulated in sewage systems and is then transported to WWTFs for treatment. The young, elderly and immunocompromised are generally at greatest risk from these pathogens, with higher incidence of death amongst these demographics in relation to intestinal disease (Käferstein, *et al.* 1997; Gerba, *et al.* 1996). This increased incidence can be attributed to nonspecific host factors, such as a lack of pre-existing immunity, nutrition, or a reduced ability to elicit an immune response (Gerba, *et al.* 1996).

Enteric pathogens undergo several growth phases during their life cycle, whether they are inside the primary host environment or outside of it. It can be outlined as followed (Figure 2.1; Buchanan, 1918; Roszak and Colwell, 1987):

(1) An initial stationary or lag phase, where bacterial numbers remain constant.
(2) Logarithmic or exponential growth phase, which is a constant rate of growth per microbe with a corresponding minimal generation time per microbe.

(3) Maximum stationary phase, where there is no observable increase in microbial numbers.
(4) Logarithmic death phase, where the rate of death becomes constant.

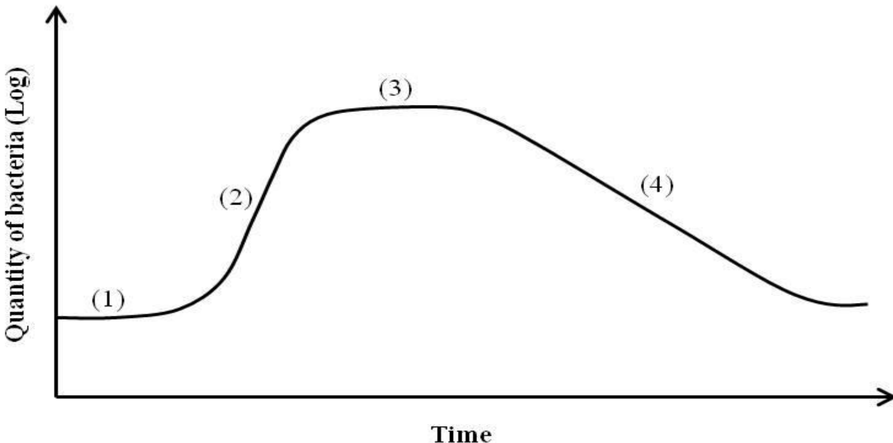

Figure 2.1. Simplified graph representing the growth kinetics of bacteria (adapted from Buchanan, 1918; Roszak and Colwell, 1987).

Additionally, bacterial cells can survive or persist without growing/replicating, effectively remaining in a state of stasis. This stasis usually corresponds to conditions that are adverse to propagation, such as when they are exposed to varying environmental conditions or limited nutrients. Propagation/growth then occurs when conditions become more tenable, such as with the input of additional nutrients from fertiliser application. This would be similar to the variable but non-culturable (VBNC) theory, outlined in Section 3.2.3, and is one of the mechanisms with which they survive the treatment processes at WWTFs.

Some of the most well known pathogenic strains associated with outbreaks of foodborne disease are found within the genera: *Escherichia*, *Salmonella* and *Campylobacter*. They cause disease through the use of two substances: exotoxins, a protein actively secreted by cells, or endotoxins, a components of the outer membrane of Gram negative bacteria, released when these cell lyses (Campbell and Reece, 2005). There are several strains of *E. coli* which causes disease, including: enterotoxigenic (ETEC), Enteropathogenic (EPEC) and Enteropathogenic (EHEC) strains (Prescott, *et al.* 2005a). A well-known form of EHEC is *E. coli* 0157:H7. These strains generally cause diarrhoea, haemorrhagic colitis or haemolytic uremic syndrome (which is

characterised by kidney failure and anaemia). Furthermore, strains of *Salmonella* contain can cause typhoid fever or food poisoning (Prescott, *et al.* 2005b). There is an estimated 93.8 million cases of gastroenteritis caused by strains of *Salmonella* each year; the majority of which are food-borne illnesses (Majowicz, *et al.* 2010). *Campylobacter* species also cause gastroenteritis, eliciting disease through several mechanisms including binding/entering cells and producing a cytolethal distending toxin which can cause apoptosis (van Vliet and ketley, 2001; WHO, 2011).

Due to the prevalence of these disease causing strains, and their excretion in waste, these pathogens are monitored to ensure the safety. For example, the Environment Agency, UK, (2002) classifies them as coliform bacteria and uses them as indicators of contamination in wastewater, sewage sludge and food stuffs. The majority of coliform bacteria are defined as lactose-fermenting, Gram negative, *Enterobactericeae* (Ishii and Sadowsky, 2008). Within research, model pathogens are occasionally used in their stead as they are non-pathogenic strains, thus posing significantly less risk. Model pathogens are often well studied (e.g. *E. coli*) microorganisms, which have lost the ability to reproduce in the intestinal tract (Tatum and Lederberg, 1946). It is generally accepted that knowledge gained from using such microorganisms will provide insight into the behaviour and biological mechanisms associated with their pathogenic counterparts.

Exposure to enteric pathogens can occur through faecal-oral transmission, contact with contaminated produce or water, and by direct contact (Buchholz, *et al*, 2011; Davis, *et al*, 2005; Hrudey, *et al*, 2003). It is generally presumed that contamination of water and produce occurs through the application of contaminated fertilisers, such as sewage sludge, to agricultural land. However, there is sparse evidence to support this, with a large proportion of outbreak studies only 'implicating' produce and related agricultural practices. For example, in the US in 2002, approximately 500 patients across several states were infected with *Salmonella* Newport, associated with the consumption of contaminated tomatoes (Greene, *et al.* 2008). The pathogen was later tracked to contaminated irrigation water on farmland. Similarly, in 2005, an outbreak of verotoxin-producing *E. coli* (VTEC) occurred in Sweden affecting 135 individuals (Söderström, *et al.* 2008). This included 11 cases of

haemolytic uremic syndrome. Contaminated lettuce was implicated as a vector. Across the UK in 2007, 55 cases of *S.* Senftenberg were associated with contaminated basil from Israel (Pezzoli, *et al.* 2008). Due to this lack of significant or definitive evidence, inconsistent reporting and difficulty with pinpointing sources of outbreaks, it makes it difficult to assess the full risk posed by these transmission routes.

Furthermore, these cases and other well publicised outbreaks, can elicit strong, negative, responses from the general public. Such a response can lead to a drop in sales of related produce and a decrease in trust in relation to both shops and their suppliers. Consequently, it is estimated that the agricultural sector loses £169 million per year through bacterial or viral outbreaks related to food-borne illness (Pretty, *et al.* 2000). It is further estimated that the related economic cost of sick leave and healthcare due to such illnesses was £743 million in England as of 1994/1995 prices (Roberts, *et al.* 2003). Additionally, Rayner and Scarborough (2005) also estimated that food-borne illnesses cost the NHS, UK, £6 billion each year, using data provided by the National Health Executive relating to the 1992/1993 financial year. More recent data is also available, though such research tends to be more focused on either incidence of disease or specific pathogens. For example, Tam, *et al,* (2012) estimated the overall incidence of infectious intestinal disease (IID) by pathogen in local communities that presented at general practices. They monitored 88 practices across the UK for up to 52 weeks. From this, they estimated that the overall incidence of IID within these communities was 274 cases per 1000 persons annually. Norovirus was found to be the most common infectious organism, whilst *Campylobacter* was found to be the most common bacterial pathogen.

Additionally, Santos, *et al*, (2011) compared the cost of *Salmonella Typhimurium* and *Salmonella* Enteritidis in England. Using cases of both *S. Typhimurium* and *S.* Enteritidis confirmed between July and November of 2008, they estimated that direct costs (those associated with medical provision) were £1282 and £993 per case, for *S. Typhimurium* and *S.* Enteritidis respectively. Furthermore, indirect costs (those associated with sick leave and carers) were estimated to be £409 and £228 per case, for *S. Typhimurium* and *S.* Enteritidis respectively. However, the current global burden of food-borne disease is unknown, with the WHO expected to provide

new estimates in 2015 (WHO, 2015). Whilst these estimated economic and social incursions vary, they illustrate the potential socio-economic threat posed by pathogens. Therefore, research into their persistence in the environment is both valid and essential to future socio-economic security. In light of this, the following section discusses one of the apparent sources of the problem.

2.2. Sewage sludge

Sewage sludge is a highly variable, semi-solid material, containing large quantities of OM, nutrients, heavy metals, pathogens and other microorganisms (Alloway and Jackson, 1991). It is the residue generated from the primary (physical/chemical), secondary (biological) and tertiary (often additional, primarily nutrient removal) treatment of wastewater (Hammer and Hammer, 2004b; Fytili and Zabaniotou, 2008). Due to the potentially hazardous composition of sewage sludge, a number of treatment practices have been implemented. These aid in mitigating risks of exposure to such harmful substances/pathogens and also stabilises the residues involved. These treatment processes significantly influence the composition of sewage sludge and wastewater, as well as the persistence of enteric pathogens found therein. However, their efficacy is reliant upon factors such as: temperature, pH and moisture content (EC, 2001a).

2.2.1. Treatment

Sewage sludge is produced from the primary, secondary and tertiary treatment of wastewater (Figure 2.2). Wastewater itself is the liquid effluent accumulated from residences, businesses and institutions at WWTFs (Hammer and Hammer, 2004c). It is comprised of faeces, urine and water, as well as any other objects or liquids disposed of via sewer pipes. Its composition and volume can vary with season, input, infiltration and treatment (Hammer and Hammer, 2004c).

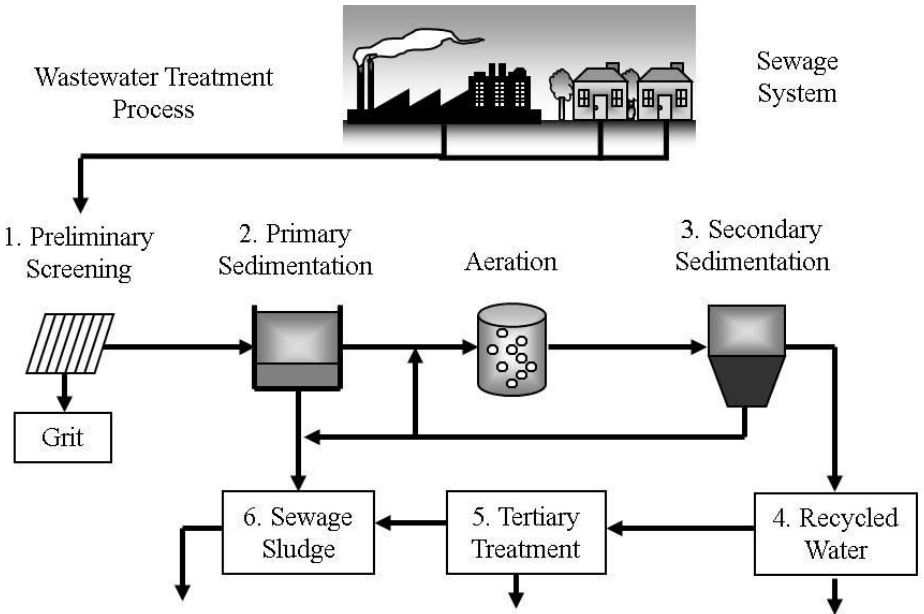

Figure 2.2. Simplified schematic of wastewater treatment process (simplified adaptation from AWA, 2009).

Current treatment processes begin with preliminary filtration, which involves the filtering or screening of large solids from wastewater (Madigan, *et al.* 2006a). This is followed by primary sedimentation, which removes suspended solids (Grey, 2004). Sedimentation occurs through prolonged settling of the wastewater, where the velocity of the water does not contain enough energy to hold larger solids in suspension. Thus the solid matter settles to the bottom. Any microbial cells, including enteric pathogens attached to the surface of these solids are also removed at this point. Following this, the remaining effluent is aerated and then undergoes secondary treatment, also known as activated sludge treatment. It converts the unsettleable solids, still suspended in the liquid effluent, into biological cells (Grey, 2004). These cells are further removed via sedimentation. Again this will include the removal of enteric pathogens. Three processes occur simultaneously during biological treatment (Grey, 2004):

(i) Oxidation: The formation of mineralised end-products that are removed in effluent

(ii) Biosynthesis: Removal of organic matter by conversion into new biomass

(iii) Auto-oxidation: Occurs in limiting environmental conditions and causes microbial cell tissue to be endogenously respired

Tertiary treatment is the final stage in the wastewater treatment process. It is often an optional step as it improves the standard of good quality effluents by removing nutrients and further stabilising the material. The methods used include prolonged settling, irrigation onto grassland or filtration through a fine mesh (Grey, 2004). The solids produced from each sedimentation phase are termed sewage sludge.

At this point, sewage sludge can be unsafe, due to the presence of hazardous chemicals and pathogenic microorganisms. To remove or minimise these components, as well as to stabilise the OM content, sewage sludge itself, undergoes a series of treatments (EC, 2001a). These treatments include centrifugation, liming, pasteurisation and anaerobic digestion. Centrifugation reduces the water content of the sewage sludge, whilst addition of lime alters the pH to 12+, leading to a reduction in odour and pathogens (FWR, 2012). Pasteurisation involves heating sewage sludge to 70°C for an extended period of time, which aids in the breakdown of OM and again reduces pathogen load (FWR, 2012). This is followed by anaerobic digestion, where sludge is held at 35° for 12 to 20 days whilst microorganisms break down the OM, converting it into gas (approximately 65% methane). These processes produce a product that is classified as treated sewage sludge (FWR, 2012). The composition of this sewage sludge can vary regionally, temporally and due to changes in environmental conditions. The following sections discuss the associated characteristics of sewage sludge. However, it should be noted that this is a generalised example and is not necessarily representative of all waste effluents.

2.2.2. Physicochemical properties

The OM content of sewage sludge is normally composed of hydrocarbons, amino acids, small proteins and lipids, with nitrogen predominantly found in an organic form (EC, 2001b). Approximately 30% to 90% of phosphorus content is found in mineral form (EC, 2001b). The macronutrients, such as nitrogen and phosphorus, within sewage sludge can promote plant growth and benefit soil characteristics (Logan and

Harrison, 1995). However, varying quantities of heavy metals, including copper (Cu^{2+}), lead (Pb^{2+}), nickel (Ni^{2+}) and zinc (Zn^{2+}), may also be present and can originate from industrial dyes, cosmetics and mining (Binkley and Simpson, 2003). The presence of these elements can pose a significant problem in treating wastewater and sewage sludge, as they can impede anaerobic digestion; depending upon their solubility (Lin, 1992; Mueller and Steiner, 1992). They also pose a major risk to public health, with exposure leading to kidney damage, encephalopathy or cancer (Järup, 2003). Similarly, they can cause environmental problems, expressed as phytotoxicity or ecotoxicity (McLaughlin, *et al.* 2000).

The treatment processes implemented can significantly alter the composition of sewage sludge (Table 2.1). These impacts can be caused by increased concentration of molecules through dewatering, or breakdown of molecules during digestion. For example, untreated primary sewage sludge has a similar Total Dry Solids (TS) to digested sewage sludge. This is in contrast to activated sludge, which has comparatively small TS. Similar patterns are also followed for lipid content of sewage sludge, where greater variation in quantity is observed in untreated sewage sludge as opposed to primary or activated sewage sludge. The range of nitrogen and phosphorus, in contrast, generally increases with primary and activated sewage sludge. This can be attributed the breakdown of larger molecules, such as proteins, during the treatment process.

External factors such as temperature, humidity and pH can also enhance the physicochemical variability of sewage sludge (EC, 2001b). For instance, increasing temperature (up to the point of enzyme denaturation) can lead to increased reaction rates and cell growth, thus leading to increased breakdown of organic matter in sewage sludge. Similarly, changes in pH can significantly alter the efficacy of treatment chemicals (Hammer and Hammer, 2004d). It can also significantly impede biological interactions (Prescott, *et al.* 2005c). Furthermore, the biological properties can also be influenced by the treatment processes involved.

Table 2.1. Chemical composition of sewage sludge (simplified adaptation from Fytili and Zabaniotou, 2008).

	Treatment		
	Untreated	**Primary**	**Activated**
Total dry solids (TS), %	2.0-8.0	6.0-12.0	0.83-1.16
Volatile solids (% of TS)	60-80	30-60	59-88
Grease and fats (% of TS)			
Ether soluble	6-30	5-20	-
Ether extract	7-35	-	5-12
Protein (% of TS)	20-30	15-20	32-41
Nitrogen (N, % of TS)	1.5-4	1.6-6.0	2.4-5.0
Phosphorous (P_2O_5, % of TS)	0.8-2.8	1.5-4.0	2.8-11.0
Cellulose (% of TS)	8.0-15.0	8.0-15.0	-
Iron (not as sulphide)	2.0-4.0	3.0-8.0	-
Alkalinity (mg l^{-1} as $CaCO_3$)	500-1500	2500-3500	580-1100
Organic acids (mg l^{-1} as acetic acid)	200-2000	100-600	8000-10000
pH	5.0-8.0	6.5-7.5	6.5-8.0

2.2.3. Biological properties

The composition of the microbial community within sewage sludge is influenced not only by the health of the individuals within the catchment areas of the WWTF but also by the procedures and treatments implemented by the WWTF itself (EC, 2001b). It can contain high quantities of pathogenic organisms, such as bacteria (10^2-10^6 g^{-1} fresh weight), viruses (10^2-10^4 g^{-1} fresh weight), protozoa (10^2-10^3 g^{-1} fresh weight), and helminths (10-10^3 g^{-1} fresh weight) (Table 2; EC, 2001a). They are shed in excrement and whilst treatment processes reduce their quantity, their ability to adapt to changing environmental conditions can lead to their survival (Kearney, *et al.* 1994).

Their presence within sewage sludge can also arise from recontamination, regrowth or re-activation during treatment, transport and subsequent application to land. For example, recontamination can arise after from pasteurisation and anaerobic digestion through contact with unsterile vehicles during transport or storage areas (Bagge, *et al.* 2005). Similarly, regrowth, where relatively low numbers of microbes re-populate the sewage sludge can also occur (Bagge, *et al.* 2005). Reactivation, which is linked to the Viable but Non-Culturable (VBNC) theory, denotes microorganisms which survive in a dormant form during periods of intensified stress, and are then 'reactivated'

(Steinert, *et al.* 1997). The stress in this instance is the treatment process. For example during centrifugation of sewage sludge, where the shear stresses that are associated with this process could cause cell lysis. During VBNC periods they are undetectable by standard culture methods, with return to optimal environmental conditions leading to their reactivation and concurrent detection (Steinert, *et al.* 1997). The ability of enteric pathogens to survive and adapt during these treatment processes is a topic of concern, particularly because of the volume of sewage sludge produced and its' valuable nature as a fertiliser.

Table 2.2. Pathogens associated with wastewater and sewage sludge, with associated diseases or symptoms (reproduced from Gerba and Smith, 2005).

Category	Pathogen	Related disease or symptoms
Bacteria	*Salmonella* spp.	Salmonellosis (food poisoning), typhoid
	Shigella spp.	Bacillary dysentery
	Vibrio cholerae	Cholera
	Campylobacter jejuni	Gastroenteritis
	Escherichia coli	Gastroenteritis
Viruses	Poliovirus	Poliomyelitis
	Coxsackievirus	Meningitis, pneumonia, hepatitis, fever
	Echovirus	Meningitis, paralysis, encephalitis, fever
	Hepatitis A virus	Infectious hepatitis
	Rotavirus	Acute gastroenteritis with severe diarrhoea
	Hepatitis E virus	Hepatitis
	Adenoviruses	Respiratory tract infections, gastroenteritis
Protozoa	*Cryptosporidium*	Gastroenteritis, cryptosporidiosis
	Entamoeba histolytica	Acute enteritis
	Giardia lamblia	Giardiasis (diarrhoea and abdominal cramps)
	Balantidium coli	Diarrhoea, dysentery
	Toxoplasma gondii	Toxoplasmosis
Helminth worms	*Ascaris lumbricoides*	Digestive disturbances, abdominal pain
	Ascaris suum	Symptoms including coughing and chest pain
	Trichuris trichiura	Abdominal pain, diarrhoea, anaemia
	Toxocara canis	Fever, abdominal discomfort, muscle aches
	Necator americanus	Hookworm disease

2.2.4. Legislation

Approximately 10.13 million tons of sewage sludge is produced in Europe annually (RPA, *et al.* 2008). Additionally, the UK produced approximately 1.4 million tons of sewage sludge as of 2008, whilst Ireland produced 42.1 thousand tons as of 2003 (RPA, *et al.* 2008; Water UK, 2010). Due to the hazardous composition and quantity of this sewage sludge, its management continues to be one of the most difficult and expensive issues surrounding the treatment of wastewater (Metcalf and Eddy, 1991). In response to these hazards, several directives, regulations and voluntary programmes across Europe have been developed to mitigate or eradicate exposure to these hazards within wastewater and sewage sludge. These practices provide a framework which monitors and regulates wastewater discharges, as well as their treatment, re-use or disposal (EC, 2012, Vinceviciene, 2007). Current EU targets hope to reduce waste disposal, equivalent to the levels in the year 2000, by 50% by 2050 (EC, 2007). They aim to do this by preventing and recycling waste, improving treatment processes and handling. This is further highlighted in the following EU directives:

- The Waste Framework Directive 2008/98/EC which defines the basic concepts and definitions of waste management, whilst encouraging the safe disposal of waste (CEC, 2008). The subsections Articles 17 to 20, which relate to hazardous waste, also specifically deals with sewage sludge. They require the implementation of stricter control measures, such as labelling, record keeping, monitoring and tracking the material from the waste producer to final disposal or recovery.
- The Sewage Sludge Directive 86/278/EEC encourages the use of sewage sludge in agriculture, whilst regulating it to prevent harmful effects on the environment and man (CEC, 1986). It also limits the use of untreated sewage sludge on agricultural land and defines the specifications which designate treated sewage sludge; "*biological, chemical or heat treatment, long-term storage or any other appropriate process so as significantly to reduce its fermentability and the health hazards resulting from its use*". Two sets of regulations transpose the SSD into UK legislation. These regulations are The Sludge (Use in Agriculture) Regulations 1989 and The Sludge (Use in Agriculture) Regulations 1990

(Amendment) (SI, 1989; SI, 1990). They provide relevant physicochemical standards, such as pH and nutrient content, for both sewage sludge and soil.

- The Urban Wastewater Directive 91/271/EE amended by 98/15/EC, which seeks to provide more stringent quality standards for wastewaters by instigating a requirement for secondary treatment of wastewater (CEC, 1998). It also requires the re-use of sewage sludge "*whenever appropriate*". This Directive also phased out the disposal of sewage sludge to surface waters by 1998.

It should be noted that the treatments implemented from these schemes were originally designed to mitigate exposure to harmful chemicals, such as heavy metals; with only a few EU member states setting allowable limits for pathogen content (Mininni, *et al.* 2014). Several EU member states have also implemented their own sets of regulations, such as The Code of Practice for Agricultural Use of Sewage Sludge and the voluntary Safe Sludge Matrix (ADAS, 2001; DEFRA, 2006). These regulations are often more stringent than the core policies outlined by the EU itself. For example, in the UK, the Safe Sludge Matrix (ADAS, 2001) outlines acceptable application rates that correspond to the treatment history of the sewage sludge and also the crop type to be harvested (Table 2.3). However, these schemes are voluntary and are not strictly monitored, making it difficult to fully assess their effectiveness.

The development of this network of directives, regulations and voluntary programmes, has driven the management of sewage sludge for more than two decades. They have contributed to the increased health of both the general public and the environment. This is most noticeable in the improvement of watercourses following the abolition of sewage sludge being added to waterways (DEFRA, 2012a). However, continuous improvement and better enforcement is required, especially in regards to regulations pertaining to pathogen content in sewage sludge applied to land (Inglezakis, *et al.* 2012). The Soil Framework Directive (SFD) (CEC, 2006) attempted to respond to this omission by providing a more precise and focused framework to mitigate exposure to pathogens (Creamer, *et al.* 2010). However, this has recently been withdrawn (EC, 2015a). It is integral that mitigating exposure to pathogens be incorporated into the handling of sewage sludge. By limiting pathogen content prior to application to land,

the likelihood of persistence in soil and infection from contaminated produce would presumably decrease.

Table 2.3. Use of sewage sludge in agriculture (reproduced from ADAS, 2001). ✔ All applications must comply with the Sludge (Use in Agriculture) Regulations and Department of Environment Transport and Regions (DETR). ✘ Applications not allowed (except where stated conditions apply).

Crop type	Untreated sludge	Conventionally treated sludge		Enhanced treated sludge	
Fruit	✘	✘		✔	10 month harvest interval
Salad	✘	✘ 30 month harvest interval		✔	
Vegetables	✘	✘ 12 month harvest interval		✔	
Horticulture	✘	✘		✔	
Combinable & animal feed crops	✘	✔		✔	
Grass & forage					
Grazed	✘	✘ Deep injected or ploughed down only	3 week no grazing or harvest interval	✔	3 week no grazing or harvest interval
Harvested	✘	✔ No grazing in season applied		✔	

2.2.5. Management

There are several handling routes for sewage sludge, including disposal to landfill, incineration and as an agricultural fertiliser (EC, 2012). Approximately 39% of sewage sludge produced in Europe is re-used in agriculture, with 80% and 63% of UK and Irish sewage sludge spread to land respectively (RPA, *et al.* 2008; DEFRA, 2012b). In recent years, with the drive towards more sustainable options, disposal to landfill has decreased whilst use in agriculture and incineration has increased (Table 2.4).

Table 2.4. The re-use or disposal route for sewage sludge within Europe (reproduced from DEFRA, 2012b).

	Re-use of sludge		Disposal of sludge			
Year	**Soil and agriculture**	**Other**	**Landfill**	**Incineration**	**Other**	**Total**
1992	440,137	32,100	129,748	89,800	24,300	997,673
2008	1,241,639	90,854	10,882	185,890	1,523	1,530,779
2010	1,118,159	23,385	8,787	259,642	2,863	1,412,836

The disposal of sewage sludge to landfill poses a significant environmental and public health risk. Leachate, or liquid waste, produced from landfills can contain high quantities of organic matter, toxic substances and enteric pathogens (Taylor, 2003). Due to this risk, landfill disposal is currently being phased out by the Landfill Directive 99/31/EC, which has set targets to reduce the amount of biodegradable waste going to landfill (RPA et al. 2008). In contrast, disposal of sewage sludge via incineration can prove useful, as it can provide fuel to generate energy (Werther and Ogada, 1999). Use as an agricultural fertiliser is the primary management route for sewage sludge. It is one of the most cost effective and sustainable management practices, as it is both a cheaper alternative to chemical fertilisers and decreases the volume of material entering disposal routes (Grey, 2004). Re-use as an agricultural fertiliser is also actively promoted by EU legislation (see section 2.2.4). However, this route does pose its own set of problems with the potential introduction of pathogens and other contaminants to the soil environment. There are two main modes of sewage sludge application to land, namely (i) tilling which involves subsequent incorporation and (ii) surface spreading (Figure 2.3). This current legislative and managerial drive, supporting the re-use of sewage sludge as fertiliser, further highlights the importance of studying pathogen survival in sludge applied to land.

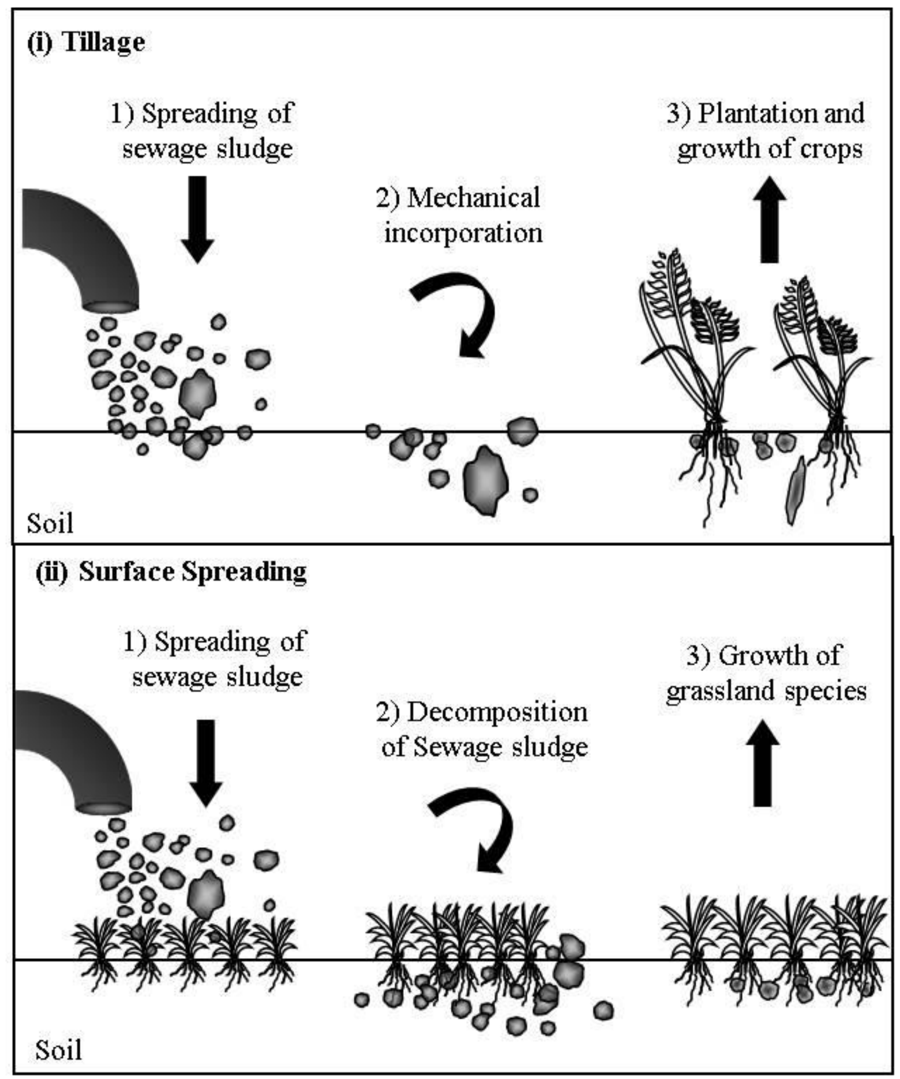

Figure 2.3. Schematic showing (i) sewage sludge tilled into crop land and (ii) sewage sludge spread onto grazing land.

2.3. Persistence of pathogenic bacteria in soil

The soil environment is a markedly different niche from that of a homeostatically regulated organ in a warm-blooded animal, with nutrients and temperature varying both spatially and temporally. A review by Winfield and Groisman, (2003), suggested that *E. coli* survived to a lesser extent outside of the primary host environment. This was attributed to strong abiotic and biotic interactions. In contrast, they propose that *Salmonella* were more capable of surviving such conditions. However, several other studies have shown that the survival of pathogens in the soil environment can vary quite significantly. For example, *E. coli* that persisted for between

29 days and 231 days have been observed (Fenlon, *et al.* 2000; Jiang, *et al.* 2002). Environmental strains of *E. coli*, purportedly hardier than model or lab strains, have also been shown to persist for upwards of 9 years (Brennan, *et al.* 2010). Similarly, *Salmonella* species have been show to survive for approximately 200 to 400 days in soil when co-introduced with manure, irrigation water or slurry (Islam, *et al.* 2004; You, *et al.* 2006). Furthermore, Avery, *et al.* (2004a), found that when *E. coli* was sub-surface injected into soil, it survived for 8 weeks whilst it survived for up to 6 weeks when spread onto grassland vegetation. A follow-on field study, where cattle, sheep and pigs were penned on grassland, found that natural *E. coli* populations persisted for approximately 5 to 6 months (Avery, *et al.* 2004b). Jäderlund, *et al.* (2010) also found that two strains of *Campylobacter jejuni* survived for 21 days when co-introduced in soil and on spinach plants. It should be noted that this is a relatively short period of time to track survival in soil. Overall, this apparent variety within and between bacterial species suggests that varying physicochemical and biological conditions, methods and microbial physiologies play a significant and complex part in persistence. Their varied survival also makes it challenging to accurately predict the likelihood of exposure to such harmful microorganisms, with prolonged survival increasing the possibility of ingestion and/or infection.

2.3.1. Physicochemical factors

The physicochemical factors which govern the survival of enteric pathogens in soil include; moisture content, nutrient availability, temperature, pH, soil type and management practices. Nutrient availability is arguably the most important factor as all cells need a specific set and ratio of nutrients to survive. For example, Aldén, *et al.* (2001), found that carbon and phosphorus availability limited microbial growth in soils, with phosphorus availability only limiting growth in certain calcareous soils. Similarly, Erickson, *et al.* (2009), found that altering the C:N ratio in manure-based composts could inactivate species of *Salmonella*. Furthermore, Mallmann and Litsky, (1951), found that the survival of streptococci and *S. typhosa* across several soils was increased when organic matter, in the form of raw, treated and activated sewage sludge was introduced.

Moisture content can also influence nutrient availability. For example, Chandler and Craven, (1980) found that *E. coli* persistence in soil decreased with increasing water content; attributed to a lack of organic carbon in more dilute mixtures. The decline also corresponded to the wilting range of moisture contents for the soils that were studied. Similarly, Drenovsky, *et al.* (2004), found that manipulating organic carbon inputs and soil water content, determined the composition of the microbial community after noting changes in fatty acid composition. However, the type of carbon introduced to the samples had no effect. This lack of difference is probably because of a lack of some other essential nutrient. Microorganisms require a balanced mixture of elements to both survive and propagate (including: oxygen, carbon, hydrogen, and nitrogen), with shortages in any one leading to limited growth, no matter the abundance of the remaining nutrients (Prescott, *et al.* 2005d; Horton, *et al.* 2006). Similarly, temperature and pH can influence both enteric pathogen survival and availability of nutrients. There is generally an inverse relationship between increasing temperature and bacterial survival, with increasing temperature leading to greater bacterial die-off (Reddy, *et al.* 1981; Howell, *et al.* 1996). Enteric bacteria also appear to survive for shorter periods of time under acidic conditions, attributed to a lack of nutrient availability (Ellis and McCalla, 1976). Furthermore, Castro, *et al.* (2010), found that aspects of climate change, e.g. increased precipitation, changed the overall relative abundance of specific bacterial phyla, including *Proteobacteria* and *Acidobacteria. Proteobacteria* were more abundant in wetter treatments, whilst *Acidobacteria* were more abundant in dry treatments. This change in microbial community, brought about from climatic changes, could influence the predominant phyla present within the soil as well as which phyla may compete with enteric pathogens co-introduced with sewage sludge. Additionally, the survival of such enteric pathogens may also be influenced by such climatic changes further affecting their persistence in the soil environment.

Soil type and management practices can also significantly affect the survival of enteric pathogens. For instance, Mubiru, *et al.* (2000), assessed the survival of pathogenic and non-pathogenic strains of *E. coli* O157:H7 in two silt loam soils with different physicochemical characteristics. Both strains showed a higher mortality in the silt loam soil containing higher clay content. It was suggested that this higher clay content lead to a lower availability of water, which caused the greater die-off.

Conversely, Brennan, *et al*, (2014) found that clay mineral addition altered the cation exchange capacity and surface area of the soil, as well as differentially affecting the survival of *Listeria monocytogenes*, *S.* Dublin, and *E. coli* O157. The amendment of montmorillonite to soil promoted the greatest survival of *L. monocytogenes*, whilst the amendment of illite to soil promoted the greatest survival of *S.* Dublin, and *E. coli* O157. They propose that this increase in survival could have been related to a greater provision of minerals from the clay amendments or the increased cation exchange capacity promoting greater bacterial respiration. Additionally, Franz, *et al.* (2008), studied 36 Dutch soils and different management type on the survival of *E. coli* O157:H7. They concluded that *E. coli* O157:H7 declined at a greater rate under soil with less available nutrients, while management practices had little effect on survival. The lack of management effect was attributed to a lack of chemical and biological differences in the soils studied. Similarly, Lauber, *et al.* (2008), also found that changes in specific soil properties, such as pH and soil texture, altered soil microbial communities to a greater extent than specific land-use types. Conversely, Wang, *et al.* (2014), found that *E. coli* O157:H7 survival varied with different management practices; forest (loam) and tea plantation (clay loam) practices showed a greater die-off than vegetable garden (silt loam) and bamboo grove (clay loam) practices. These differences were associated with nutrient availability, pH and sand content. Furthermore, Topp, *et al.* (2003), studied the strain-dependent variability in *E. coli* survival in response to soil conditions. They found that natural *E. coli* communities in swine slurry changed when incubated in soil, suggesting that community composition could change profoundly in soils as fitter *E. coli* populations take over.

2.3.2. Biological factors

Microorganisms constitute less than 0.5 % of the total mass of soil but are responsible for the majority of nutrient cycling, energy flows and reactions that shape habitats (Tate, 1995, Wiegert and Owen, 1971). The biotic interactions which occur within the soil environment not only dictate the predominance of these nutrient cycles but also the structure of populations and the survival of individuals within the given

community. Biological interactions which influence the survival of enteric pathogens include: competition, antagonism and predation.

Competition occurs when organisms compete for the same resources. Intra-specific competition denotes competition between individuals within the same species. This is best exemplified in the die-off phase of bacterial growth (Figure 2.1), where a high proportion of bacterial cells compete for a continually depleting pool of resources. One response to this is quorum sensing, where bacteria regulate internal pathways, in response to the density of cells within their own population (Madigan, *et al.* 2006b). For example, Moynihan, *et al.* (2012), found that *E. coli* concentrations within soil microcosms increased in the absence of the local microbial community, whilst concentrations decreased in with their presence. These responses were, however, temperature dependent, with *E. coli* concentrations remaining constant at a lower temperature, regardless of treatment. As microbial activity is impeded at lower temperatures, this would imply that the treatment which contained the local microbial community was in some way inactive. Or that the *E. coli* was not propagating rapidly enough to illicit a "competitive response" from the microbial community. Additionally, Yao, *et al.* (2014), found that Gram negative bacteria suppressed the survival of *E. coli* O157:H7 to a great extent, whilst the presence of *E. coli 0157* also changed the composition of the resident soil microbial community. Furthermore, the microbial diversity of the soil can determine the invasion of soil by a bacterial pathogen (Ibekwe, *et al.* 2010; van Elsas, *et al.* 2012). For example, van Elsas, *et al.* (2007), found that the survival of *E. coli* 0157:H7 in a loamy sand soil increased in correspondence with a decrease in the complexity of soil microbial community.

Antibiosis, which is the detrimental interaction between at least two or more species, is another form of interaction which can impede the survival and growth of introduced pathogens. For example, the production of antibiotics can kill or impede the growth of microorganisms (Lartey, 2006). Raaijmakers, *et al.* (1997), found that two antibiotics; phenazine-1-carboxylic acid (PCA) and 2, 4-diacetylphloroglucinol (Phl) determined the survival of soil borne plant pathogens. Similarly, parasitism can impede the survival of pathogens. A parasite is an organism that lives in or on its host, and whilst deriving nutrients from it does not provide any beneficial contributions in return

(Lartey, 2006). Bacteriophages are one example; they are viruses which invade and reproduce within bacterial cells (Chibani-Chennoufi, *et al.* 2004).

Antagonism, where one species benefits at the expense of another, is yet another example of biotic interactions; a prime example of which is predation. For example, Acea and Alexander, (1988), also found that three species of bacteria, *Agrobacterium tumefaciens, Pseudomonas* sp. and *Corynebacterium* sp., declined in soil in the presence of protozoa; suggesting that predation is a major factor limiting their growth. Selective grazing by protozoa may have also promoted a diverse selection of grazing-resistant phenotypes and species; leading to adaptive mechanisms such as formation of biofilms, as well as promoting the evolution of multicellularity and pathogenesis (Carsten and Kjalleberg, 2005). However, whilst protozoa affect bacterial populations they may also act as vectors or reservoirs of pathogens. Barker, *et al.* (1999), even found that *E. coli* 0157 was able to survive inside the amoeba *Acanthamoeba polyphaga*, which may have implications for their further dissemination in the environment. Species of *Legionella* and *Listeria* have also been shown to survive and multiply in amoebae (King, *et al.* 1988; Barker and Brown, 1994; Brown and Barker, 1999). Furthermore, *Salmonella enterica* was shown to survive when sequestered in food vacuoles of *Tetrahymena* (Brandl, *et al.* 2005). The survival of these bacteria has implications for food safety also, with the presence of these protozoa on produce potentially acting as a vector for the transmission of disease (Gourabathini, *et al.* 2008).

2.4. A hot spot of activity

These biological and physicochemical interactions, as well as the input of sewage sludge combines to produce a complex and dynamic system with each factor playing a significant role in determining the survival of enteric pathogens. As most microorganisms within the soil are found in a state of semi-starvation, an input of new nutrients, such as with sludge amendment, can lead to a burst in microbial activity (Dilly, 2005). This burst in microbial activity can be seen as a hot spot, which are generally small, spatially isolated niches with increased biological activity (Bundt, *et al.*

2001). They can be zones of animal manure, accumulated OM or the rhizosphere (Parkin, 1987; Petersen, *et al.* 1996; Griffiths, 1994).

Therefore, it is proposed that with the addition of sewage sludge to soil, a nutrient- and OM rich niche may form, encouraging enhanced activity of the local soil microbial community. This in turn may lead to a lower survival rate for the enteric pathogenic co-introduced with the sewage sludge, arising from an increase in antagonistic effects associated with the indigenous soil microbial community. Furthermore, the interface between the two matrices, soil and sludge, may be an important consideration as their physicochemical characteristics could affect the extent of exposure or interaction with the indigenous soil microbial community.

2.5. Aims and research questions

This research intends to clarify our understanding of the mechanisms which affect the persistence of enteric pathogens in soil, when co-introduced with sewage sludge. In particular the impact of physicochemical properties of sewage sludge on microbial interactions and how they impact the persistence of such pathogens. The main question posed by this research is: How will the input of organic carbon and nutrients and, associated with sewage sludge applied to land, affect soil microbial activity and the persistence of pathogenic bacteria found within sewage sludge? In light of this question and the aforementioned theory described in Section 2.4, a set of questions was developed over the course of the project:

1. Does the proportion of sewage sludge to soil affect the persistence of bacterial pathogens? (Chapter 4).
2. Does the proportion of sludge to soil affect the persistence of indigenous sewage sludge *E. coli*? (Chapter 5).
3. How will the addition of supplementary substrate (yeast extract and glucose) to differential phases of sludge and soil mixtures affect the survival of sewage sludge-derived *E.coli*? (Chapter 6).

4. How will the repeated additions of supplementary substrate (glucose), mimicking the recurrent input of carbon that occurs in plant/soil systems, affect the survival of sewage sludge-derived *E. coli*? (Chapter 7).

2.5.1. Objectives

From these questions, a set of aims and objectives were then developed to aid in focusing each experiment:

1. To elucidate how the loading rate of sewage sludge influences the survival and interactions of bacterial pathogens co-introduced with the sewage sludge, to soils.
2. To elucidate how the loading rate of sewage sludge influences the survival and interactions of indigenous sewage sludge *E. coli* co-introduced with the sewage sludge, to soils.
3. To clarify the effect of supplementary nutrients on the survival of sewage sludge-derived *E. coli* and general coliforms.
4. To elucidate how repeated input of supplementary nutrients will affect the survival of sewage sludge-derived *E. coli* and general coliforms.

3. General methods

3.1. Introduction

This chapter describes the main protocols used throughout this thesis. Fundamental techniques such as basic physicochemical and biological analyses are detailed, as well as method development for the extraction of bacteria from environmental matrices. Respiration analysis, adapted from the substrate induced respiration protocol, and microbial carbon analysis are also described. These protocols will be referred to where necessary throughout the thesis, with specific steps for each experiment outlined in the Materials and Methods Section of the relevant Chapter.

3.2. Collection, preparation and analysis of samples

3.2.1. Site description: soil

The soil used within each experiment was a loamy, brown earth soil and was collected from a cattle-grazed pasture (principally comprised of clover, *Trifolium repens,* and ryegrass, *Lolium perenne*). The site is predominantly exposed to a mild, oceanic climate. Average annual rainfall varies between 800 mm to 1200 mm, with mean daily temperatures ranging from 4 - 18°C (IMS, 2013a; IMS, 2013b). Fresh samples were collected as needed.

3.2.2. Site description: sewage sludge

Fresh samples of anaerobically digested and dewatered sewage sludge cake were provided by United Utilities, Ellesmere Port, UK when required for each experiment. The WWTF at Ellesmere Port supports the Gowy sub-catchment, whilst also receiving waste from Helsby WWTF and Sutton Hall and Huntington WWTF which are comprised of primarily domestic and industrial sites.

3.2.3. Sample preparation and analysis

For each experiment, three top-soil samples (0 - 10 cm) were collected from the same cattle grazed pasture within one month of the onset of the experiment. The same pasture was used throughout. The soil was sampled from points randomly within the pasture. The samples were then manually sieved to a particle size of 4 mm and coned/quartered to produce a homogeneous composite sample (de Zozi, *et al.* 2005). Sewage sludge was homogenised to produce a composite sample, with specific preparation steps detailed in relevant experimental chapters. Both soil and sewage sludge composites were stored at 4°C until use.

The preparation of each treatment cohort was specific to each experiment and is therefore discussed within respective Chapters. The moisture content for each cohort of treatments was also monitored throughout each experiment. The weight of each sample was taken at the beginning of the experiment and subsequent randomised checks occurring on a weekly basis thereafter on 10% of the total number of samples. If weight varied by >5% of the starting weight during this time then sterile water was added to adjust to the original volume.

Selected physicochemical analyses were performed in triplicate at Brookside Laboratories Inc., New Knoxville, Ohio, US within one month of collection (Table 2.1; Table 2.2). Soil and sewage sludge composites were also assessed in triplicate, for levels of indigenous microorganisms at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland; these protocols are outlined in Sections 3.3.3, 3.3.4., and 3.3.

Table 3.1. Soil analyses and corresponding references.

Analysis	Protocol
pH (1:1 in H_2O)	(McLean, 1982)
Total Exchange Capacity (MEQ $100g^{-1}$)	(Ross, 1995)
Moisture content (%)	(Rowell, 1994)
Organic matter (loss on ignition, %)	(Schulte and Hopkins, 1996)
Bray I phosphorus (mg l^{-1})	(Bray and Krutz, 1945)
Nitrogen (mg l^{-1})	(Dahnke, 1990)
Total	
Nitrate (NO_3)	
Ammonium (NH_4)	
Carbon (mg l^{-1})	(Nelson and Sommers, 1996)
Total	
Organic	
Mehlich III Extractable (mg l^{-1})	(Mehlich, 1984)
Phosphorus (P)	
Manganese (Mn)	
Zinc (Zn)	
Boron (B)	
Copper (Cu)	
Iron (Fe)	
Aluminium (Al)	
Sulphur (S)	
Calcium (Ca)	
Magnesium (Mg)	
Potassium (K)	
Sodium (Na)	

Table 3.2. Sewage sludge analyses and corresponding references.

Analysis	Protocol
pH (1:1 in H_2O)	(EPA 150.1, 2001)
Total solids (g kg^{-1})	(EPA 160.3, 1971)
Moisture content (%)	(Rowell, 1994)
Total phosphorus/potassium (g kg^{-1})	EPA 3050B (1996) for digestion and 6010 (1996) for analysis
Potassium as K_2O (g kg^{-1})	
Phosphorus as P_2O_5 (g kg^{-1})	
Nitrogen (g kg^{-1})	(EPA 300.0, 1984; EPA 350.1, 1997, EPA 1687, 1997)
Total Kjeldahl	
Organic	
Nitrate (NO_3)	
Ammonia (NH_4)	
Carbon (mg l^{-1})	(Nelson and Sommers, 1996)
Total	
Organic	
Potentially Toxic Element (mg kg^{-1})	
Arsenic (As)	(EPA 7061, 1992)
Cadmium (Cd)	(EPA 6010, 1996)
Chromium (Cr)	(EPA 6010, 1996)
Copper (Cu)	(EPA 6010, 1996)
Lead (Pb)	(EPA 6010, 1996)
Mercury (Hg)	(EPA 7471, 1994)
Molybdenum (Mo)	(EPA 6010, 1996)
Nickel (Ni)	(EPA 6010, 1996)
Selenium (Se)	(EPA 7741, 1994)
Zinc (Zn)	(EPA 6010, 1996)

3.3. Preparation, extraction and enumeration of pathogens

3.3.1. Pathogens

Two model pathogens, *Salmonella* Dublin and an environmentally-persistent *Escherichia coli* isolate (lys 9), were selected and used in Chapter 4. Though both laboratory strains (i.e. non-pathogenic), they were perceived to be environmentally relevant and representative of bacterial strains which are potential threats to public health. Isolates of sewage sludge-derived *E. coli* were also used to assess how native

species of pathogens survive in their local environment. Specific details of which experiments used such isolates are given in future chapters.

3.3.2. Inoculum preparation

Isolates of *S.* Dublin, *E. coli* and sewage sludge-derived *E. coli* (contingent on specific experiments, as explained in relevant sections) were incubated at 37ºC in 50 ml Lysogeny Broth (LB) broth for 24 hours, on an orbital shaker (120 rev min^{-1}). Aliquots (100 µl) of each culture were then transferred to another vial containing 50ml of fresh LB broth and incubated following the same procedures for another 24 hours. The culture was then centrifuged and washed 3 times. The remaining procedure, which is specific to each isolate/protocol that was implemented, is provided in the respective experimental chapters.

Samples of the *S.* Dublin and *E. coli* cell suspensions were then diluted 10-fold in solution, and tested for absorbance (250 nm) using a Modulus Microplate reader. This dilution series was also plated onto relevant agar. Optical density (OD) values were obtained from the dilution series and compared to plate counts, confirming an accurate cell count for the cell suspension. OD was then used in each subsequent experiment that involved the aforementioned pathogens. This protocol was performed within each experiment that required the inoculation of a given pathogen, with corresponding data shown in Appendix 2.

Aliquots (0.1 ml) of the cell suspension were then inoculated into the samples containing soil and sewage sludge. The agar used was dependent upon which strains were used and are outlined in Sections 3.3.4 and 3.3.5, whilst the solution used is outlined in relevant Chapters due to protocol specificity.

3.3.3. Extraction of pathogens

A modified protocol, derived from Troxler, *et al*. (2012), was used to extract microbes from environmental samples. Modifications were adopted following method-

development studies (Appendix 1). A diluent (50 ml) was added to each sample and shaken gently by end-over-end rotation (100 rpm) for 30 minutes. The samples were then vortexed for 10 seconds and a 10- fold dilution made up from the resulting extract. Where model pathogens and their corresponding enumeration protocol were used, quarter strength Ringer's solution was used as the diluent. Where indigenous sewage sludge or sewage sludge-derived *E. coli were* used and their corresponding enumeration protocol were used, maximum recovery diluent (MRD) was used as the diluent.

3.3.4. Enumeration of model pathogens

To enumerate *E. coli* and *S.* Dublin, aliquots (0.1 ml) were taken from three dilutions of sample suspension and plated on to pathogen-specific agar; Sorbitol MacConkey agar (SMAC), to enumerate *E. coli*, and Xylose Lysine Deoxycholate agar (XLD), to enumerate *S.* Dublin. Plates were then incubated for 24 hours at 37°C**.** Controls for sterility of plates and media used, including quarter strength Ringer's and ambient conditions were also assessed at each time point within the experiments which used these protocols.

SMAC is a selective and differential medium that isolates *Escherichia coli* O157:H7, a pathogenic strain of *E. coli* (Thermo Scientific, 2012a). Most strains of *E. coli* ferment sorbitol causing the development of pink colonies, whilst *E. coli* 0157:H7 is unable to ferment sorbitol, leading to the formation of colourless colonies. XLD agar is used for the isolation of *Salmonella* and *Shigella* species (Oxoid Ltd, 2014a). Species of *Salmonella* metabolise thiosulfate which produces hydrogen sulphide, leading to the formation of red colonies with black centres, in contrast *Shigella* are unable to metabolise thiosulphate, producing red colonies.

3.3.5. Enumeration of indigenous sewage sludge and sewage sludge-derived pathogens

For the enumeration of indigenous or sewage sludge-derived *E. coli* , aliquots (1 ml), were taken from three dilutions and plated onto Membrane Lactose Glucuronide

Agar (MLGA) using chromogenic membrane filtration (Environment Agency, UK, 2003). These aliquots were filtered with 5 ml of MRD in order to better disperse the suspension across the sterile filter paper, improving the readability of the resulting cell counts. Controls for sterility of plates and media used, including MRD and ambient conditions were also assessed at each time point within the experiments which used this protocol.

Chromogenic membrane filtration required the use of a Microsart® e.jet pump, Sartorius Stedim Biotech (Figure 3.1a) and 3 place Combisart stainless steel manifold, Sartorius Stedim Biotech (Figure 3.1b). A sterile membrane filter (0.45 µm pore size) was placed on the manifold apparatus (Figure 3.1c) by removing the detachable funnels (Figure 3.1d). The funnels were then reattached and the liquid sample was pumped through the membrane filter, using the spigot to open/close an inner valve (Figure 3.1e). Any microorganisms within the sample remained on the membrane filter, whilst the liquid passed through and was disposed of. The membrane filter was then removed from the manifold and placed onto plates containing MLGA. To sterilise the funnels, they were placed in a hot water bath (90ºC) for 10 minutes and rinsed with ethanol before being allowed to dry. They were then run through a Bunsen burner flame prior to reattachment to the manifold.

MLGA differentiates between general coliforms and *E. coli* through two reactions: lactose fermentation detected by red phenol dye, which gives yellow colonies, and the breakdown of chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (BCIG) by the glucuronidase enzyme, which leads to a build-up of blue chromophore in bacterial cells (Oxoid Ltd, 2014b). As coliforms are lactose positive they produce yellow colonies while *E. coli* are both lactose positive and possess glucuronidase and so produce green colonies.

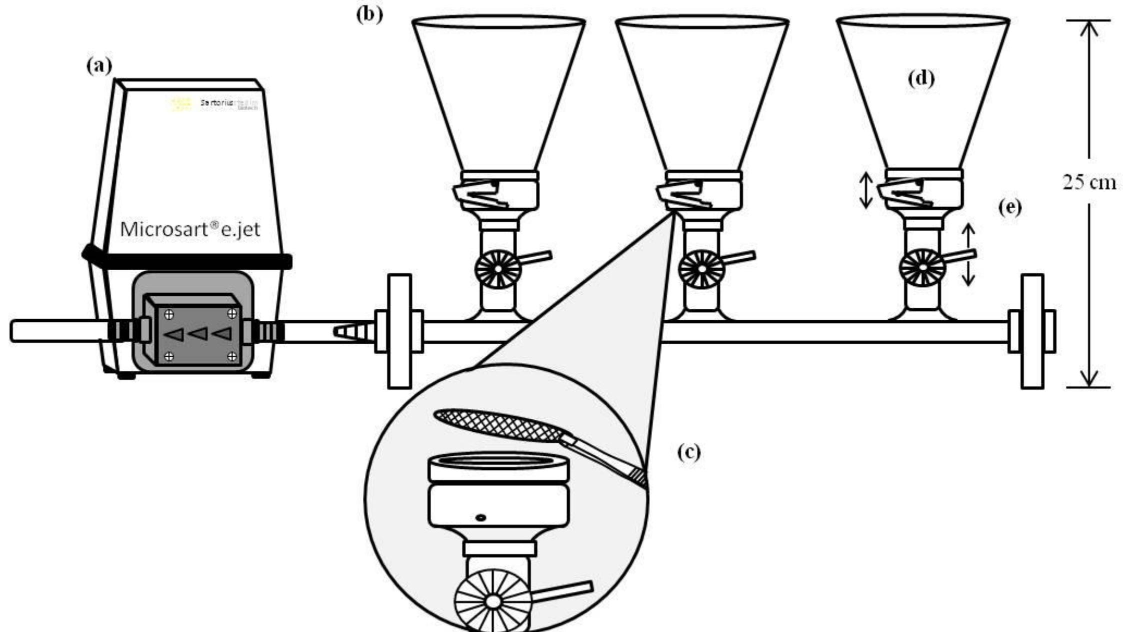

Figure 3.1. Equipment used for chromogenic membrane filtration protocol (a) Microsart® e.jet pump; (b) Combisart stainless steel manifold apparatus; (c) placement of membrane filter; (d) removable funnels; (e) latch to secure funnels to the main body, with spigot to open/close inner valve to allow liquid through.

3.4. Respiration

3.4.1. Background

This protocol was adapted from West and Sparling (1986) and the International Standard 14240-1:1997 (ISO, 1997), which was originally modified from Anderson and Domsch (1978). The original method, substrate induced respiration (SIR), quantifies active microbial biomass in relation to experimental variables. It requires the saturation of the sample with readily available substrate in liquid form, such as glucose, and adjustment of water content, normally to 60 %. The addition of substrate stimulates a peak in respiration which is proportional to the quantity of microbial biomass present and thus can be used as an indicator of microbial activity. It is generally operated over a short time period, with several head space samples taken within approximately 24 hours, or longer, depending on the requirements of the given experiment. The experiments within this project required the analysis of respiration and enumeration of surviving microbes over an extended period (>1 month), therefore the previous protocol was not feasible. This was because the addition of glucose in liquid form and

attenuation of the sample to 60% water content would not be comparable to actual environmental conditions over an extended period of time. Therefore, samples were not attenuated to 60% moisture content. The possibility of decreased diffusion of substrate due to this lack attenuation was considered to be unlikely because of the high moisture content of sewage sludge. In the case of treatments containing only soil, this was accepted as a potential but acceptable limitation, as the treatments were intended to act as controls for identification of potential pathogens within soil. Powdered substrate was also supplemented for liquid substrate and was diluted with quartz sand (50-70 mesh particle size) to a ratio of 1:5 substrate:sand prior to addition to samples. The substrate type and quantity is also outlined within relevant experimental chapters. Where there were control treatments, requiring the absence of substrate, the equivalent weight of sand was added to ensure continuity of sample weight. Following this, an 18 hour incubation period at experimental temperatures was followed by acquisition of one headspace sample at each time point for the duration of the experiment.

3.4.2. Sample preparation

Wet samples, of known dry weight, were placed in 100ml sterile, plastic, screw-cap containers and covered with Parafilm to ensure aerobic conditions. They were then stored at 10°C for the duration of each experiment. Prior to each time point (18 hours), lids modified with rubber septa were screwed on to create an air tight seal. Headspace samples were then taken and the samples destructively analysed for microbial enumeration.

3.4.3. Headspace sampling

Headspace samples were obtained by inserting a needle, attached to a 10 ml syringe (Figure 3.2a), through the septum located in the lid of the sample (Figure 3.2b). The syringe was then flushed three times with the headspace gas and a 10 ml sample taken. This sample was then transferred to the Supelco 7 ml glass GC vial with screw cap and rubber septum, which had been decontaminated in an oven (104°C) and

evacuated in advance. The full 10 ml of headspace gas was transferred to over pressurise the vial, ensuring enough sample would remain for analysis in the presence of any leaks. At each time point, three samples of ambient air conditions were also taken to determine background levels of CO_2. Headspace samples were then stored at room temperature (approximately 21ºC) until analysis.

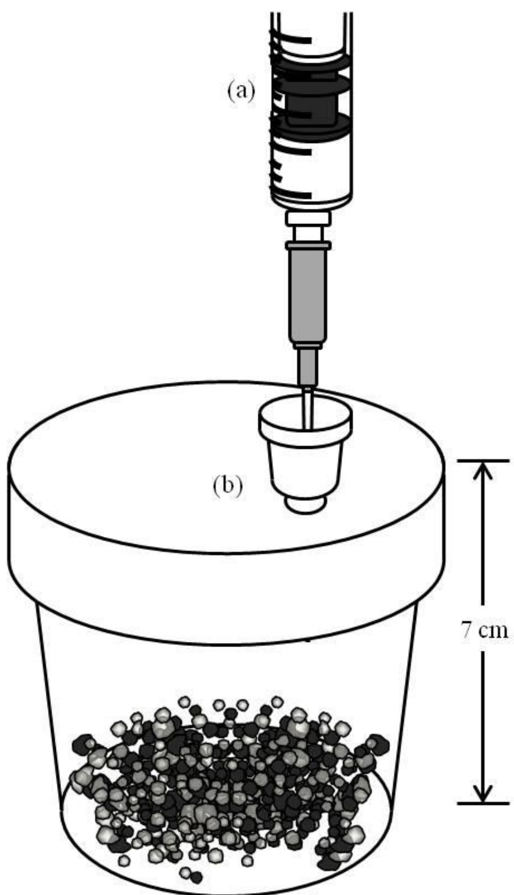

Figure 3.2. Apparatus used to hold and obtain headspace samples; (a) 10 ml syringe with needle attached, inserted through rubber septum; (b) 100ml sterile, plastic, screw-cap container holding sewage sludge and soil samples.

3.4.4. Gas chromatography procedure

CO_2 analysis was performed on the Varian CP-3800 gas chromatograph (GC), using a CTC Analytics Combi-pal auto sampler. This was calibrated using certified Argo CO_2 gas in a range from 500-1000 ppm. The headspace sample (700 µl) was injected into a column injector (60°C). The sample was then carried through the column

(60°C), packed with Porapak[tm] packing (80-100 mesh), by the carrier gas (Argon) at a flow rate of 35 ml min^{-1}. The rate of CO_2 evolution was then calculated as follows:

$$RCO_2 - C\ (\mu g\ CO_2 - Cg^{-1}h^{-1}) = (V_0 \times \frac{12}{22.4 \times t \times m_{sd}})1000 \quad (1)$$

Where $RCO_2 - C$ is the $CO_2 - C$ formation on a dry mass basis ($\mu g\ CO_2 - Cg{-}1h{-}1$, $V0$ enrichment volume of CO_2 in standard temperature and pressure conditions (ml), 12 is the molar mass of $CO_2 - C$, 22.4 is the molar volume of CO_2 under standard conditions, t is incubation time (hours) and m_{sd} is the mass of dry soil or sewage sludge; depending upon treatment composition.

3.5. Microbial carbon

3.5.1. Background

Soil microbial biomass represents the living component of soil organic matter (SOM). It accounts for approximately 1-4 % of total SOM and is representative of soil health, due to the rapid turnover of fungal and bacterial cells in response to changes in environmental conditions (Sparling, 1992; Schloter, *et al.* 2003; Andrews, *et al.* 2004). In this protocol, according to the British Standard Institute 7755-4.4.2:1997 (BSI, 1997) and adapted from Vance, *et al.* (1987), ethanol-free chloroform is used to lyse cells by fumigation. This also leaves non-biomass soil OM unaltered (Vance, *et al.* 1987). The difference between the carbon content of the fumigated and non-fumigated samples represents the soil microbial biomass. This procedure can also be used to quantify other elemental components of biomass, including phosphorus and nitrogen (Brookes, *et al.* 1985; Brookes, *et al.* 1982). The microbial biomass of sewage sludge was also measured using the protocol described above.

3.5.2. Fumigation procedure

Two samples (10 g dry weight) of each treatment were weighed into glass jars. One sample remained un-fumigated and was immediately extracted, whilst the other was placed into an implosion-resistant desiccator (Corning Pyrex Desiccator ®, UK) to be fumigated. The desiccators also contained damp tissue paper, a glass beaker of soda lime (3 g) to absorb CO_2 and a glass beaker of ethanol-free chloroform (50 ml) with anti-bumping granules (<5 g). The lid of the desiccator was then replaced and the desiccator evacuated for 2 minutes using a vacuum pump. The desiccator was then sealed and the pump turned off. After 24 hours, the desiccators were vented and the beaker of chloroform removed. The lid was replaced and the pump was used to evacuate the desiccator a further six times (2 minutes each) to ensure that all chloroform was removed.

3.5.3. Extraction procedure

All samples were extracted using 0.5 M K_2SO_4 and shaken for 45 minutes on an orbital shaker. They were then filtered through sterile Whatman No.42 filter paper and stored (-20ºC) until analysis. Blank 0.5 M K_2SO_4 was also run through sterile Whatman No.42 filter paper and stored until analysis, to determine background levels.

3.5.4. Calculation of microbial carbon

Microbial carbon (MC) was determined using a Shimadzu TOC-V_{eph} analyser. It employs combustion catalytic oxidation which oxidises the samples at 700°C. The extracts were acidified (pH 2-3) and bubbled with sparge gas to remove inorganic carbon. MC was then quantified by subtracting extractable carbon in the un-fumigated samples from the extractable carbon in the fumigated samples using the following equation:

$$\mathrm{MBC} = (\mathrm{C_{fum}} - \mathrm{C_{non-fum}})$$

(2)

Where C_{fum} is carbon extracted from fumigated samples, $C_{non-fum}$ is carbon extracted from un-fumigated samples.

3.6. Statistical analysis

Selected data, including cell count, respiration and microbial carbon, were analysed using a two-way factorial ANOVA in Statistica, version 11 for Windows (Stats Soft, 1984 – 2015), using independent variables: treatment and time. A one-way ANOVA was then implemented to test the significance of treatment effects for each time point. A *post hoc* analysis, the Bonferroni correction, was used to assess means for homogeneity with a significance level of 95%. The tables for the post-hoc analyses, implemented for each experimental chapter, can be found in Appendix 3.

A non-linear estimation was also used on selected cell count data to calculate death rates (k-value). A first order decay function was then fitted to the data using Statistica version 11 for Windows (Stats Soft, 1984 – 2013), as follows:

$$x = (a + b)\exp^{-kt}$$

(3)

Where x is the population at a given time point, t; a+b is the initial concentration (CFU gDS^{-1}); k is the exponential rate of decline or death rate (d^{-1}) and a is the asymptote of the final concentration (Mubiru, *et al.* 2000; Oliver, *et al.* 2006; Moynihan, *et al.* 2012). The difference between these death rates was then assessed using Students t-test analysis with a significance level of 95% (Dytham, 2006).

4. The significance of sewage sludge loading on the persistence of pathogenic bacteria in soil

4.1. Introduction

Several studies provide quantitative data on the microbial diversity of soil; ranging from 4,000 bacterial genomes (Torsvik, *et al.* 1990) to 8.3 million unique sequences per gram (Gans, *et al.* 2005). Roesch, *et al.* (2007), discredits this latter prediction, as well as providing a more conservative range of 10,000 to 50,000 operational taxonomic units (OTU, analogous to "species") per gram of soil. This lack of consensus is in part due to the inherent bias in DNA extraction, as well as the dynamic and changeable nature of the soil environment itself. Therefore, whilst the actual number associated with soil microbial diversity varies, it can be taken from the above research that the soil harbours a diverse range of life. Consequently, it is theorised that such diversity and/or quantity of microorganisms within the soil would interact with enteric pathogens and sewage sludge. The extent microbial diversity would impact on the persistence of enteric pathogens would depend on which microbes were predominant within the soil, as well as the likelihood of interactions that could occur, such as competition or predation, and their level of fitness in comparison to the introduced pathogens. Moynihan, *et al*, (2015) found that the phenotypic structure of the microbial community associated with different land uses, significantly affected the survival of three pathogens, *S.* Dublin, *L. monocytogenes*, non-toxigenic *E. coli* O157. Similarly, Vivant, *et al,* (2013) uaing demonstrated that the persistence of *L. monocytogenes* was greatly impeded within microcosms containing highly diverse microbial communities, with the potential phylogenetic composition contributing to such effects. Furthermore, with a greater quantity of microorganisms present it would be expected that there would be a greater likelihood of interactions between the soil microbial community and enteric pathogens, with the presence of such microorganisms potentially bolstered with nutrient input.

There are several studies which incorporate the use of fertiliser or manure amendments that also support the above theory. For example, Jiang, *et al.* (2002), determined the rate of *E. coli* O157:H7 decline, in autoclaved/unautoclaved sandy loam soil, under ratios of manure to soil (wt/wt; 1:10, 1:25, 1:50, or 1:100), held at three

temperatures (5°C, 15°C and 21°C). They postulated that the survival of *E. coli* O157:H7 was influenced by the manure-to-soil ratio, soil temperature, and indigenous soil microorganisms. They subsequently found that the smallest ratio, or a greater quantity of manure to soil (1:10 manure to soil versus 1:25, 1:50 or 1:100), resulted in a greater decline in *E. coli* O157:H7. They also found that *E. coli* O157:H7 survived for up to 231 days in such microcosms before becoming undetectable. Additionally, Schwarz, *et al.* (2014), demonstrated a greater decline *E. coli* and *S. enterica* under anaerobically-digested dewatered biosolids applied to soils, in comparison to un-amended soils. They postulated that the greater decline in *E. coli* and *S. enterica* in amended soils could have been caused by enhanced antagonistic activity of the indigenous soil microorganisms. This enhanced activity of the indigenous microorganisms was attributed to greater availability of nutrients and improved moisture retention; which would allow for a greater diffusion of such nutrients.

Furthermore, when fertilisers, such as sewage sludge or animal manure are incorporated into soil, it can significantly alter the composition and/or the activity of the local microbial community. For example, Acea and Carballas, (1988), found that slurry amendment to a acidic soil led to a proliferation in microflora, which subsequently returned to their initial concentrations. The initial rise in biomass was attributed to the nutrients supplied by the slurry, whilst the ensuing fall was attributed to the rapid consumption of essential nutrients, antibiosis or predation. Following a second addition of slurry, however, they found that such effects were not repeated. They attributed this to rapid nutrient consumption and dry climate conditions; which may have slowed incorporation of slurry into the soil. Similarly, Fernandes, *et al.* (2005), studied the effect of sewage sludge application on microbial biomass, basal respiration metabolic quotient and enzyme activity in a tropical soil. Their application regime consisted of four applications of sewage sludge at five different concentrations. They found that microbial biomass, basal respiration, metabolic quotient and enzyme activity increased with sewage sludge amendment; and was positively correlated with the dosage of sewage sludge. Overall, this increase in quantity of microbial biomass and overall microbial activity could lead to increased microbial interactions and thus increase the potential for competition for remaining resources (Sidhu, *et al.* 2001). This could

effectively instigate a hot spot of activity that may prompt the die-off of enteric pathogens, co-introduced with sewage sludge.

It is also possible that the interface between the fertiliser amendment and the soil will be the primary sites of interaction between microbes. For example, Petersen, *et al.* (1993), examined the microbial processes in and around a sandy-loam soil core which had a 16 mm thick section of manure inserted into its centre. They found that concentrations of phospholipid-P, used to estimate microbial biomass, were unaffected ≥4 mm away from the soil-manure interface. Furthermore, they found that oxygen penetrated only 2-2.5 mm of the manure after a 21 day period. This depth of oxygen penetration corresponded with the distribution of phospholipids which exhibited a sharp gradient in biomass between the oxygenated manure layer and the soil matrix. Similarly, Frostegård, *et al.* (1997), examined microbial community dynamics associated with manure hot spots in soil. They found that microbial biomass doubled within a 2 mm distance of the soil-manure interface after a 3 day period. This also occurred alongside a change in PLFA composition; indicating a change in the structure of the microbial community. These changes were attributed to the diffusion of dissolved organic carbon from the manure and did not occur within soil layers further away from the manure amendment.

From these studies it can be taken that fertiliser amendment, whether manure, slurry or sewage sludge, both increase and change the local microbial community; due to increased availability of nutrients and likely diffusion of such from increased moisture content. Additionally, the interface between the fertiliser and soil matrices exhibits the greatest change. Consequently, it is theorised that such activity and change could drive the exclusion or decline of introduced enteric pathogens when co-introduced with sewage sludge. Furthermore, it is theorised that the proportion of sewage sludge to soil (i.e. loading rate) would alter the extent or mixing between the two matrices, thus altering the extent and nature of the interfaces between the two matrices. Consequently, this would alter the degree of interaction between the two microbial communities involved; with a greater portion of sludge to a smaller quantity of soil leading to a greater survival in enteric pathogens.

Therefore, the research reported here aims to further clarify the factors controlling the survival of enteric pathogens in soil, by elucidating the importance of sewage sludge loading (i.e. proportion of sludge to soil) on the persistence of model pathogenic bacteria in soil. The hypothesis for this experiment is that there will be a positive correlation between increasing ratios of sludge to soil and the survival of model bacterial pathogens. To test this hypothesis, two microcosm-based studies were developed, where *E. coli* or *S*. Dublin were added to treatments containing soil or sewage sludge, with microbial numbers quantified periodically over a 42 day period.

4.2. Aims, objectives and hypotheses

4.2.1. Aim

This work aims to elucidate how the loading rate of sewage sludge influences the survival and interactions of bacterial pathogens co-introduced with the sewage sludge, to soils.

4.2.2. Objectives

- Demonstrate how sewage sludge loading affects the persistence of model pathogenic bacteria in soil.
  - By varying the proportion of sewage sludge to soil
  - By using two model pathogens; *Salmonella* Dublin and *Escherichia coli* lys 9, to assess the consistency of treatment effect between differing microorganisms.

4.2.3. Hypothesis

H_1 Pathogen survival in sewage sludge will decline with increasing proportions of soil to sewage sludge.

4.3. Materials and methods

4.3.1. Sample collection and analysis

Composite samples of soil and sewage sludge were used for these experiments, as outlined in Chapter 3.2.1 and 3.2.2. The sewage sludge was air-dried to a fixed moisture content of 60% and pasteurised (70°C) for 24 hours to increase friability and to decrease the quantity of indigenous microorganisms. The quantity of *E. coli* within the sewage sludge at this point was approximately 1.0 x 10^1 CFU gDS^{-1} to 1.0 x 10^3 CFU gDS^{-1}. It was then sieved to a particle size of 4 mm. Physicochemical and biological analyses were also assessed using the protocols referenced in Chapter 3.2.3. Viable or total microbial counts were also performed on both matrices, using Plate Count Agar, which was then incubated at 37°C for 24 hours (Thermo Scientific, 2014b).

4.3.2. Treatment preparation

Two sets of microcosms containing soil and sewage sludge were established, containing either *E. coli* or *S.* Dublin isolates. The weights for each treatment were prescribed on a volume to volume ratio, with a standardised volume of 16 ml per sample used throughout. The fresh weights required to give this volume are given in Table 4.1 and are further explained in Figure 4.1. Aliquots (0.1 ml) of *E. coli* and *S. Dublin*, grown in LB broth and washed with quarter strength Ringer's, were subsequently inoculated into sterile 100 ml, plastic, screw-cap containers containing sewage sludge, as outlined in Chapter 3.3.3. A dilution series was created from the culture and used to assess absorbency and cell count, using quarter strength Ringer's (see Chapter 3.3.3, Appendix 2). The un-inoculated soil was then added to the spiked sewage sludge. The sewage sludge and soil were then gently shaken by end-over-end rotation for 60 seconds, to encourage even dispersal of both matrices. Where there were treatments without sludge (spiked control soil), the bacteria were added directly to the soil. The two control treatments, control soil and control sludge, were inoculated with sterile quarter strength Ringer's solution, allowing for the analysis of background levels of microorganisms. The control treatment for the model pathogen (control pathogen), contained no matrices and was only used at the outset of the experiment (Day 0), to

ascertain the impact of the extraction protocol on their survival. The matrices for the mixed treatments were then combined manually, using end-over-end rotation. This minimised the loss of inoculated pathogen through transfer. The microcosms were then incubated at 10°C over a 42 day period.

Table 4.1. Treatment outline (n = 3) based on volume basis (%), with equivalent weight of sewage sludge and soil (± 0.05 g).

		Fresh weight (g)		Dry weight (g)	
No.	**Contents**	**Soil**	**Sludge**	**Soil**	**Sludge**
1	Control pathogen	0	0	0	0
2	Control soil	24.22	0	8.53	0
3	Inoculated control soil	24.22	0	8.53	0
4	Mixed, 25% sludge	18.17	3.56	6.4	2.43
5	Mixed, 50% sludge	12.11	7.12	4.27	4.85
6	Mixed, 75% sludge	6.05	10.68	2.13	7.28
7	Control sludge	0	14.24	0	9.71
8	Inoculated control sludge	0	14.24	0	9.71

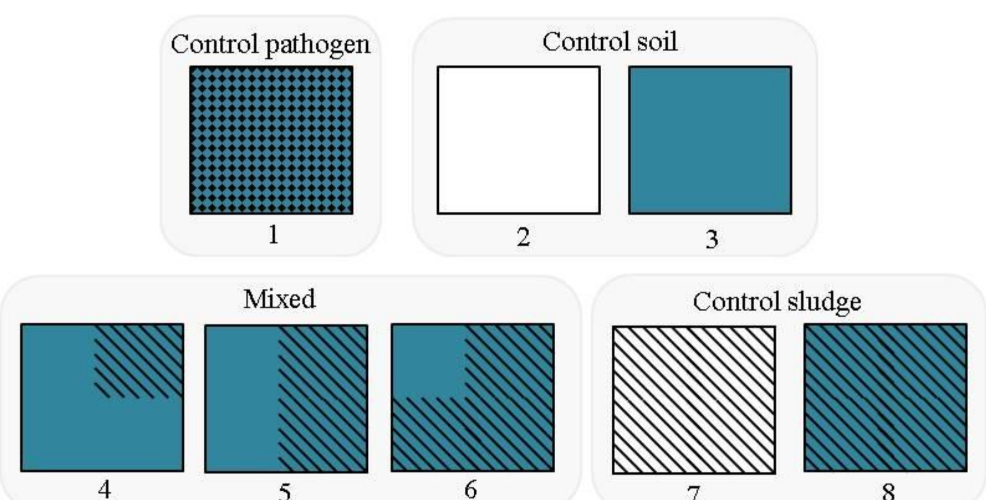

Figure 4.1. Treatment outline, detailing amendment strategy. Key: [empty box] soil; [hatched box] sludge; [solid box] inoculated with model pathogen; [dotted box] inoculated with model pathogen only, i.e. no soil or sewage sludge present within treatment.

4.3.3. Survival of model pathogens *E. coli* and *S.* Dublin

At each time point (0, 1, 3, 7, 14, 28, 42 days), one full cohort of treatments for each pathogen (n = 3) were removed from the incubator. *E. coli* and *S.* Dublin were then extracted and enumerated using the protocols outlined in Chapter 3.3.3 and 3.3.4. For each replicate, aliquots (0.1 ml) of the extracted solution were plated in duplicate. Duplicate plating was undertaken in this experiment to act as a quality control.

4.3.4. Statistical analysis

The resulting cell count data was analysed, as outlined in Chapter 3.6, using a two-way factorial ANOVA using treatment and time as independent variables, followed by a one-way ANOVA to test the significance of treatment effects for each time point. Death rates were also calculated using the cell count data and were subsequently compared using Students t-test analysis, again outlined in Chapter 3.6.

4.4. Results

4.4.1. Background analysis

The soil and sewage sludge exhibited pHs of 6.37 and 7.54, respectively (Table 4.2, Table 4.3). The soil was found to have a moisture content half that of sewage sludge. Additionally, the soil contained tenfold more nitrate than it did ammonium. In contrast, sewage sludge had a lower nitrate content than it did ammonium. Both the soil and sewage sludge showed low numbers of presumptive *E. coli*, though high numbers of *Salmonella* species in the soil (Table 4.4). A viable microbial count also showed that the soil contained approximately 4.50 x 10^4 CFU gDS^{-1}, whilst the sewage sludge had a far larger general microbial count of 7.00 x 10^5 CFU gDS^{-1}.

Table 4.2. Chemical characteristics of soil (Mean ± SEM, n = 3)

Analysis	Soil		
pH (H_20) 1:1	6.37	±	0.11
Total exchange capacity (MEQ 100 g^{-1})	12.3	±	0.23
Moisture content (%)	31.8	±	0.3
Organic matter (humus) %	6.92	±	0.18
Bray I phosphorus (mg l^{-1})	111	±	0.7
Nitrogen (mg l^{-1})			
Nitrate	49.6	±	0.51
Ammonia	6.07	±	0.25
Mehlich III Extractable (mg l^{-1})			
Phosphorus	108	±	0.77
Potassium	228	±	0.89

Table 4.3. Chemical characteristics of the sewage sludge (Mean ± SEM, n=3)

Analysis	Sewage sludge (Fresh weight)		
pH (H_20) 1:1	7.54	±	0.8
Total solids (g kg^{-1})	247	±	0.56
Moisture content (%)	64.8	±	0.41
Total phosphorus (g kg^{-1})	203	±	6.17
Phosphorus as P_2O_5 (g kg^{-1})	476	±	9.43
Potassium (g kg^{-1})	22.2	±	2.04
Potassium as K_20 (g kg^{-1})	26.8	±	2.23
Nitrogen (g kg^{-1})			
Total Kjeldahl	11.3	±	0.13
Organic	8.96	±	0.16
Nitrate	<0.1	±	0
Ammonia	2.35	±	0.1

Table 4.4. Biological characteristics of the sewage sludge and soil, prior to inoculation of model pathogenic bacteria (Mean ± SEM, n=3) (* n=2)

Analysis	Soil (CFU gDS^{-1})	Sewage sludge (CFU gDS^{-1})
E. coli O157	283 ± 6	5.00 x 10^{+3} ± 24
Presumptive Salmonella	233 ± 7	0 ± 0
Viable bacterial count	4.50 x 10^{+4} ± 42*	7.00 x 10^{+5} ± 176

4.4.2. Survival of model pathogen *E. coli*

The quantity of indigenous *E. coli* indicated by the un-inoculated control sludge, generally remained between 1.00 x 10^3 CFU gDS^{-1} and 1.00 x 10^5 CFU gDS^{-1} for the duration of the experiment. Due to this, the un-inoculated control sludge treatment was omitted from Figures 4.2 and 4.3, as well as from further statistical analysis. The un-inoculated control soil was also omitted from Figures 4.2, 4.3 and further statistical analysis as it did not contain detectable concentrations of *E. coli*. Initial concentrations of *E. coli* in the inoculated control sludge treatment were 3.32 x 10^6 CFU gDS^{-1}. This remained constant for the first week, subsequently dropping to approximately 2.9 x 10^5 CFU gDS^{-1}, on Day 42. A Two-way factorial ANOVA of *E. coli* concentrations showed highly significant main effects and sludge-loading by time interaction effects ($p<0.001$; Figure 4.2).

Implementing a one-way ANOVA to compare the differences between treatments at each time point, *E. coli* concentrations in the inoculated control soil were shown to be significantly lower than in the inoculated control sludge, apart from on Days 0 and 7 ($p<0.001$). There were no significant differences between the *E. coli* concentrations within the mixed treatments. Nor were there any significant differences between the mixed treatments and the inoculated control sludge within the first two weeks of the experiment. However, on Days 29 and 42, inoculated control sludge exhibited significantly higher concentrations of *E. coli* than all mixed treatments ($p<0.001$). Additionally, when implementing a one-way ANOVA of *E. coli* death rates in the remaining treatments, mixed 75% sludge was significantly greater than in the remaining mixed treatments ($p<0.001$; Figure 4.3). The remaining death rates did not significantly differ.

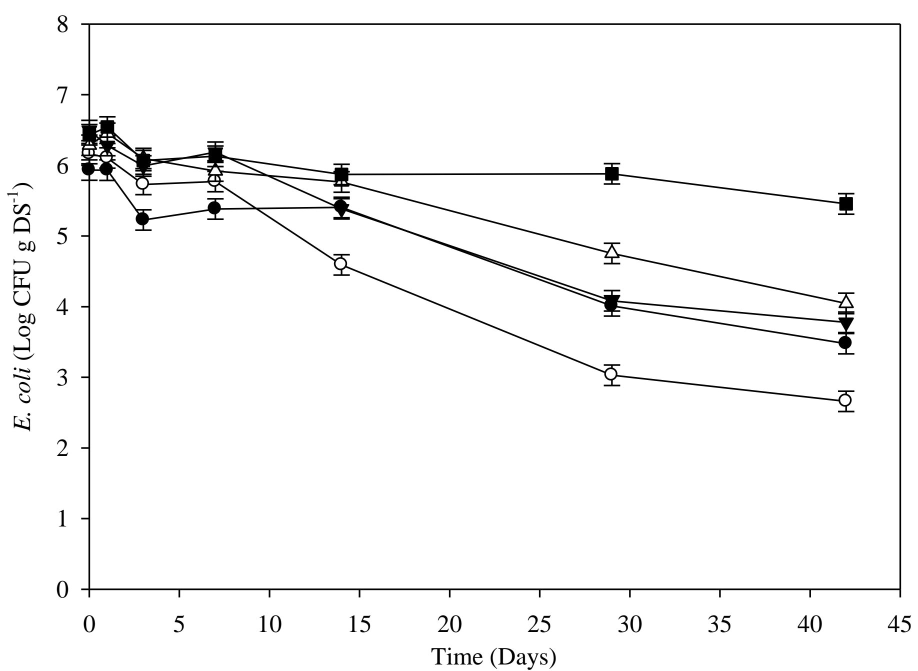

Figure 4.2. Survival of *E. coli* within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE). Key: —●— inoculated control soil; —○— mixed 25% sludge; —▼— mixed 50% sludge; —△— mixed 75% sludge; —■— inoculated control sludge.

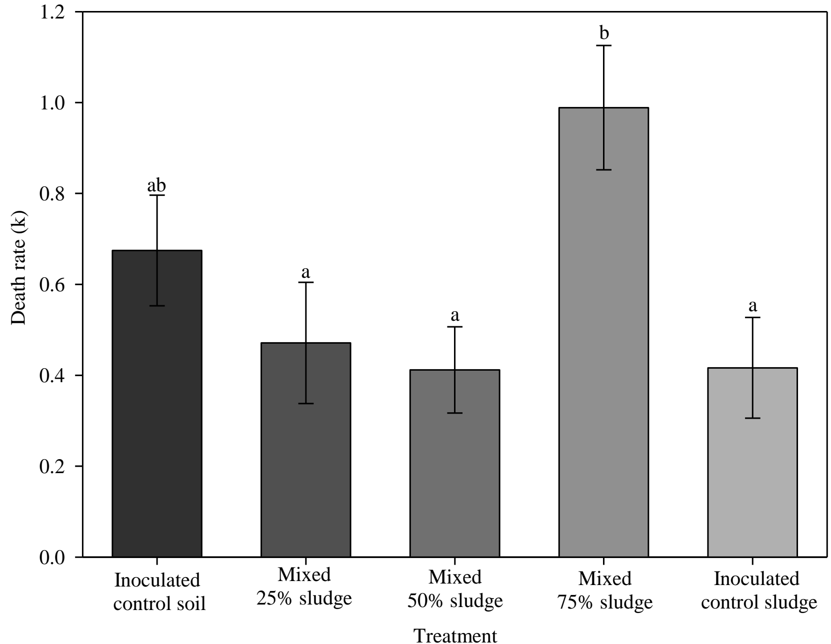

Figure 4.3. Death rates for *E. coli* in microcosms containing mixtures of sewage sludge and soil, calculated using CFU gDs^{-1} (n=3, Mean ± SEM). Letters denote homogeneous means at 5% significance level.

4.4.3. Survival of model pathogen *S.* Dublin

The un-inoculated control sludge and un-inoculated control soil treatments did not contain detectable levels of *Salmonella*, and were therefore omitted from Figures 4.4, 4.5 and further statistical analysis. Initial concentrations of *S.* Dublin in the inoculated control sludge treatment were 7.54×10^6 CFU gDS^{-1}. This remained constant for the first week, subsequently dropping to approximately 1.14×10^5 CFU gDS^{-1}, on Day 42. Following this, a two-way factorial ANOVA of *S.* Dublin concentrations highlighted highly significant main effects and sludge-loading by time interaction effects ($p<0.001$; Figure 4.4).

Implementing a one-way ANOVA found that *S.* Dublin concentrations in the inoculated control soil were significantly lower than in inoculated control sludge for the entirety of the experiment ($p<0.01$). However, there were no significant differences between the *S.* Dublin concentrations within the mixed treatments, aside from on Day 7. Furthermore, there were significant differences between the mixed treatments and the inoculated control sludge treatment on Days 42, 7 and 1; where inoculated control sludge contained significantly higher concentrations of *S.* Dublin ($p<0.001$). Additionally, using a one-way ANOVA to assess *S.* Dublin death rates showed that there were no significant differences between treatments; apart from the death rate for the mixed 75% sludge treatment, which was significantly lower compared with inoculated control soil ($p<0.05$; Figure 4.5).

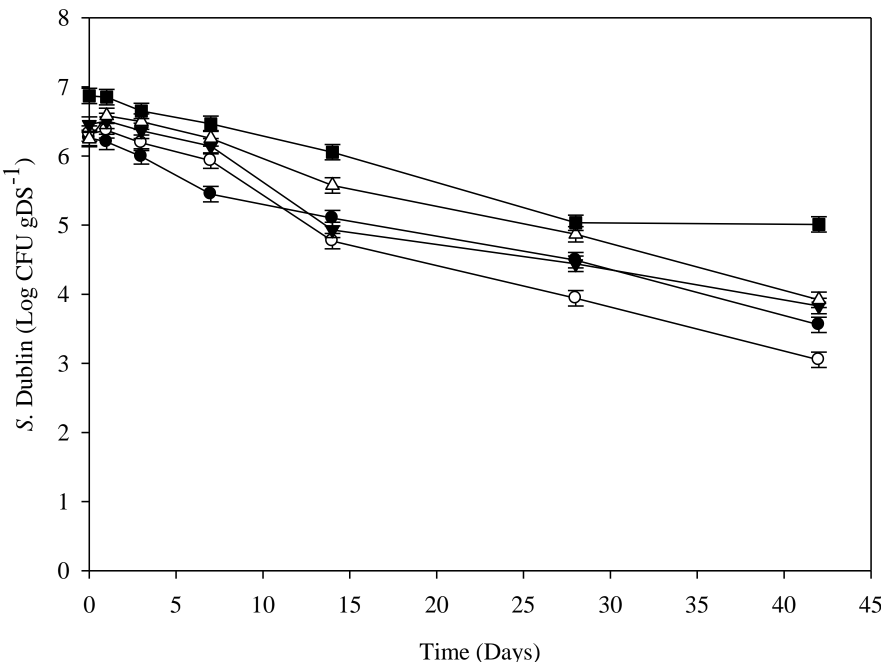

Figure 4.4. Survival of *S.* Dublin within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE). Key: —●— inoculated control soil; —○— mixed 25% sludge; —▼— mixed 50% sludge; —△— mixed 75% sludge; —■— inoculated control sludge. *errors are smaller than symbols

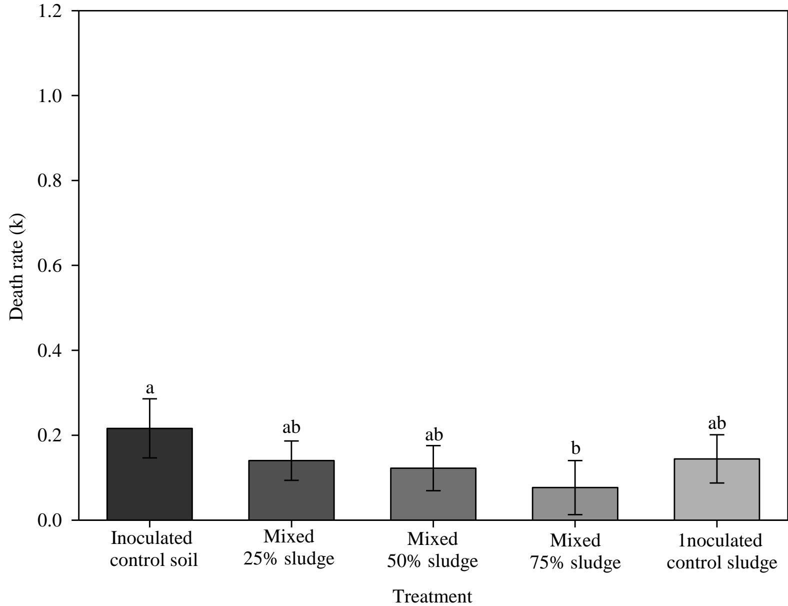

Figure 4.5. Death rates for *S.* Dublin in microcosms containing mixtures of sewage sludge and soil, calculated using CFU gDs^{-1} (n=3, Mean ±SEM). Letters denote homogeneous means at 5% significance level.

4.5. Discussion

This experiment considered the importance of sewage sludge loading on the persistence of inoculated model pathogenic bacteria in soil. The attempt at eradicating the indigenous *E. coli* population in the sewage sludge, prior to the start of the experiment by pasteurisation, failed. However, this did not become apparent until the experiment was started (Section 4.3.1). Counts made straight after elimination attempts were in the range of tens to hundreds of *E. coli* per gDS, indicating acceptably low numbers of indigenous *E. coli*. Initially, this lead to a query hanging over the work as to whether the results could be relied upon. Upon re-assessing the data, it was considered that these numbers were included in the count for the other treatments, as they were detectable using the agar medium indicated in Chapter 3.3.4. Therefore making the *E. coli* CFU data a total *E. coli* count rather than model *E. coli* count.

Overall, within treatments inoculated with *E. coli*, there were no significant, or consistent, correlations between varying proportions of sludge to soil and the survival of the model bacterial pathogens. Therefore, the initial hypothesis stating that there would be a positive correlation between increasing ratios of sludge to soil and the survival of inoculated bacterial pathogens was rejected for treatments inoculated with *E. coli*. For example, *E. coli* prevailed at greater numbers when inoculated directly into soil, whilst *E. coli* declined to the greatest extent within mixed microcosms containing 25% sludge. *E. coli* numbers also prevailed in the inoculated control sludge treatment, implying that interaction with the soil and its microbial community may have some form of detrimental effect on survival. This could be through antibiosis, antagonism, predation or competition for resources, including space and nutrients.

The relationship, where the smallest quantity of sewage sludge to soil causes an overall greater decline in *E. coli* numbers, is similar to the relationship observed by Jiang, *et al.* (2002). They found that the smallest ratio (1:10) of manure to soil caused a greater decline in *E. coli* O157:H7. This minimal ratio could have caused such a reaction due to a balance of factors which may favour the soil microbial community. The additional nutrients provided in the sewage sludge/manure would bolster the local microbial community, whilst the minimal amendment strategy would not alter the environment to favour the sewage sludge/manure microbial community. Additionally,

the greater survival of *E. coli* in pure soil was in contrast to the findings of Schwarz, *et al.* (2014); who demonstrated a greater decline *E. coli* and *S. enterica* under anaerobically-digested dewatered biosolids applied to soils, in comparison to un-amended soils. This could be attributed to a difference in state or activity between the soil microbial communities in this work and in the work of Schwarz, *et al.* (2014). Furthermore, when considering the death rates calculated for *E. coli*, it is difficult to reconcile the apparent lack of treatment effect for these death rates. This disparity could possibly be attributed to the nature of the death rate calculation, which represents the overall exponential decline. As the overall survival of *E. coli* was not strictly a 'linear' exponential decline, apparent by the change in the slope of the lines within Figure 4.3, the model adopted may not be appropriate as a comparative tool. Furthermore, a straightforward mathematical function describing the time courses in relation to the decline of *E. coli* could not be identified.

Similarly, within treatments inoculated with *S.* Dublin, there were no significant, or consistent, correlations between varying proportions of sludge to soil and the survival of the model bacterial pathogens. All treatments containing *S.* Dublin appeared to decline at a similar rate, which was more linear that the decline observed within treatments inoculated with *E. coli*. Therefore, the initial hypothesis stating that there would be a positive correlation between increasing ratios of sludge to soil and the survival of inoculated bacterial pathogens was rejected for treatments inoculated with *S.* Dublin. However, there was a similar trend to that which was observed with the *E. coli* inoculated treatments. The mixed treatment containing 25% sludge showed the greatest overall decline in *S.* Dublin numbers, whilst the control soil inoculated directly with *S.* Dublin, showed greater quantities of *S.* Dublin. *S.* Dublin also persisted to a greater extent in the inoculated control sludge treatment. Furthermore, whilst the query over the calculation of death rates remains, there appears to be no significant differences between the death rates calculated for the decline of *S.* Dublin. It would also appear that the more linear nature of the *S.* Dublin data set is better suited to such a function.

4.5.1. Conclusion

From these findings, it can be concluded that there are no direct relationships between the proportion of sludge to soil and its affect on pathogen survival. However more complex and content-dependent relationships appear to be occurring, for example, a smaller amount of soil appeared to result in lower eventual numbers. Furthermore, whilst partitioning effects were discussed in the introduction it became apparent that a more precisely designed experiment to test such interactions would be required. This is due to the design of the experiment, which assumed that the majority of energy would be provided by the sewage sludge. In order to fully test such this, a comparison between microbial responses to energy within either soil or sewage sludge would be appropriate. Initially, however, it will be important to clarify if the use of indigenous sewage sludge *E. coli* will provide more salient results. The use of this microbe may provide data which is more suited to the original premise of this work, as under such scenarios it would be indigenous sewage sludge *E. coli* that would be of concern. This strain would also be better acclimatised to conditions within the sewage sludge, and may therefore be more capable at surviving the change in conditions when mixed with soil.

5. The significance of sewage sludge loading on the persistence of indigenous sewage sludge pathogens in soil

5.1. Introduction

Previous work considered the importance of sewage sludge loading on the persistence of inoculated model pathogenic bacteria in soil. It was found that sewage sludge loading, or the proportion of sludge to soil, did not significantly affect the survival of model pathogens in the soil environment. High concentrations of indigenous *E. coli* were also observed in the un-inoculated sewage sludge treatment, even after attempts were made to eradicate them. This was both an unexpected and interesting development, as the diversity of *E. coli* and its ability to adapt to varying conditions could influence their survival in soil. Therefore, indigenous strains of *E. coli*, prevalent within sewage sludge, could possibly better adapted for survival in soil in comparison to strains of model pathogens. Furthermore, they are potentially more relevant due to the source material they inhabit.

Whittam, (1989), found that gastro-intestinal isolates of *E. coli*, which can be found in sewage sludge, were genetically distinct from environmental strains. Additionally, Franz, *et al.* (2011), studied the persistence of 18 *Escherichia coli* O157 strains; 8 animal, 1 food and 9 human isolates, in manure-amended sandy soil. They found that there was a high degree of variation in survival across all of the strains analysed. Generally, human isolates survived for significantly greater periods (median 211 days) when compared to animal isolates (median 70 days) ($p=0.025$). They also assessed metabolic capacity, which could be a contributing factor in their differing survival rates. For example, microorganisms with a greater metabolic capacity, or the ability to oxidise a wide range of substrates, could persist in the soil environment as they would be able to avail of a wider range of resources and be less likely to become limited than those microorganisms that have a smaller metabolic capacity. It was found that the metabolic profiles of each strain which survived for longer than 200 days was markedly different than those that survived for less than 200 days. Similarly, Topp, *et al.* (2003), demonstrated changes in natural *E. coli* communities in swine manure slurry when combined with soil; with a strain designated as C279 becoming more prevalent in the presence of manure, when compared to another strain, designated C278. These

studies demonstrate the different survival patterns that can occur between different strains of *E. coli*, showing how making generalisations regarding survival patterns even at a species level can be dangerous.

Therefore, this experiment proposed to study the persistence of naturally occurring, or indigenous, sewage sludge *E. coli* in soil. It followed the same approach as the previous experiment, which assessed the importance of sewage sludge loading (i.e. proportion of sludge to soil) on such persistence. The hypothesis for this experiment postulated that the survival of indigenous sewage sludge *E. coli* would decline with increasing proportions of soil to sewage sludge. To test this, a microcosm-based study was developed, where varying quantities of soil was added to a consistent quantity of sewage sludge, which contained indigenous *E. coli*. Microbial numbers were then quantified periodically over a 56 day period and subsequently analysed.

5.2. Aims, objectives and hypotheses

5.2.1. Aim

This work aims to elucidate how the loading rate of sewage sludge influences the survival and interactions of indigenous sewage sludge *E. coli* co-introduced with the sewage sludge, to soils.

5.2.2. Objectives

- Demonstrate how sewage sludge loading affects the persistence of indigenous sewage sludge *E. coli* in soil.
    - By varying the proportion of sewage sludge to soil
    - By using indigenous *E. coli*, that is already present in the sewage sludge, to further assess consistency of treatment effect between differing microorganisms.

5.2.3. Hypothesis

H_1 Indigenous sewage sludge *E. coli* survival will decline with increasing proportions of soil to sewage sludge.

5.3. Materials and methods

5.3.1. Sample collection and analysis

Composite samples of soil and sewage sludge were used for these experiments, as outlined in Chapter 3.2.1 and 3.2.2. The sewage sludge was manually crumbled to an aggregate size of approximately 0.5 cm to ensure homogeneity. Its moisture content was unaltered. Physicochemical and biological analyses were also assessed using the protocols referenced in Chapter 3.2.3.

5.3.2. Treatment preparation

One set of microcosms containing soil and sewage sludge was established (Table 5.1; Figure 5.1). The sewage sludge contained high concentrations of indigenous sewage sludge *E. coli*, thus no additional isolates were used for this study. The initial cell count was taken from the first reading (Day 0) of the control sludge treatment. The weights for each treatment were prescribed on a dry weight ratio. A consistent weight of sewage sludge was used throughout all treatments which contained sewage sludge. Maintaining a consistent quantity of sewage sludge ensured a consistent concentration of *E. coli* was present across all treatments containing sewage sludge. For mixed treatments, soil and sewage sludge were weighed out separately into sterile 100 ml, plastic, screw-cap containers. The soil was then incorporated into the containers holding the sewage sludge, through manual end-over-end rotation for 60 seconds. This minimised the loss of pathogen through transfer. When adding sewage sludge to plastic containers, the transfer of *E. coli* or other pathogen to the inner face of the container is a possibility. By adding the soil to the container holding the sewage sludge we minimised the loss of any pathogen that would otherwise remain behind, if the procedure were done the other way round. The microcosms were then incubated at 10°C over a 56 day period.

Table 5.1. Treatment outline (n = 3), with equivalent weight of sewage sludge and soil (± 0.02g).

		Fresh weight (g)		Dry weight (g)	
No.	**Contents**	**Soil**	**Sludge**	**Soil**	**Sludge**
1	Control soil	1.35	0.00	1.02	0.00
2	Mixed, 15% sludge	7.63	4.00	5.75	1.02
3	Mixed, 25% sludge	4.04	4.00	3.05	1.02
4	Mixed, 50% sludge	1.35	4.00	1.02	1.02
5	Mixed, 75% sludge	0.45	4.00	0.34	1.02
6	Control sludge	0.00	4.00	0.00	1.02

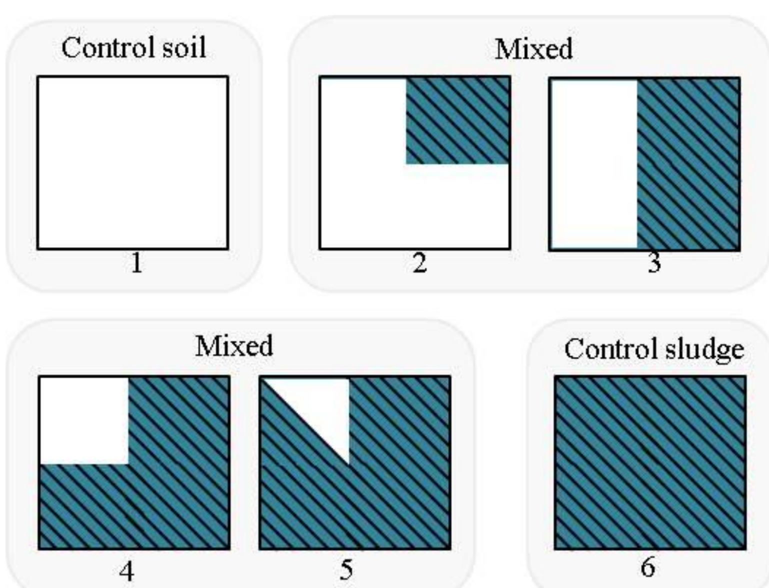

Figure 5.1. Treatment outline, detailing proportion of soil to sewage sludge. Key: soil; sludge; presence of indigenous sewage sludge *E. coli*.

5.3.3. Survival of indigenous sewage sludge *E. coli*

At each time point (0, 1, 3, 7, 14, 28, 56 days), one full cohort of treatments (n = 3) was removed from the incubator (10°C, Table 5.2). Indigenous sewage sludge *E. coli* were then extracted and enumerated using the protocols outlined in Chapter 3.3.3 and 3.3.5.

5.3.4. Statistical analysis

The resulting cell count data was analysed, as outlined in Chapter 3.6, using a two-way factorial ANOVA using treatment and time as independent variables. A one-way ANOVA was also implemented to test the significance of treatment effects for each time point.

5.4. Results

5.4.1. Background analysis

The soil exhibited a pH of 6.1, whilst the sewage sludge exhibited a pH of 7.3 (Table 5.2, Table 5.3). Additionally, the soil was found to have a moisture content less than half that of sewage sludge. Furthermore, the soil contained tenfold more nitrate than it did ammonium. Sewage sludge also contained a greater quantity of nitrate than it did ammonium. The soil contained no *E. coli* but relatively high quantities of general coliform species (Table 5.4). In contrast, sewage sludge contained far more *E. coli* than soil, though it harboured fewer general coliform species.

Table 5.2. Chemical characteristics of soil (Mean ± SEM, n = 3).

Analysis	Soil		
pH (1:1 in H_2O)	6.1	±	0.06
Total Exchange Capacity (MEQ 100 g^{-1})	9.34	±	0.27
Moisture content (%)	24.5	±	0.04
Organic Matter (loss on ignition, %)	4.95	±	0.07
Bray I Phosphorus (P) (mg l^{-1})	64.3	±	1.2
Nitrogen (mg l^{-1})			
Nitrate (NO_3)	20.1	±	1.19
Ammonium (NH_4)	1.13	±	0.09
Mehlich III Extractable (mg l^{-1})			
Phosphorus (P)	66.7	±	0.25
Manganese (Mn)	12.3	±	0.27
Zinc (Zn)	4.03	±	0.16
Boron (B)	22.2	±	2.03
Copper (Cu)	4.48	±	0.19
Iron (Fe)	300	±	0.98
Aluminium (Al)	610	±	1.25
Sulphur (S)	11.7	±	0.25
Calcium (Ca)	1214	±	2.26
Magnesium (Mg)	137	±	0.33
Potassium (K)	96.3	±	0.67
Sodium (Na)	27.3	±	0.6

Table 5.3. Chemical characteristics of the sewage sludge (Mean ± SEM, n = 3).

Analysis	Sewage sludge (Fresh weight)		
pH (H_20) 1:1	7.39	±	0.17
Total solids (g kg^{-1})	245	±	0.44
Moisture content (%)	74.6	±	0.04
Total phosphorus (g kg^{-1})	5.68	±	0.06
Phosphorus as P_2O_5 (g kg^{-1})	13	±	0.09
Potassium (g kg^{-1})	195	±	6.12
Potassium as K_20 (g kg^{-1})	0.72	±	0.04
Nitrogen (g kg^{-1})			
Total Kjeldahl	9.73	±	0.16
Organic	9.58	±	0.16
Nitrate	44.3	±	2.35
Ammonia	0.16	±	0.03
Carbon (mg l^{-1})			
Organic	3.06	±	0.08

Table 5.4. Biological characteristics of the sewage sludge and soil (Mean ± SEM, n = 3).

Analysis (CFU gDS^{-1})	Soil			Sewage sludge		
E. coli	0	±	0	$6.57 \times 10^{+5}$	±	$9.39 \times 10^{+4}$
General coliforms	$4.43 \times 10^{+3}$	±	$5.70 \times 10^{+2}$	$1.13 \times 10^{+5}$	±	$2.82 \times 10^{+4}$

5.4.2. Survival of indigenous sewage sludge *E. coli*

The control soil treatment did not contain detectable concentrations of indigenous *E. coli* during the experiment and so were omitted from Figures 5.3, as well as from further statistical analysis. A two-way factorial ANOVA of indigenous sewage sludge *E. coli* concentrations in the remaining treatments showed highly significant main effects and treatment by time interactions ($p<0.01$; Figure 5.2).

Initial concentrations of indigenous sewage sludge *E. coli* in the control sludge treatment were approximately 1.13×10^5 CFU gDS^{-1} (Figure 5.2). This declined gradually over a period of 56 days to 6.38×10^2 CFU gDS^{-1}. At the onset of the experiments, the mixed treatments did not vary from the control sludge treatment in terms of *E. coli* numbers. However, from Day 3, the quantity of *E. coli* significantly decreased in all of the mixed treatments when compared to the control sludge treatment ($p<0.001$). Similarly, on Day 7, all of the mixed treatments, apart from the 50% sludge mixed treatment, harboured significantly fewer *E. coli* than the sludge control treatment ($p<0.001$). Following this, on Day 14, only the 25% sludge mixed treatment exhibited significantly lower quantities of *E. coli* than the sludge control ($p<0.02$). In contrast, on Day 42, the mixed treatments containing 50% and 75% sludge showed significantly decreased levels of *E. coli*, in comparison to the sludge control treatment ($p<0.001$). By Day 56, all mixed treatments exhibited significantly lower quantities of *E. coli* in comparison to the control sludge treatment.

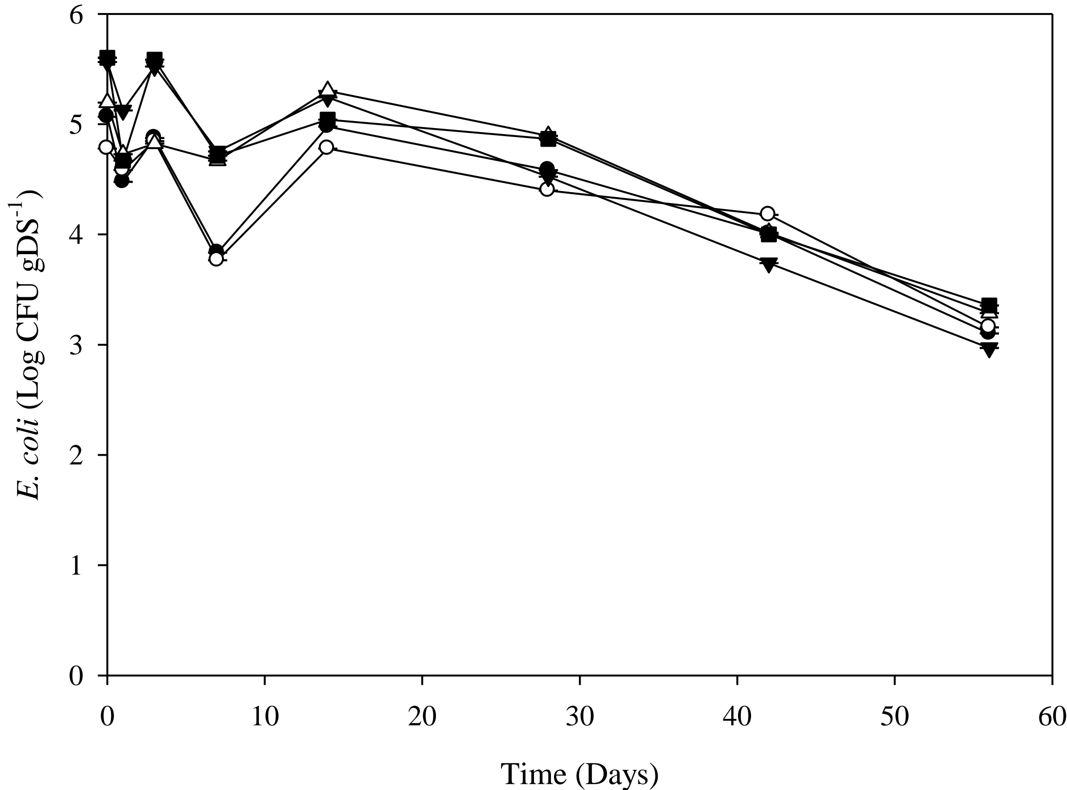

Figure 5.2. Survival of indigenous sewage sludge *E. coli* within microcosms comprising soil/sludge mixtures (n=3, ± pooled SE; error bars fall within confines of points in most instances). Key: —●— mixed, 15% sludge; —○— mixed, 25% sludge; —▼— mixed, 50% sludge; —△— mixed, 75% sludge; —■— Control sludge. *errors are smaller than symbols

5.5. Discussion

This experiment considered the importance of sewage sludge loading on the persistence of indigenous sewage sludge *E. coli* in soil. As with previous work (Chapter 4), there were no significant, or consistent, correlations between varying proportions of soil to sludge and the survival of *E. coli*. Thus the initial hypothesis proposing that the survival of indigenous sewage sludge *E. coli* in sewage sludge would be adversely affected by increasing the proportion of soil to sewage sludge was rejected. Additionally, there was an unexpected decline in *E. coli* numbers, followed by an increase around Day 7. The possible causes of this shift are unknown. However, it could have occurred due to natural variations and interactions within the system or more likely due to an error within the experimental protocol performed on this day. This latter reason is more likely due to the shift in cells occurring throughout the entire cohort of treatments. Furthermore, there were no observable trends between the mixed treatments and survival of *E. coli*, as which occurred with both model pathogens as described within Chapter 4. Moreover, the indigenous *E. coli* also exhibited a more consistent linear decline after the first week, when compared to the model *E. coli*, which exhibited a more varied response. These findings are in contrast to those of Jiang, *et al.* (2002), who found that the proportion of manure to soil did affect the survival of *E. coli* O157:H7; especially in regards to the smallest proportion of manure to soil, which saw the greatest die-off.

This lack of significant treatment effect in both the model strains and that of the indigenous sewage sludge *E. coli* may have been caused by unforeseen factors. For example, a lack of available or labile substrate could have impeded microbial activity thus leading to minimal competition for resources that would not instigate significant or differential die-off. This could have arisen due to depletion of nutrients during storage for this work or low initial nutrient levels arising from WWTF treatment practices. For example, the sewage sludge used within this experiment contained relatively low levels of nitrogen and high level of phosphorus when compared to the soil. The ratio of such concentrations, as well as carbon, is important in the development of biomass and cell organelles. This potential, or theorised, lack of available resources then begs the question; does the addition of sewage sludge to soil actually provide enough energy to

create a hot spot of activity? The answer to which would probably be dependent upon the physicochemical properties of the sewage sludge to be utilised, as well as the state in which both soil and sewage sludge microbial communities are to be found in (i.e. are they 'active' or 'dormant'). For example, Chaudri, *et al.* (2008), assessed the impact of heavy metals in sewage sludge on soil microbial processes and subsequent long-term effect on agricultural productivity. The experiment consisted of nine field sites amended with sewage sludge cake, metal-amended liquid sludge's and inorganic metal salts. They found that whilst copper and cadmium had little effect on the population size of *Rhizobium leguminosarum* biovar *trifolii*, zinc caused significant die-off when it was added in concentrations close to the UK limit of 300 mg kg $^{-1}$ at a pH of 6.0-7.0 (SI, 1989). They postulated that the free-living rhizobia were directly exposed to high concentrations of zinc in and around sludge cake particles when mineralising the OM present within the sludge. So whilst the presence of OM may attract and promote microbial activity, the presence of zinc or other metals could cause toxic effects with the amendment of sewage sludge acting as a form of a 'poisoned chalice'. This is indirect evidence that sewage sludge particles can act as 'hotspots' for microbial activity, but such eventual activity would be predicated on a range of factors beyond the potential energy contained within them. There is also the possibility that the microbial communities within the sludge and in the soil are isolated and don't interact.

5.5.1. Conclusion

From these findings, it can be concluded that the proportion of sludge to soil does not significantly affect the survival of pathogens in the soil environment. Considering that an increasing quantity of soil would be expected to mean a proportional increase in the rate and extent of encounters between the native microbial community and the introduced organisms, this lack of treatment effect is confounding. It would be expected that a greater quantity of soil would naturally lead to a greater total interface between sewage sludge and the native soil microorganisms which would attenuate the introduced pathogens. To counter these results, it was theorised that the lack of treatment effects could have arisen from a lack of available substrate to promote enough microbial activity to instigate the die-off of *E. coli*. Similarly, the substrate

within the sewage sludge may not have been as immediately available for use within the soil microbial community, allowing the *E. coli* time to acclimatise/establish without increased competition from the local microbial communities. Additionally, the physicochemical composition of sewage sludge; namely the heavy metal content, could further impede microbial activity and subsequent interactions. Future work, will therefore need to analyse the heavy metal content of the sewage sludge as well as the usual physicochemical parameters. Therefore, the following experiment will assess the effect of supplementary substrate on both *E. coli* survival and overall microbial activity. It will also attempt to consider the extent of partitioning effects on such microbial interactions, should any in fact occur.

6. The effect of supplementary nutrients on the survival of pathogens in soil, co-introduced with sewage sludge

6.1. Introduction

Previous work investigated the survival of both model and indigenous sewage sludge pathogens within microcosms containing varying proportions of sewage sludge to soil. In Chapter 4, the model pathogen *S.* Dublin declined over time with little treatment effect whilst the model pathogen *E. coli* showed a similar pattern, though there was greater variation in overall decline. There were also high levels of background *E. coli* within the sewage sludge. The lack of treatment effect was unexpected as it was rationalised that varying inputs of sewage sludge would instigate different rates of decline due to varying provisions of energy triggering microbial activity. Further work, in Chapter 5, examined the survival of indigenous sewage sludge *E. coli*, which was present in high concentrations within the sewage sludge at the onset of the experiment. Again, there was little variation in decline of indigenous *E. coli* among treatments over a 56 day period, with respect to the relative proportions of soil to sewage sludge. Therefore, it was shown that the origin of the pathogen and the variation in sewage sludge to soil had little effect on the persistence of pathogens.

The original concept postulated that the sewage sludge would create a hotspot of microbial activity, through the provision of readily available organic carbon and other nutrients, leading to a more rapid decline of pathogens. However, this decline in pathogens did not occur as there were no consistent treatment affects within any of the previous experiments. It was postulated that this lack of variation in response to varying proportions of sewage sludge to soil may have been associated with a lack of microbially-available substrate or energy. Furthermore, some form of partitioning effect between the soil and sewage sludge matrices could have impeded the interaction between the microbial communities. Therefore, this chapter considers how the effect of supplementary nutrients and the phase in which they are located (either sewage sludge or soil) influences the survival of sewage sludge-derived *E. coli*.

As the majority of microorganisms within the soil prevail in a state of semi-starvation, the input of new substrate can promote a short-term increase in microbial

activity (Hobbie and Hobbie, 2013). This induction of microbial activity, followed by any subsequent degradation of local soil organic matter (SOM) is known as a priming effect (Kuzyakov, *et al.* 2000). It involves four steps; (i) preferential use of substrate, where simpler, more readily available molecules are utilised in preference to more complex molecules, (ii) stimulation of microbial biomass by readily utilizable substrate, (iii) subsequent use of other remaining substrates according to utilizability, and (iv) decline to original state (Kuzyakov and Bol, 2006). This response to substrate amendment has been utilised to study a variety of factors. For example, Kouno, *et al.* (2002) studied the impact of substrate input (glucose or ryegrass) on the turnover of biomass carbon (C) and phosphorus (P). They found that biomass C had a turnover time of approximately 82 days and 95 days for glucose and ryegrass, respectively. In contrast, biomass P had a turnover of approximately 37 days for glucose and 42 days for ryegrass. The shorter turnover of biomass P, in comparison to biomass C, was attributed to P being more labile within microbial cells. Henderson, *et al.* (2010) studied the influence of biomass on changes in denitrifier abundance in anoxic soils. They found that denitrification and respiration increased in soil independently amended with plant residues (red clover, soybean or barley straw) and glucose compared to un-amended soil. However, denitrifier communities responded similarly to the different plant residues. Priming effects have also been observed in the rhizosphere, with live roots significantly controlling SOM decomposition leading to elevated decomposition; the extent of which varies with plant species and seasonality (Cheng, *et al.* 2003). This induction of microbial activity through substrate application is analogous to the initial premise of the work relating to the addition of sewage sludge as fertiliser, and its induction of microbial activity, effectively creating a hot spot of activity. This increased activity could subsequently initiate a decline in recently introduced pathogens through competition for resources. However, this addition could alternately promote the survival of such pathogens, especially in the case of *E. coli* due to its fast growth rate.

Furthermore, there is debate over the interactions belying the idea of priming effects and what could cause them. Substrate composition and availability may not be the only factor involved in the priming effect, with Fontaine, *et al.* (2003) postulating that this effect arises due to competition between microorganisms for energy and nutrient acquisition. Blagodatskaya and Kuzyakov, (2008) theorised that the addition of

substrate could stimulate the growth of r strategists, the most active part of the microbial community. Subsequently, they went on to study the effect of substrate amendment on growth kinetics (Blagodatskaya, *et al.* 2009). They demonstrated shifts in functional properties of soil microorganisms after substrate addition; the input of readily available substrate (glucose or root exudates) stimulated r- strategists whilst the addition of less available substrate (plant residues) stimulated slow-growing K-strategists. The physical structure and texture of the soil is another factor which may govern decomposition of substrate, mechanisms of which include adsorption of organics to clay surfaces and entrapment in aggregates (van Veen and Kuikman, 1990). Furthermore, partitioning between microbial communities may occur due to the physical structure, texture and moisture content of both the soil and sewage sludge, which can affect motility, adhesion and formation of biofilms. This could be likened to macro-scale ecological theory, where the interaction and distribution of larger organisms can be influenced by physical barriers such as lakes or mountains (Prosser, *et al.* 2007).

Consequently, it was postulated that enhancing microbial activity, through the addition of substrate and any associated priming effects, may further promote competition for available resources and thus may trigger a decline in sewage sludge-derived *E. coli*. The addition of readily biodegradable organic carbon would also boost the energy levels within the system that were apparently missing in Chapters 4 and 5. This increase in competition is attributed to the stimulation of the local microbial community, raising them from a putatively semi-starved state to a more active state. It was also postulated that matrix partitioning, i.e. structural or physical hindrance, may also play a significant part in such interactions. Therefore, it was hypothesised that increasing the soil microbial activity, via the application of substrate, would cause a significantly decline in sewage sludge-derived *E. coli*. Conversely, increased microbial activity in sewage sludge, via the application of substrate, was hypothesised to significantly increase microbial activity and the survival of sewage sludge-derived *E. coli*. This hypothesis it attempting to consider the system in a holistic manner, as the substrate added will potentially be available to the whole soil/sewage sludge microbial community not just a subset. So the hypothesis essentially poses the question of what will happen if the *E. coli* are able to access the substrate? Additionally, considering *E. coli* are already part of the sewage sludge microbial community, and how quickly they

are able to grow, it would be reasonable to assume that by providing substrate in closer proximity (i.e. directly to the sewage sludge) they would be more likely to survive due to quicker or easier access to it. The increased survival of *E. coli* could then also be counted as part of the overall microbial activity.

To test these hypotheses, two microcosm-based studies were performed where glucose or yeast extract were added to soil, sewage sludge, or both matrices, with microbial activity and *E. coli* numbers quantified periodically. Glucose, a simple carbon monosaccharide, is readily utilised by a high proportion of microorganisms, whilst yeast extract is a complex set of molecules, containing carbon, nitrogen, minor elements and complex organics such as vitamins (Stotzky and Norman, 1961; Grant and Pramer, 1962; Harrison and de Mora, 2001; Prescott, *et al.* 2005e).

6.2. Aims, objectives and hypotheses

6.2.1. Aim

This work aims to clarify the effect of supplementary nutrients and the importance of matrix partitioning in the survival of sewage sludge-derived *E. coli*.

6.2.2. Objectives

- Determine if supplementary nutrients affect the survival of sewage sludge-derived *E. coli*.
    - By adding a labile substrate, glucose, and a less labile and more complex substrate, yeast extract to promote microbial activity in soil and sewage sludge matrices.
- Determine if matrix partitioning is important in the survival of sewage sludge-derived *E. coli*.
    - By adding substrate to either soil, sewage sludge, or both.

6.2.3. Hypotheses

H_1 The addition of labile substrate to soil/sewage sludge matrices will significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli*.

H_2 The location of labile substrate will significantly affect microbial activity and the persistence of sewage sludge-derived *E. coli*.

$H_{2.1.}$ The addition of substrate to soil will cause a significant decline in sewage sludge-derived *E. coli*.

$H_{2.2.}$ The addition of substrate to sewage sludge will cause a significant increase in the persistence of sewage sludge-derived *E. coli*.

6.3. Materials and methods

6.3.1. Sample collection and analysis

Composite samples of soil and sewage sludge were used for these experiments, as outlined in Chapter 3.2.1 and 3.2.2. The sewage sludge was manually crumbled to an aggregate size of approximately 0.5 cm to ensure homogeneity. Its moisture content was unaltered. Physicochemical and biological analyses were also assessed using the protocols referenced in Chapter 3.2.3.

6.3.2. Treatment preparation

Two sets of microcosms were established containing samples of sewage sludge, inoculated with sewage sludge-derived *E. coli*, and soil. They were amended with either glucose or yeast extract (Table 5.1; Figure 5.1). To form these microcosms, soil and sewage sludge were weighed out separately into sterile 100 ml, plastic, screw-cap containers. Isolates of sewage sludge-derived *E. coli* were then grown in culture media, washed with MRD and subsequently inoculated into all containers comprising sewage sludge, as outlined in Chapter 3.3.3. The following dilution series of the culture, used to assess absorbency and cell count, were also produced using MRD. Glucose or yeast extract (5000 mg C kg^{-1}), diluted (1:5 ratio) with quartz sand (particle size: 210 - 297 µm), was then added to each set of containers associated with amended treatments. Further description of this protocol can be found in British Standards Institution, (1997). Pure quartz sand of equivalent mass was then added to the containers associated with un-amended treatments/matrices (control), to ensure that all microcosms underwent the same form of disturbance. The containers associated with the mixed treatments were then combined; with the soil poured into the containers holding the sewage sludge. The matrices were then mixed by hand, using end-over-end rotation for 60 seconds. This minimised the potential loss of inoculated *E. coli* through transfer. The microcosms were then incubated at 10°C over a 42 day period. A description of the containers can be found in Chapter 3.4.2.

Table 6.1. Treatment formulations (n = 4), with equivalent dry weight (± 0.02 g) of sewage sludge and soil.

No.	Treatment	Contents	Substrate	Soil (g)	Sewage sludge (g)
1	Control	100% soil	Un-amended	10	0
2	Control	100% soil	Amended	10	0
3	Mixture	70% soil to 30% sludge	Both un-amended	7	3
4	Mixture	70% soil to 30% sludge	Soil amended	7	3
5	Mixture	70% soil to 30% sludge	Sludge amended	7	3
6	Mixture	70% soil to 30% sludge	Both amended	7	3
7	Control	100% sludge	Un-amended	0	10
8	Control	100% sludge	Amended	0	10

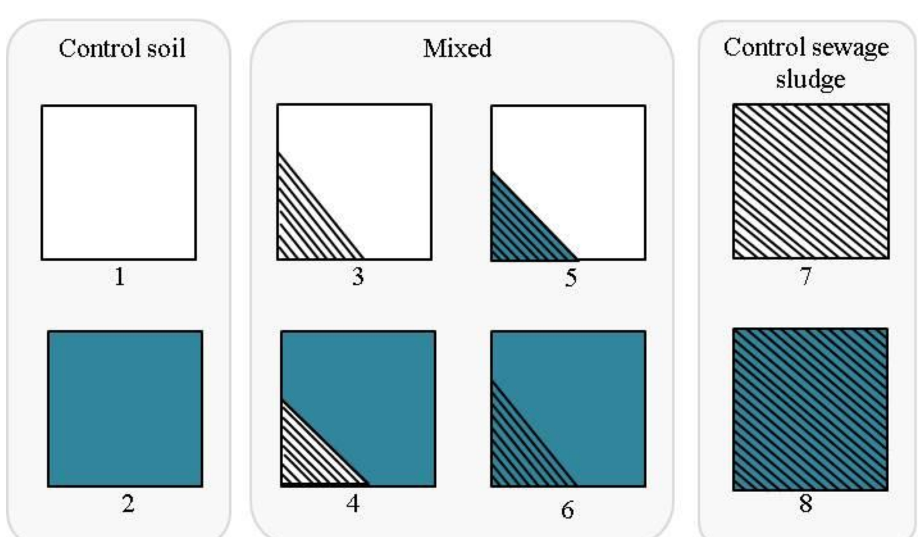

Figure 6.1. Treatment outline, detailing substrate amendment strategy. Key: ☐ soil; ▧ sludge; ■ amended with substrate. Sewage sludge-derived *E. coli* was present within sludge in this instance.

6.3.3. Analysis of microbial activity and survival of sewage sludge-derived *E. coli*

At each time point, (Table 6.2), one full set of treatments (n = 3) was removed from the incubator (10°C). Head-space samples were then taken to assess evolved CO_2, followed by the extraction and enumeration of sewage sludge-derived *E. coli*; as outlined in Chapter 3.3.3, 3.3.5 and 3.4. Microbial carbon was also assessed at the

beginning (D0); 18 hours after substrate addition, and end (D42) of the experiment, following the protocol detailed in Chapter 3.5.

Table 6.2. Time points and respective analyses.

Days	Analysis		
	Respiration	**Enumeration**	**Microbial carbon**
	Substrate amendment (-18hrs)		
0	×	×	×
1	×	×	
3	×	×	
6	×	×	
13	×	×	
28	×	×	
42	×	×	×

6.3.4. Statistical analysis

For each cohort (either glucose or yeast), the cell count, respiration and microbial carbon data was analysed as described in Chapter 3.6. Cell count data was assessed using a two-way factorial ANOVA in Statistica, version 11 for Windows (Stats Soft, 1984 – 2015), followed by a one-way ANOVA was also implemented to test the significance of treatment effects for each time point. The respiration data was similarly assessed after it was log transformed, in order to normalise it. The microbial carbon was analysed using two, one-way ANOVAs to test the significance of individual treatment effects between time points, and to test for significant differences between treatments at a given time point.

6.4. Results

6.4.1. Background analysis

Physicochemical and biological data for both sewage sludge and soil are shown in Tables 6.3, 6.4 and 6.5. The soil was mildly acidic (pH 5.7), with a moisture content of 27.7%. In comparison, sewage sludge exhibited a neutral pH of 7.6 and a typical moisture content of 75.7%. Nitrate levels were marginally greater than ammonium concentrations within the soil. Sewage sludge showed similar levels of nitrate and ammonium. Macro and micro nutrients were present within soil, with minerals ranging from: phosphorus, potassium and zinc, to aluminium and sulphur. In contrast, the sewage sludge contained smaller quantities of minerals. *E. coli* and general coliform concentrations were relatively high in the sewage sludge; $1.17 \times 10^{+6}$ CFU gDS^{-1} and $1.50 \times 10^{+8}$ CFU gDS^{-1} respectively, whilst the soil contained relatively low concentrations of such microorganisms.

Table 6.3. Physicochemical characteristics of soil (Mean ± SEM, n = 3).

Analysis	Soil		
pH (1:1 in H_2O)	5.67	±	0.03
Total Exchange Capacity (MEQ 100g $^{-1}$)	11.4	±	0.39
Moisture content (%)	27.6	±	0.04
Organic matter (loss on ignition, %)	6.3	±	0.56
Bray I phosphorus (mg l^{-1})	2.08	±	1.2
Nitrogen (mg l^{-1})			
Total	3.4	±	0.07
Nitrate (NO_3)	53.6	±	1.99
Ammonium (NH_4)	9.7	±	0.78
Carbon (mg l^{-1})			
Total	23.5	±	0.35
Organic	89.9	±	2.56
Mehlich III Extractable (mg l^{-1})			
Phosphorus (P)	50.7	±	2.22
Manganese (Mn)	141	±	1.08
Zinc (Zn)	3.76	±	0.13
Boron (B)	0.89	±	0.15
Copper (Cu)	5.3	±	0.08
Iron (Fe)	400	±	0.86
Aluminium (Al)	689	±	2.09
Sulphur (S)	15.3	±	0.41
Calcium (Ca)	1268	±	3.06
Magnesium (Mg)	141	±	1.08
Potassium (K)	87	±	0.88
Sodium (Na)	31.3	±	0.58

Table 6.4. Physicochemical characteristics of sewage sludge (Mean ± SEM, n = 3).

Analysis	Sewage sludge (Fresh weight)		
pH (1:1 in H_2O)	7.56	±	0.03
Total solids (g kg^{-1})	259	±	0.8
Moisture content (%)	75.7	±	0.05
Total Phosphorus (g kg^{-1})	5.2	±	0.09
Phosphorus as P_2O_5 (g kg^{-1})	11.9	±	0.14
Potassium (g kg^{-1})	0.66	±	0.03
Potassium as K_2O (g kg^{-1})	0.8	±	0.03
Nitrogen *(g kg^{-1})*			
Total Kjeldahl	0.25	±	0.003
Organic	9.77	±	0.24
Nitrate (NO_3)	1.00	±	0
Ammonia (NH_4)	0.59	±	0.07
Carbon (mg l^{-1})			
Total	218	±	1.432
Organic	203	±	7.55
Potentially Toxic Element (mg kg^{-1})			
Arsenic (As)	2.69	±	0.12
Cadmium (Cd)	0.4	±	0.03
Chromium (Cr)	2680	±	22.7
Copper (Cu)	45	±	0.33
Lead (Pb)	24.6	±	0.27
Mercury (Hg)	0.01	±	0
Molybdenum (Mo)	1.34	±	0.1
Nickel (Ni)	26.5	±	0.31
Selenium (Se)	0.53	±	0.04
Zinc (Zn)	138	±	0.65

Table 6.5. Biological characteristics of soil and sewage sludge (Mean ± SEM, n = 3).

Analysis	Soil (CFU gDS^{-1})			Sewage sludge (CFU gDS^{-1})		
E. coli	2	±	2	$1.17 \times 10^{+6}$	±	$1.69 \times 10^{+5}$
General coliforms	83	±	17	$1.50 \times 10^{+8}$	±	$1.67 \times 10^{+7}$

6.4.2. Survival of sewage sludge-derived *E. coli*; glucose amendment

Both control soil treatments, amended and un-amended with glucose, did not contain detectable concentrations of *E. coli*. Consequently, they were omitted from Figure 6.2, as well as further statistical analysis. When implementing a two-way factorial ANOVA of *E. coli* concentrations in the remaining treatments, highly significant main effects and glucose-treatment by time interactions were observed ($p<0.001$; Figure 6.2). Initial concentrations of *E. coli* in un-amended, control sludge were 1.0×10^6 CFU g DS^{-1}. This remained constant for the first 14 days, and was followed by a decline in several orders-of-magnitude, to approximately 1.0×10^2 CFU g DS^{-1} at Day 42. In comparison, the addition of glucose to sludge instigated a significant decline in *E. coli* concentration within the first 6 days ($p<0.01$). This was followed by a more gradual decline and subsequent convergence in concentration with un-amended, control sludge on Day 42.

Within mixed treatments, the addition of glucose to soil, sludge, or both matrices caused a significant decline in *E. coli* concentrations when compared to the un-amended mixed and control sludge treatments, up to Day 28 ($p<0.01$). Furthermore, there were no significant variations between the mixed treatments, either soil amended or sludge amended, throughout the entirety of the experiment ($p<0.01$). The mixed treatment where both matrices were amended with glucose also showed little deviation from the other mixed treatments after the first week, aside from on Day 42 where a significant rate of decline prevailed in comparison to all other treatments ($p<0.01$). The incorporation of soil within all mixed treatments also prompted a significant decline in concentration, in comparison to the un-amended control sludge treatment, between Days 3 and 28 ($p<0.01$).

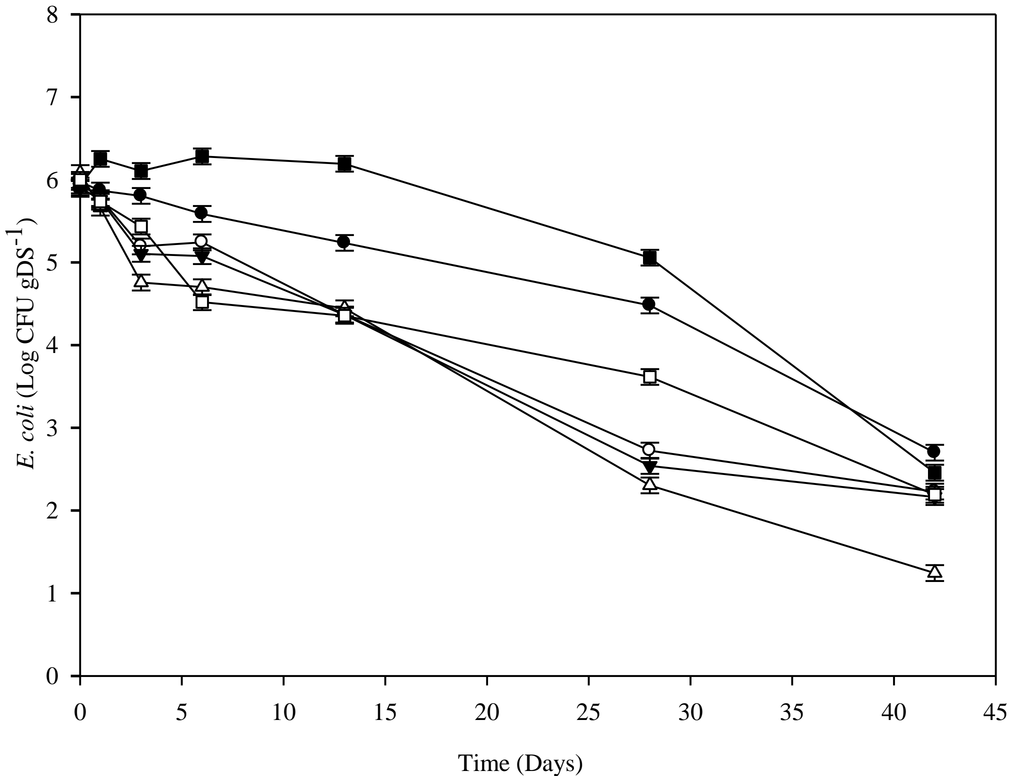

Figure 6.2. Survival of sewage sludge-derived *E. coli* across all treatments, amended with glucose (n=4, ± pooled SE). Key: —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge.

6.4.3. Survival of sewage sludge-derived *E. coli*; yeast extract amendment

Both control soil treatments, amended and un-amended with yeast extract, did not contain detectable concentrations of *E. coli*. Therefore, they were omitted from Figure 6.3, as well as from further statistical analysis. A two-way factorial ANOVA of *E. coli* concentrations in the remaining treatments indicated highly significant main effects ($p<0.001$) and yeast extract-treatment by time interactions ($p<0.02$; Figure 6.3). Initial concentrations of *E. coli* in un-amended, control sludge were approximately 1.0 x 10^6 CFU g DS^{-1}. This declined after the first 6 days, to approximately 1.0 x 10^4 CFU g DS^{-1} on Day 42. Similarly, the addition of yeast extract to sludge instigated a decline after the first 6 days. However, this treatment did not significantly differ from the un-amended control sludge for the majority of the experiment; up to Day 28. This was followed by a significant decline in *E. coli* concentration on Day 42, which was significantly lower than both un-amended control sludge and un-amended mixed treatments ($p<0.001$).

Within mixed treatments, the addition of yeast extract to soil did not cause a significant decline in *E. coli* concentrations when compared to the un-amended mixed and control sludge treatments, between Days 6 and 28. Similarly, from Day 3, the addition of yeast extract to sludge in mixed treatments did not cause a significant decline when compared to its un-amended counterpart. However, sludge amended mixed treatments were significantly different from the un-amended control sludge treatment for the majority of time points, excluding Days 0 and 13 ($p<0.01$). Where yeast extract was added to both matrices within the mixed treatments, *E. coli* concentrations were significantly lower on Days 1, 3 and 42 when compared to its un-amended counterpart ($p<0.01$). Similarly, *E. coli* concentrations were also significantly lower in the both amended mixed treatment from Day 1, onwards when compared to the un-amended control sludge treatment ($p<0.01$). Finally, the incorporation of soil within all mixed treatments did not cause a significant decline in *E. coli* concentration, in comparison to un-amended control sludge treatment, apart from on Days 3 and 13 ($p<0.01$).

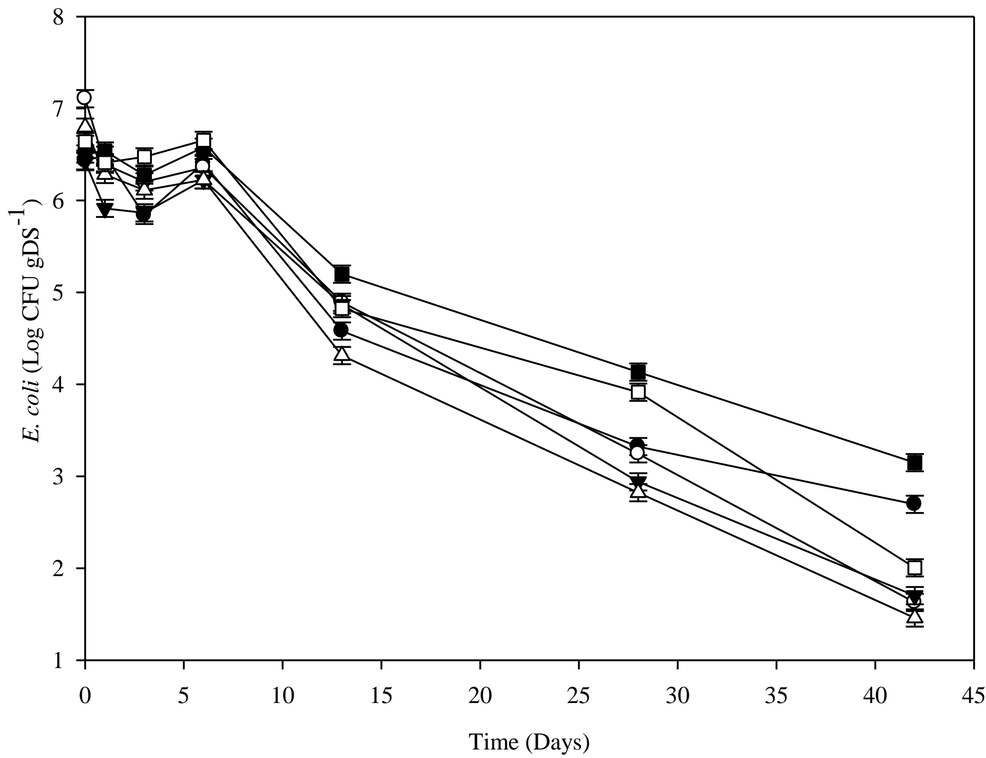

Figure 6.3. Survival of sewage sludge-derived *E. coli* across all treatments, amended with yeast extract (n=4, ± pooled SE). Key: —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge.

6.4.4. Respiration; glucose amendment

Both control soil treatments, amended and un-amended with glucose, did produce detectable levels of CO_2 and thus were included in further analyses. A two-way factorial ANOVA of CO_2 respiration confirmed significant main effects and glucose-treatment by time interactions ($p<0.001$; Figure 6.4). The rate of respiration increased with glucose amendment within the first week and was proportional to treatment type. For example, amended control soil showed a peak of approximately 4 µg CO_2-C g^{-1} h^{-1}, followed by a gradual decline that was significantly different from its un-amended counterpart ($p<0.001$). In contrast, amended control sludge, showed a significantly maximal peak of approximately 44 µg CO_2-C g^{-1} h^{-1} on Day 3 in comparison to all other treatments ($p<0.001$). Following this, it equilibrated with the un-amended control sludge to approximately 15 µg CO_2-C g^{-1} h^{-1}. The amended mixed treatment also exhibited varying peaks in respiration within the first week, ranging from approximately 10 µg CO_2-C g^{-1} h^{-1} to 15 µg CO_2-C g^{-1} h^{-1}, that were significantly greater than their un-amended counterparts ($p<0.001$).

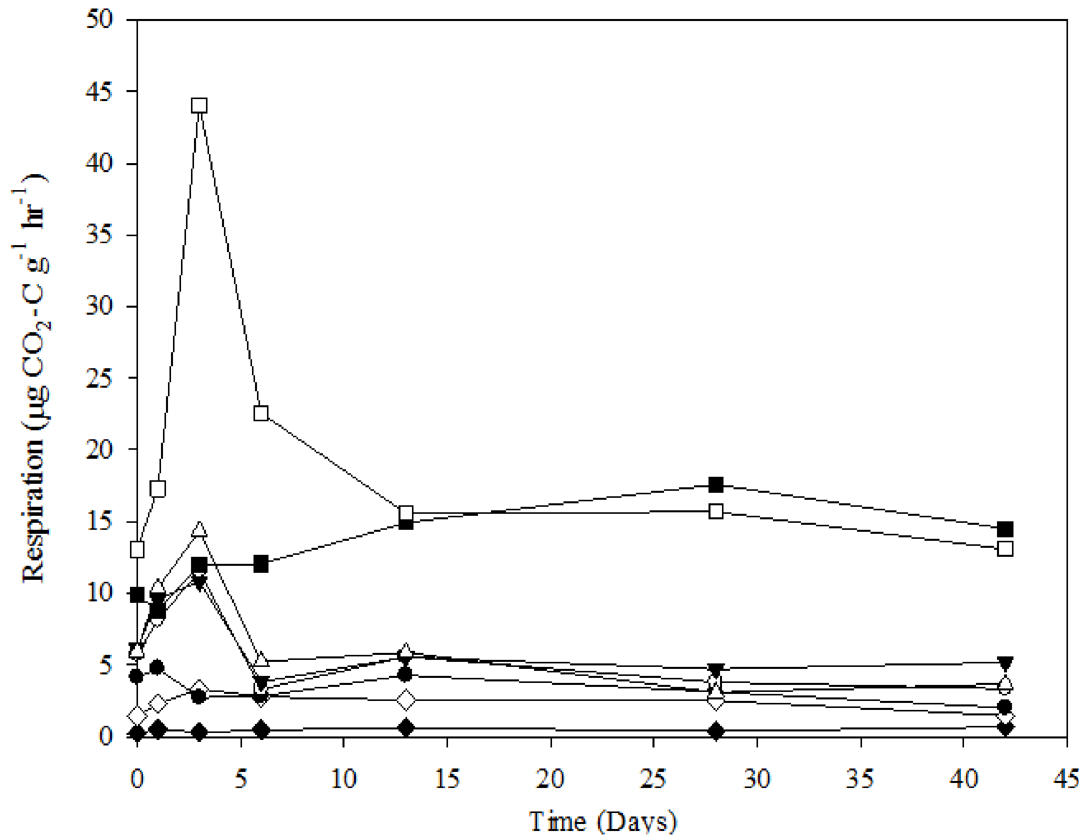

Figure 6.4. Respiration in microcosms amended with glucose (n=4, mean). Key: —◆— un-amended control soil; —◇— amended control soil; —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge. Pooled standard error of log transformed data: 0.06.

6.4.5. Respiration; yeast extract amendment

Both control soil treatments, amended and un-amended with yeast extract, produced detectable levels of CO_2 and thus were included in further analyses. A two-way factorial ANOVA of respired CO_2 confirmed significant main effects and yeast extract-treatment by time interactions ($p<0.001$; Figure 6.5). The rate of respiration increased with yeast extract amendment within the first two weeks after application. Un-amended control soil demonstrated consistently lower respiration rates than all other treatments, up to Day 28 ($p<0.01$). In comparison, amended control soil showed a significantly increased and sustained response to application, with a peak of approximately 10 μg CO_2-C g^{-1} h^{-1} on Day 3 ($p<0.01$). Amended control sludge also had significantly higher respiration rates on Day 13, when compared to all other treatments ($p<0.001$). In contrast, the amended mixed treatments initially underwent a decline in respiration, followed by a gradual increase between Days 6 and 13. None of these treatments were significantly different from each other or their un-amended counterpart, from Day 1 onwards ($p<0.001$).

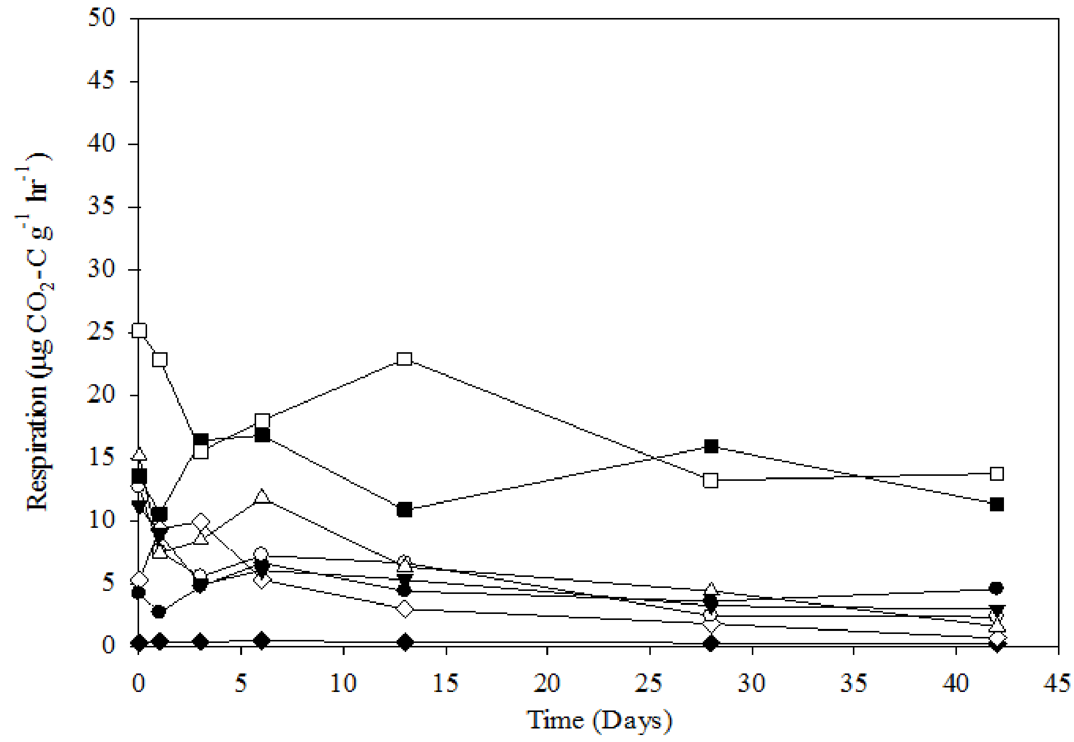

Figure 6.5. Respiration in microcosms amended with yeast extract (n=4, mean). Key: —◆— un-amended control soil; —◇— amended control soil; —●— un-amended mixed; —○— soil amended mixed; —▼— sludge amended mixed; —△— both amended mixed; —■— un-amended control sludge; —□— amended control sludge. Pooled standard error of log transformed data: 0.21.

6.4.6. Microbial carbon; glucose amendment

A one-way ANOVA revealed significantly lower microbial carbon concentrations on Day 42, when compared to Day 0 for soil amended and both amended mixed treatments, as well as amended control sludge ($p<0.05$). The remaining treatments did not significantly differ between days.

On Day 0, (Figure 6.6), un-amended treatments (control soil, mixed and control sludge) and amended control soil did not exhibit significantly different microbial carbon concentrations. In comparison, soil amended mixed and both amended mixed treatments had significantly higher microbial carbon concentrations, when compared to their un-amended counterpart on Day 0 ($p<0.001$). However, the mixed treatment with sludge amended did not significantly differ from the un-amended form on the same day. Furthermore, on Day 0, amended control sludge exhibited significantly higher microbial carbon concentrations than its un-amended counterpart. Additionally, microbial carbon levels within the amended control sludge treatment were not significantly different from soil amended and both amended mixed treatments at the same time point.

By Day 42, (Figure 6.6), the microbial carbon content within the majority of treatments had attenuated, thus they were not significantly different. Only the control sludge treatments had microbial carbon content that was significantly higher that the un-amended control soil and amended mixed treatments ($p<0.001$).

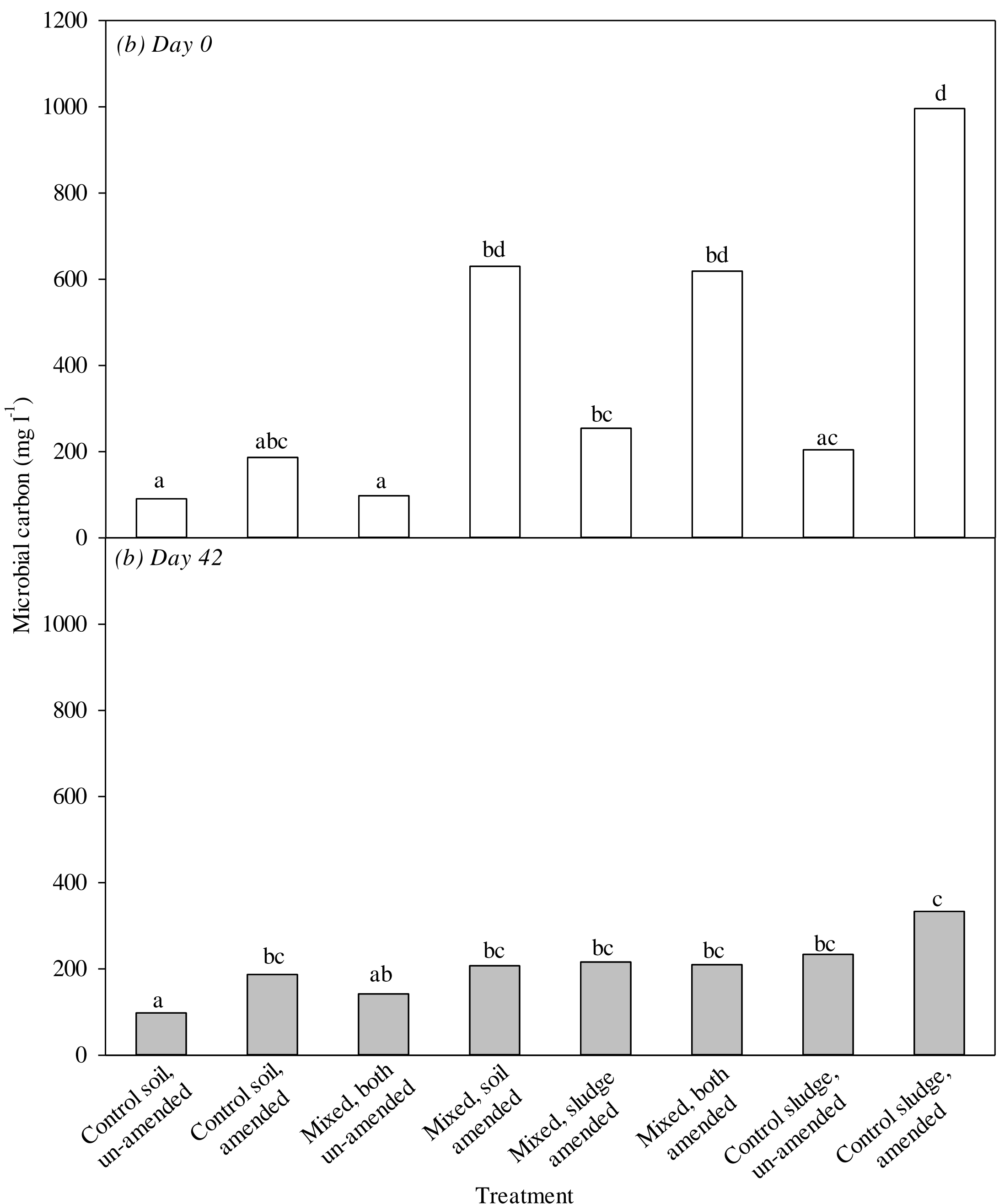

Figure 6.6. Microbial carbon content of microcosms amended with glucose, at (a) Day 0 and (b) Day 42 (n=3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.06.

6.4.7. Microbial carbon; yeast extract amendment

Using a one-way ANOVA, the microbial carbon across all treatments, except control soil and mixed treatments, un-amended, was shown to contain significantly higher microbial carbon content on Day 0, when compared to Day 42 ($p<0.02$).

On Day 0, (Figure 6.7a), the microbial carbon content in control soil, un-amended was significantly lower than all other treatments ($p<0.001$). In contrast, all of the mixed treatments and the un-amended control sludge treatment contained levels of microbial carbon that were similar to each other. Amended control sludge, contained significantly higher microbial carbon levels than all other treatments, apart from amended control soil ($p<0.001$). Additionally, amended control soil exhibited similar microbial carbon levels as those found in both sludge amended mixed and both amended mixed treatments.

On Day 42, (Figure 6.7b), the majority of treatments attenuated towards similar levels and were not significantly different. The control sludge treatments were the only cohort of treatments that were significantly different from the remaining of the treatments ($p<0.001$).

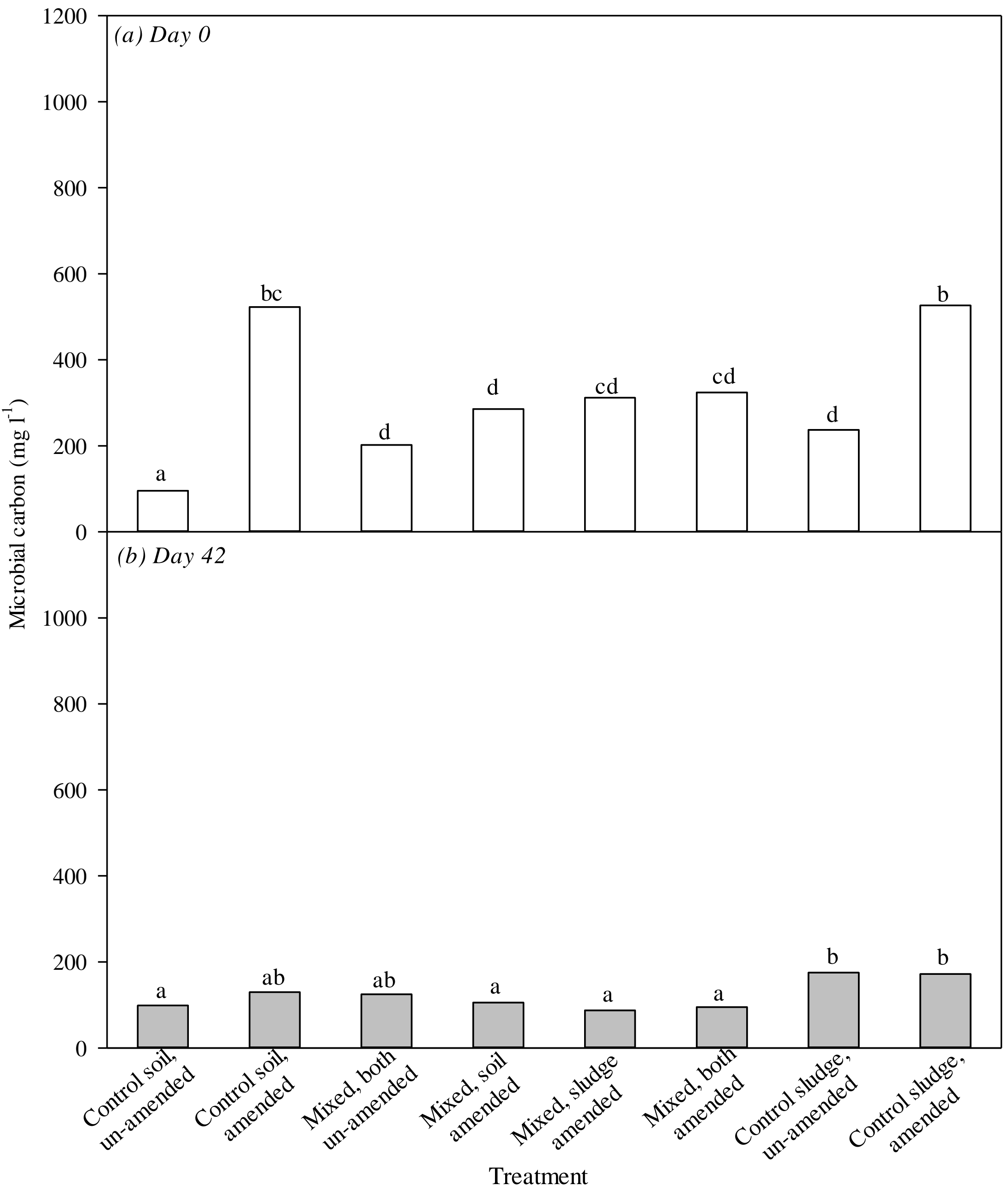

Figure 6.7. Microbial carbon content of microcosms amended with yeast, at (a) Day 0 and (b) Day 42 (n=3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.03.

6.5. Discussion

This chapter studied the response of sewage sludge-derived *E. coli* and the local microbial community to substrate amendments, when substrate was applied to sludge, soil or both. Following glucose amendment, a significant decline in sewage sludge-derived *E. coli* was observed within all amended treatments. The decline was rapid within the first week, followed by gradual attenuation across the majority of treatments. This corresponded to the rapid utilisation of glucose amongst the microbial community, exemplified by increased respiration, which presumably increased competition for other essential resources. Furthermore, the location of this substrate, i.e. whether it was added to soil, sewage sludge or both matrices, did not significantly affect survival. This discounts the theory that matrix partitioning may also play a significant part in such interactions. The high moisture content of sewage sludge, typically 70%, could have aided in cell motility or the diffusion of glucose; thus negating such an effect. Motility is affected by dispersion, filtration, adsorption/desorption, sedimentation, growth, death, and chemotaxis, as well as cell surface properties (Abu-Ashour, *et al.* 1994; Gannon, *et al.* 1991). To further distinguish between the effects of motility or diffusion, future work could consider varying the concentration of glucose amendment, which would alter the diffusion gradient. Additionally, the use of a matrix which would bind to glucose could be used, as it would cause a slower diffusion rate. Obtaining similar results for such an experiment would indicate that diffusion was not a key component. Conversely, one could consider the effects of glucose amendment on free-form microbes when compared to microbes found within/on a biofilm.

The microbial respiration was enhanced in microcosms amended with glucose when compared to un-amended treatments. This response was more immediate than with yeast extract amendment, occurring within the first week. However within one of the treatments, amended control soil, the respiratory response showed a gradual decline after an initial burst in microbial respiration. This contradicts the expected response to such amendments, where the addition of available substrate instigates a peak in microbial respiration (Anderson and Domsch, 1978). Therefore it was considered that an anomalous or missing point occurred which coincided with a higher microbial response during the first week. Overall, the increase in respiration within glucose-

amended microcosms was simultaneously mirrored by an increase in microbial carbon content. This follows the supposition that an increase in respiration is indicative of increased microbial biomass, linked to the priming effect discussed at the outset of this chapter (Anderson and Domsch, 1978).

Furthermore, the different microbial communities did potentially play a significant part in the survival of sewage sludge-derived *E. coli*. The presence of soil caused a greater die-off in sewage sludge-derived *E. coli*, suggesting interaction and competition with the soil microbial community. Die-off of sewage sludge-derived *E. coli* was also observed in the amended control sludge treatment, indicating competition from within the sewage sludge microbial community also. Additionally, the amendment of glucose to sludge instigated a greater die-off in *E. coli* than in the un-amended mixed treatment. This decline was on par with the mixed amended treatments. Finally, the un-amended mixed treatments showed marginally lower numbers than the un-amended control sludge. These findings further suggest that lack of nutrients was causing the lack of treatment effects in the previous chapters. They also suggest that competition arises both from within the local sewage sludge microbial community and from without, provided enough substrate and other resources are availabilr.

In response to these findings, the first hypothesis, which stated that the addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli* was accepted for glucose amended microcosms. In contrast the second hypothesis, which stated that the location of labile substrate would significantly affect microbial activity and the persistence of sewage sludge-derived *E. coli* was rejected.

The addition of yeast extract had little consistent effect across treatments, on the survival of *E. coli*. Lack of competition for nutrients, including, nitrogen, carbon and trace nutrients; corresponding to the slower and more varied respiratory response to yeast amendment may be one reason why the *E. coli* showed little immediate variation in decline. Additionally, respiratory response to yeast extract was slower and more varied than with the glucose amended treatments, occurring in the first two weeks. Finally, microbial carbon within yeast extract-amended microcosms followed a similar pattern to glucose amendment, with increased microbial carbon content occurring at the

onset of the experiment. In response to these findings and whilst a microbial response to amendment with yeast extract was observed, the first hypothesis, which stated that the addition of substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli* was rejected for this amendment strategy. Similarly, the second hypothesis was rejected.

The variation between amendments is most probably caused by the complexity of the substrates involved, possibly followed by responses from different sub-groups of the microbial community, i.e. fast growing r-strategists vs. slow-growing K- strategists. For example, Blagodatskaya, *et al.* (2009), studied the growth rate and substrate affinity constant of soil microorganisms in a loamy soil, under simple or complex organic amendments. They found that adding readily available substrate (glucose or root exudates) stimulated r-strategists, whilst the addition of less available substrate (plant residues) favoured k-strategists. These findings have implications in regards to the survival of sewage sludge-derived *E. coli*. Depending on the form of substrate added, r-strategists would provide a more immediate form of competition, possibly preventing the sewage sludge-derived *E. coli* from gaining a foothold in the soil environment, whilst k-strategists would perhaps provide a more long term form of competition preventing the *E. coli* from re-establishing or proliferating. Thus it could be theorised within the context of this work, that amendments with glucose stimulated the r-strategists leading to the notable decline in sewage sludge-derived *E. coli*. Similarly, it could be theorised that amendments with yeast extract stimulated the k-strategists thus eliciting the slow microbial response and minimal effects on the survival of sewage sludge-derived *E. coli*.

6.5.1. Conclusion

At the onset of this project, it was assumed that sewage sludge contained enough carbon and other nutrients to instigate an increase in microbial activity, alternately referred to as a hotspot of activity, when applied to soil. This increase in microbial activity was theorised to cause a decline in pathogens, co-introduced with the sewage

sludge. However, previous work found no correlation between sewage sludge loading rates and the decline of such pathogens. This chapter further supports this supposition, as the addition of simple substrate, glucose, instigated greater die-off than in un-amended treatments. However, this was not the case with the amendment of more complex substrate yeast extract. Relating back to the original concept belying this work, the effect that glucose amendment had on the soil and/or sewage sludge, could be viewed as a hotspot though a artificially created hotspot.

It is apparent from this work, that enteric pathogens, such as sewage sludge-derived *E. coli*, compete with the soil and/or sewage sludge microbial communities for available resources when introduced to soil via the application of sewage sludge. Though the extent of these interactions is dependent upon physicochemical parameters, such as substrate availability, with simpler substrates (glucose) causing a greater decline in *E. coli* concentrations in comparison to more complex substrates (yeast extract). However, there was no observable partitioning effect, disproving that aspect of the original concept. Finally, it is equally important to consider their survival and biological interactions within the confines of a physicochemical framework, viewing the system in a holistic manner rather than independent parts. This work attempted to accomplish this by incorporating the amendment of sewage sludge to soil, rather than simply inoculating pathogens directly into soil. However, whilst this work attempted to follow this rationale, more could be done to improve upon this design, for example, incorporating plants or crops used in agricultural systems.

To further clarify the persistence of pathogens in soil, investigation into physicochemical parameters, in relation to sewage sludge application and pathogen-microbial community interactions would be of use. Future work could consider how continuous or repeated application of substrate could influence pathogen-microbial community interactions. This would mimic the continuous flux of nutrients in natural systems, for example the presence of plants/roots and the continuous input of root exudates. Shifts in microbial community dynamics could also be monitored using molecular tools, to identify key groups or interactions. These could then be further utilised to instigate more rapid die-off of pathogens.

7. The effect of repeated, supplementary nutrients on pathogens survival in soil, co-introduced with sewage sludge

7.1. Introduction

Previous work (Chapter 6) found that the addition of simple substrate, in this instance glucose, accelerated the decline of sewage sludge-derived *E. coli*. However, amending the soil and sewage sludge microcosms with yeast extract had little effect on survival. When comparing these two findings it suggests that a lack of resources, other than carbon, is a key issue in survival with competition for these limited resources potentially leading to the decline of *E. coli*. The greater survival of *E. coli* in treatments amended with yeast extract supports this supposition, as yeast extract contains a greater complexity of resources, including carbon, nitrogen, minor elements and complex organics. Furthermore, it was also found that the phase to which the substrate was added did not affect persistence. Within the confines of this work, this suggests that there was no form of partitioning effect occurring between the soil and sewage sludge matrices. Therefore, this Chapter will seek to further elucidate the impact of substrate addition on the survival of sewage sludge-derived *E. coli* and general coliform bacteria, whilst discontinuing exploration of partition effects between the soil and sewage sludge phases.

The previous Chapter discussed the input and subsequent impact of substrate in terms of the priming effect, where the input of readily available substrate can lead to a short term increase in microbial activity which greater turnover of carbon and other nutrient sources (Kuzyakov, *et al*. 2000). This increase in microbial activity could then cause an increase in competition for resources, which relates back to the original concept of this work, which discusses hotspots of microbial activity and their effect on pathogen survival in soil, when they are co-introduced with sewage sludge. Kuzyakov, (2010), further develops the work done on priming effects by ascribing two forms of input which are related to the relative availability of the substrate being utilised; (i) pulse and (ii) continuous. The former relates to singular amendments of substrate, whilst the latter refers to substrate which is decomposed at a slower rate. Kuzyakov, (2010), also goes on to further discuss the possibility of single-pulse inputs and repeated-pulse inputs. The single-pulse inputs of carbon leading to an increase in

microbial activity. The periods of increased microbial activity after a single-pulse of substrate normally last for a few days (Nottingham, *et al.* 2009). In contrast, the repeated application of such inputs would presumably intensify or extend the period of microbial activity. The majority of priming effect studies focus on these single-pulse responses, whilst only a few focus on repeated pulses.

Hamer and Marschner, (2005), studied the effects of repeated substrate addition on the pool of soil organic carbon (SOC), which is normally the most susceptible to priming effects, in two forest soils. They found that repeatedly adding substrates (^{14}C-labelled substances d-fructose, l-alanine, oxalic acid and catechol) induced higher positive priming effects than single amendments. In this instance a 'positive priming effect' refers to acceleration in SOC mineralisation and a simultaneous increase in microbial activity and/or biomass. Similarly, Sørensen, (1974), studied the repeated addition of readily decomposable organic matter on the rate of decomposition of labelled soil organic matter (SOM). It was found that the addition of the OM increased CO_2 evolution and decomposition of labelled SOM. In contrast, Chigineva, *et al.* (2009), studied the repeated addition of labile carbon and the presence of ectomycorrhizal roots on the rate of leaf litter decomposition and on the composition of fungal communities. They found that the addition of labile carbon decreased decomposition rates whilst increasing the total micro-fungal CFU density and overall microbial biomass; an example of a negative priming effect. In general, these studies found that repeated addition induced greater priming effects, or increased microbial activity. Examining this phenomenon in relation to the original hot spot theory and its effect on pathogen survival would be of interest, as the soil system has a continual flux of resources which the repeated application of substrate would mimic.

An experiment was subsequently devised which examined the repeated addition of substrate and its effect on the survival of sewage sludge-derived *E. coli* and general coliform bacteria. It aimed to highlight the impact of sustained competition for resources on persistence, whilst mimicking the recurrent input of carbon which occurs in plant/soil systems; after an initial input of sewage sludge. It was hypothesised that increased and sustained microbial activity, via the repeated application of substrate (glucose), would cause a significant and sustained decline in sewage sludge-derived *E.*

coli and indigenous sewage sludge coliform bacteria. In response to this hypothesis, a microcosm-based study was implemented where glucose was added, with microbial activity and *E. coli/*general coliform numbers quantified periodically. After the initial substrate addition, analysis was performed to assess microbial carbon with subsequent analyses focusing on respiration and *E. coli*/general coliform colony counts. Microbial carbon was also analysed at the end of the experiment.

7.2. Aims, objectives and hypotheses

7.2.1. Aim

This work aimed to clarify the effect of repeated supplementary nutrients on the survival of sewage sludge-derived *E. coli* and general coliforms.

7.2.2. Objectives

- Elucidate how repeated input of supplementary nutrients will affect the survival of sewage sludge-derived *E. coli* and general coliforms.
    - By repeatedly adding supplementary nutrients, glucose, over 105 days to promote microbial activity in soil and sewage sludge microcosms, containing sewage sludge-derived *E. coli* and general coliforms.

7.2.3. Hypotheses

H_1 The repeated addition of supplementary nutrients to soil/sewage sludge microcosms will significantly increase microbial activity, resulting in a significant decline in the persistence of sewage sludge-derived *E. coli*.

H_2 The repeated addition of supplementary nutrients to soil/sewage sludge microcosms will significantly increase microbial activity, resulting in a significant decline in the persistence of general coliforms.

7.3. Materials and methods

7.3.1. Background analysis

Composite samples of soil and sewage sludge were used for these experiments, as outlined in Chapter 3.2.1 and 3.2.2. The sewage sludge was manually crumbled to an aggregate size of approximately 0.5 cm to ensure homogeneity. Its moisture content was unaltered. Physicochemical and biological analyses were also assessed using the protocols referenced in Chapter 3.2.3.

7.3.2. Treatment preparation

A set of microcosms were established containing samples of sewage sludge and soil, inoculated with *E. coli* and amended with glucose (Table 7.1; Figure 7.1). The sewage sludge already contained relatively high concentrations of indigenous sewage sludge coliform bacteria (see Section 7.4.1). Initially, soil and sewage sludge were weighed out separately into sterile 100 ml, plastic, screw-cap containers. Isolates of sewage sludge-derived *E. coli* were then grown in LB broth, washed with MRD and subsequently inoculated into all containers comprising sewage sludge, as outlined in Chapter 3.3.3. The containers associated with the mixed treatments were then combined; with the soil poured into the containers holding the sewage sludge. This minimised the potential loss of inoculated *E. coli* through transfer. Glucose (5000 mg C kg^{-1}), diluted (1:5 ratio by mass) with quartz sand (particle size: 210 - 297 µm), was then added to each set of containers associated with amended treatments as outlined in Table 7.1. Pure quartz sand of equivalent weight was then added to the containers associated with un-amended treatments/matrices, to ensure that all microcosms underwent the same form of disturbance. The matrices and amendments were then mixed by hand, using end-over-end rotation for 60 seconds. Glucose was added on two further occasions, following this same protocol with intervals of 14 days over a 28 day period. The microcosms were then incubated at 10°C over a 105 day period. Further description of the amendment protocol can be found in Chapter 3.4. The time points for glucose addition are also outlined in Table 7.2.

Table 7.1. Treatment formulations (n = 3), with equivalent dry weight (± 0.02 g) of sewage sludge and soil.

No.	Treatment	Contents	Substrate	Soil (g)	Sewage sludge (g)
1	Control	100% soil	Un-amended	10	0
2	Control	100% soil	Amended	10	0
3	Mixture	70% soil to 30% sludge	Un-amended	7	3
4	Mixture	70% soil to 30% sludge	Amended	7	3
5	Control	100% sludge	Un-amended	0	10
6	Control	100% sludge	Amended	0	10

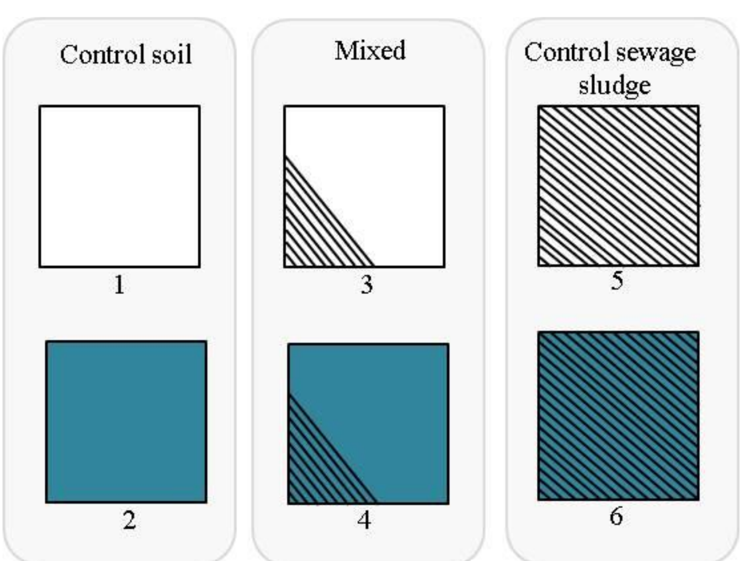

Figure 7.1. Treatment outline, detailing amendment strategy. Key: soil; sludge; amended with substrate. Sewage sludge-derived *E. coli* was present within sludge in this instance.

7.3.3. Analysis of microbial activity and survival of sewage sludge-derived *E. coli*

At each time point, (Table 7.2), one full set of treatments (n = 3) was removed from the incubator (10°C). Head-space samples were then taken to assess evolved CO_2, followed by the extraction and enumeration of sewage sludge-derived *E. coli* and coliform bacteria; as outlined in Chapter 3.3.3, 3.3.5 and 3.4. Where coliform numbers

exceeded the countable range of the dilution series filtered and subsequently plated onto MLGA, estimates were taken by using the greatest dilution factor as an indicator. Microbial biomass was also assessed at the beginning (Day 0); 18 hours after substrate addition, and end (Day 105) of the experiment.

Table 7.2. Time points, amendment regime and respective analyses

Days	Analysis		
	Respiration	**Enumeration**	**Microbial carbon**
	(i) Glucose amendment (-18hrs)		
0	×	×	×
1	×	×	
3	×	×	
7	×	×	
	(ii) Glucose amendment (-18hrs)		
14	×	×	
15	×	×	
17	×	×	
21	×	×	
	(iii) Glucose amendment (-18hrs)		
28	×	×	
29	×	×	
31	×	×	
35	×	×	
42	×	×	
49	×	×	
75	×	×	
105	×	×	×

7.3.4. Statistical analysis

The resulting cell count data was analysed using a two-way factorial ANOVA in Statistica, version 11 for Windows (Stats Soft, 1984 – 2015), using treatment and time as independent variables. A one-way ANOVA was also implemented to test the significance of treatment effects within each time point. The respiration data was also assessed by using a two-way factorial ANOVA, after the data was log transformed to normalise it. However, a one way ANOVA was not performed on this data set due to

the removal of anomalous points. The microbial carbon was analysed using two, one-way ANOVAs to test the significance of individual treatment effects between time points, and to test for significant differences between treatments at a given time point.

7.4. Results

7.4.1. Background analysis

Physicochemical and biological characteristics for both sewage sludge and soil are provided in Tables 7.3, 7.4 and 7.5. The soil had a pH of 6.53, with a moisture content of 17.9%. In comparison, sewage sludge exhibited a pH of 7.56 and a typical moisture content of 75.7%. Nitrate concentrations were similar to ammonia levels within the soil. The nitrate concentration within the sewage sludge was also similar to that of the ammonia. Macro and micro nutrients were also found within soil, with minerals ranging from; phosphorus, potassium and zinc, as well as aluminium and relatively high levels of sulphur. The sewage sludge contained smaller quantities of these minerals. *E. coli* and general coliform concentrations were relatively high in the sewage sludge; 6.50 x 10^{+6} CFU gDS^{-1} and 1.83 x 10^{+8} CFU gDS^{-1} respectively, whilst the soil did not contain such microorganisms in detectable numbers.

Table 7.3. Physicochemical characteristics of soil (mean ± SEM, n = 3).

Analysis	Soil		
Total Exchange Capacity (MEQ 100g $^{-1}$)	10.6	±	4.69
Moisture content (%)	17.9	±	0.03
Organic matter (loss on ignition, %)	5.82	±	0.19
Bray I phosphorus (mg l^{-1})	62.3	±	0.41
Nitrogen (mg l^{-1})			
Total	3.1	±	0.09
Nitrate (NO_3)	10.7	±	0.33
Ammonium (NH_4)	6.07	±	0.29
Carbon (mg l^{-1})			
Total	23.2	±	0.83
Organic	110	±	2.29
Mehlich III Extractable (mg l^{-1})			
Phosphorus (P)	60.7	±	0.25
Manganese (Mn)	115	±	0.77
Zinc (Zn)	3.23	±	0.11
Boron (B)	0.57	±	0.03
Copper (Cu)	7.73	±	0.22
Iron (Fe)	387	±	0.85
Aluminium (Al)	760	±	1.21
Calcium (Ca)	48	±	0
Magnesium (Mg)	57.3	±	0.41
Potassium (K)	37	±	0.33
Sodium (Na)	37	±	0.33

Table 7.4. Physicochemical characteristics of sewage sludge (mean ± SEM, n = 3).

Analysis	Sewage sludge (Fresh weight)		
pH (1:1 in H_2O)	7.76	±	0.07
Total solids (g kg^{-1})	251	±	0.34
Moisture content (%)	75.7	±	0.05
Total Phosphorus (g kg^{-1})	5.84	±	0.09
Phosphorus as P_2O_5 (g kg^{-1})	13.5	±	0.13
Potassium (g kg^{-1})	0.54	±	0.04
Potassium as K_2O (g kg^{-1})	0.65	±	0.04
Nitrogen *(g kg^{-1})*			
Total Kjeldahl	10.86	±	0.24
Organic	9.89	±	0.26
Nitrate (NO_3)	>1.00	±	0
Ammonia (NH_4)	0.97	±	0.1
Carbon (mg l^{-1})			
Total	200	±	0.65
Organic	533	±	20.3
Potentially Toxic Element (mg kg^{-1})			
Arsenic (As)	2.55	±	0.1
Cadmium (Cd)	0.46	±	0.05
Chromium (Cr)	263	±	7.01
Copper (Cu)	72.1	±	0.36
Lead (Pb)	28.9	±	0.15
Mercury (Hg)	>0.01	±	0
Molybdenum (Mo)	2.71	±	0.1
Nickel (Ni)	35.2	±	0.24
Selenium (Se)	>0.58	±	0.03
Zinc (Zn)	172	±	0.15

Table 7.5. Biological characteristics of both soil and sewage sludge (mean ± SEM, n = 3).

Analysis	Soil (CFU gDS^{-1})			Sewage sludge (CFU gDS^{-1})		
E. coli	0.00	±	0.00	$6.50 \times 10^{+6}$	±	$7.64 \times 10^{+5}$
General coliforms	0.00	±	0.00	$1.83 \times 10^{+8}$	±	$3.33 \times 10^{+7}$

7.4.2. Survival of sewage sludge-derived *E. coli*

Both of the control soil treatments did not contain detectable concentrations of *E. coli* or general coliforms throughout the duration of the experiment. They were, therefore, omitted from Figures 7.2, as well as from further statistical analysis. A two-way factorial ANOVA of *E. coli* concentrations in the remaining treatments showed highly significant main effects and glucose-treatment by time interactions ($p<0.01$; Figure 7.2).

Initial concentrations of *E. coli* in un-amended, control sludge were approximately 1.0 x 10^{+7} CFU gDS^{-1} (Figure 7.2a). This declined gradually over a period of 105 days to 1.0 x 10^{+2} CFU gDS^{-1}, with slightly sharper declines after the second and third sand amendments. In comparison, the repeated addition of glucose to sludge did not instigate a consistently significant decline in *E. coli* concentrations ($p<0.02$; Figure 7.2c). Following the first glucose amendment to sludge, *E. coli* concentrations declined slightly, similar to the pattern of decline observed in the Chapter 6 (Figure 7.2c). After the second amendment, *E. coli* concentrations increased above those levels found within the un-amended, control sludge treatment ($p<0.001$). After the third amendment of glucose, *E. coli* concentrations declined significantly on Days 28 and 29 ($p<0.001$) but increased thereafter, maintaining higher levels than the un-amended control sludge, with significant differences occurring on Days 42 and 105 ($p<0.001$).

Similar to the amended control sludge treatment, *E. coli* concentrations within the amended, mixed treatment declined after the first glucose amendment. This decline was significantly lower when compared to its un-amended counterpart ($p<0.001$, Figure 7.2b). After the second glucose amendment to the amended, mixed treatment, *E. coli* concentrations were significantly higher on Days 15 and 17, in comparison to the un-amended, mixed treatment. Following the third glucose amendment to the amended, mixed treatment, *E. coli* concentrations significantly declined on Days 28 and 29 ($p<0.001$). Again, similar to the pattern observed in the amended control sludge treatment. The *E. coli* concentrations subsequently peaked on Day 35 but were not significantly different from the un-amended, mixed treatment. From Day 42, onwards, *E. coli* concentrations were not significantly different from each other.

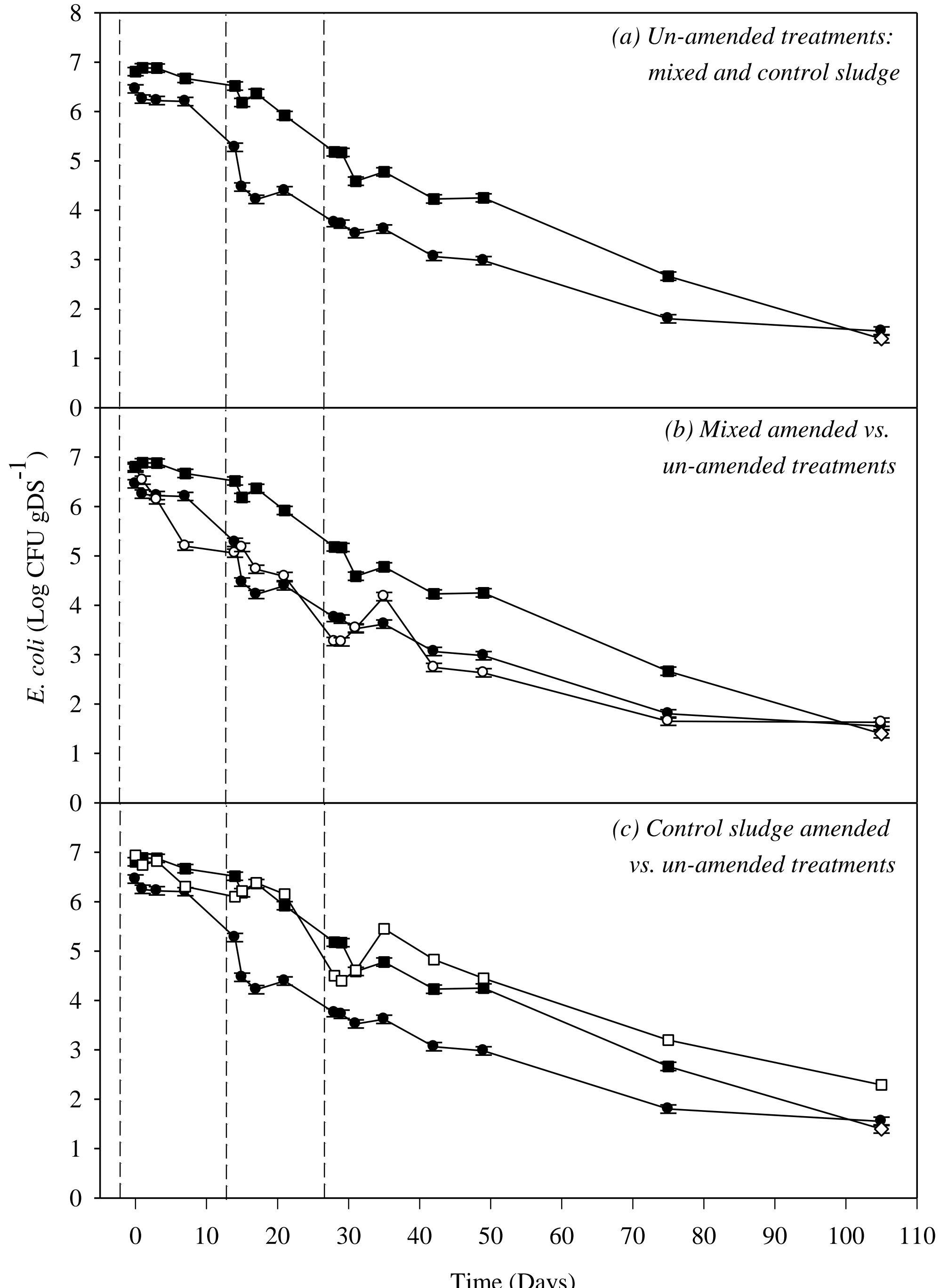

Figure 7.2. Survival of sewage sludge-derived *E. coli* in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended treatments: mixed and control sludge; (b) Amended mixed vs. un-amended treatments; (c) Amended control sludge vs. un-amended treatments (n=3, mean ± pooled SE). Key: —●— mixture, un-amended; —○— mixture, amended; —■— control sludge, un-amended; —□— control sludge, amended; – – – represents amendment points.

7.4.3. Survival of general coliforms

Concentrations of general coliforms within both control soil treatments were below detectable limits throughout the duration of the experiment. Thus both these treatments were omitted from Figure 7.3 and further statistical analysis. A two-way factorial ANOVA of general coliform concentrations in the remaining treatments showed highly significant main effects and repeated glucose-treatment by time interactions ($p<0.01$; Figure 7.3). Both days 35 and 42 are estimates of general coliforms for the un-amended control sludge, as concentrations exceeded the countable range of the dilution range analysed. Similarly, Days 29 to 42 are estimates of general coliforms for the amended mixed treatment, as concentrations exceeded the countable range of the dilution range analysed.

Initial concentrations of general coliforms in un-amended, control sludge were approximately $1.0 \times 10^{+8}$ CFU gDS^{-1} (Figure 7.3a). This declined gradually to approximately $1.5 \times 10^{+1}$ CFU gDS^{-1}, with varying responses to amendments with sand. Similarly, the repeated addition of glucose to control sludge produced a varied response from the general coliform community (Figure 7.3c). Following Day 14 and the second amendment of glucose, general coliform concentrations were significantly increased when compared to its un-amended counterpart ($p<0.001$). After the third amendment, general coliforms concentrations declined to levels that were not significantly different from un-amended control sludge on Day 28. This was followed by an increase in coliform concentrations in both control sludge treatments. After this, an overall decline was observed from Day 49 onwards, with no significant differences occurring between coliform concentrations in amended control sludge and un-amended control sludge.

General coliform concentrations within the mixed amended treatment declined after the first glucose amendment when compared to its un-amended counterpart ($p<0.001$, Figure 7.3b). Following this, a significant increase in concentrations was also observed on Day 3, again when compared to its un-amended counterpart ($p<0.001$). After the second glucose amendment, general coliform concentrations were significantly higher within amended mixed treatment between Days 14 and 17, in comparison to the un-amended mixed treatment ($p<0.001$). These concentrations increased to such an extent that they were similar to the un-amended control sludge

treatment on Day 14. Following the third glucose amendment, general coliform estimates suggest a period of equilibrium within the un-amended mixed treatment. However, it is more likely that general coliform concentrations fluctuated above the detection limits for the range of dilutions analysed. Between Day 49 and 75, coliform concentrations increased slightly in the amended mixed treatment. These concentrations were sustained up to Day 105, where all other treatments were significantly lower than the amended mixed treatment ($p<0.001$).

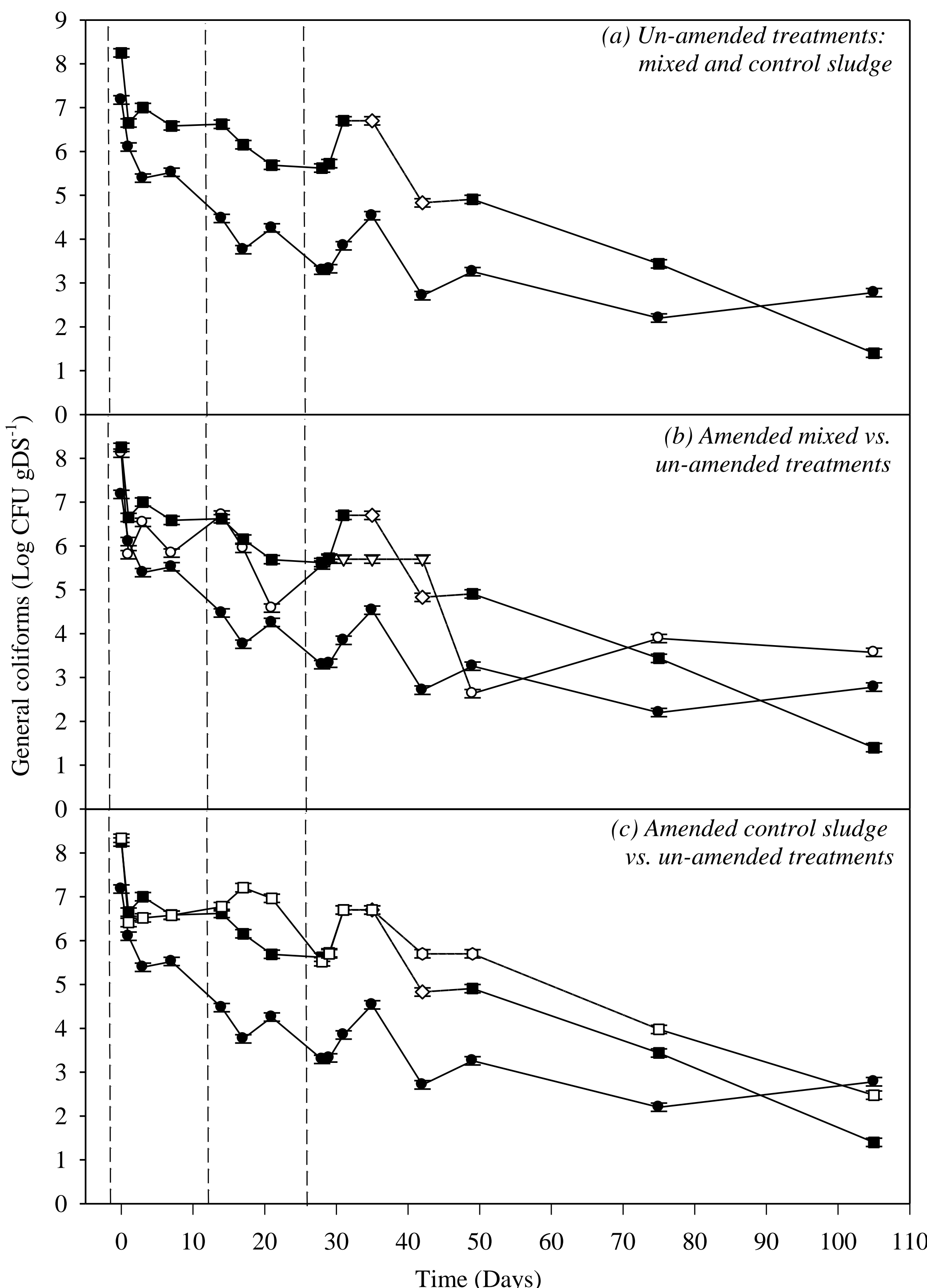

Figure 7.3. Survival of general coliforms in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended treatments: mixed and control sludge; (b) Amended mixed vs. un-amended treatments; (c) Amended control sludge vs. un-amended treatments (n=3, mean ± pooled SE). Key: —●— mixture, un-amended; —○— mixture, amended; —■— control sludge, un-amended; —□— control sludge, amended. Estimate key: ┈◇┈ control sludge, un-amended; ┈▽┈ mixture, amended; ┈⬡┈ control sludge, amended; ▬ ▬ ▬ represents amendment points. *Day 15 omitted as there was no data collected

7.4.4. Respiration

Both control soil treatments, amended and un-amended with glucose, produced detectable but low levels of CO_2 and thus were included in further analyses. Variable peaks in respiration were also observed after each glucose amendment across all amended treatments. Some relatively small responses were also observed in control treatments where sand was added. However, some of these peaks, within the amended mixture treatment and both control sludge treatments were excessively high (by orders-of-magnitude) in correspondence to their surrounding points. They also did not occur close to amendment points, and thus cannot be reconciled as being induced by glucose since it would induce respiration more rapidly. Therefore, these points; Day 42 for the amended mixture, Days 42 and 49 for amended control sludge and Day 49 for un-amended control sludge, were excluded from analysis and the figures below. The absence of these anomalous points within the figures is indicated using arrows (↓).

From the remaining data, a two-way factorial ANOVA of CO_2 respiration confirmed significant main effects and glucose-treatment by time interactions (Figure 7.4). Generally, the rate of respiration increased with each glucose amendment and was commensurate to treatment type. For example, amended control soil highest peak of 0.071 log mg CO_2 g^{-1} h^{-1} in CO_2 after the third application (Figure 7.4a). In contrast, the amended mixed treatment, showed a significantly high peak of 0.55 log mg CO_2 g^{-1} h^{-1} after the second glucose (Figure 7.4b). The amended control sludge also exhibited a peak in respiration following the second amendment, with 0.38 log mg CO_2 g^{-1} h^{-1} (Figure 7.4c).

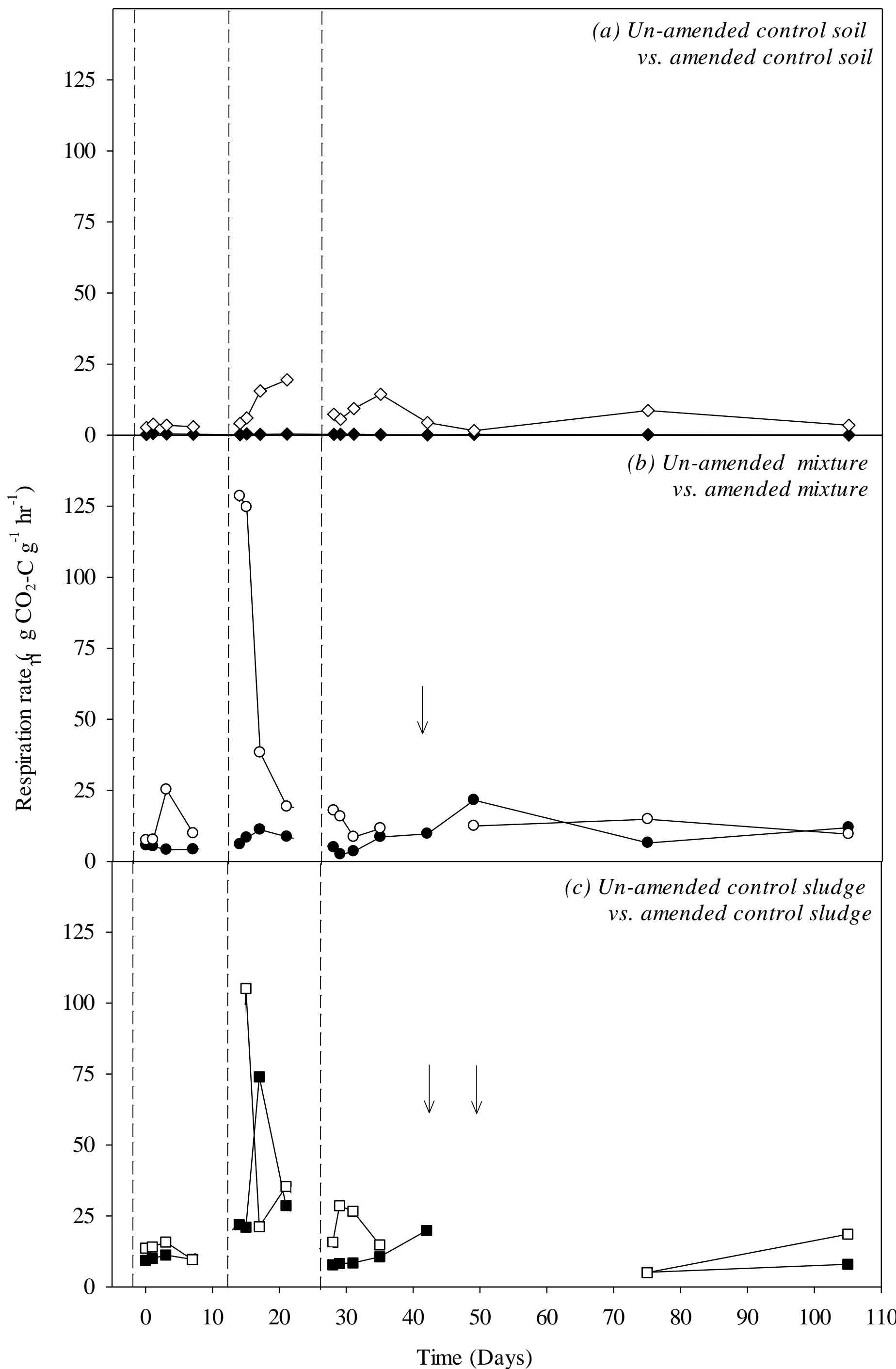

Figure 7.4. Respiration in soil/sludge microcosms, repeatedly amended with glucose; (a) Un-amended control soil vs. amended control soil; (b) Un-amended mixture vs. amended mixture; (c) Un-amended control sludge vs. amended control sludge (n=3, mean). Key: —◆— control soil, un-amended; —◇— control soil, amended; —●— mixture, un-amended; —○— mixture, amended; —■— control sludge, un-amended; —□— control sludge, amended; ↓ represents removed anomalous points; ▬ ▬ ▬ represents amendment points. Pooled standard error of log transformed data: 0.00.

7.4.5. Microbial carbon

Using a one-way factorial ANOVA, microbial carbon was shown to be significantly higher on Day 0, when compared to Day 42, for un-amended mixed, un-amended control sludge and amended control sludge treatments ($p<0.05$, Figure 7.8). The remaining treatments (both control soils and amended mixed treatments) did not significantly differ between days. On Day 0, (Figure 7.8a), microbial carbon within un-amended control soil was significantly lower than amended mixed and amended control sludge treatments ($p<0.02$). All of the other treatments did not differ significantly. By Day 105, (Figure 7.8b), the majority of treatments had attenuated to similar levels, apart from the amended mixed treatment which contained significantly greater microbial carbon levels than the two control sludge treatments ($p>0.02$) .

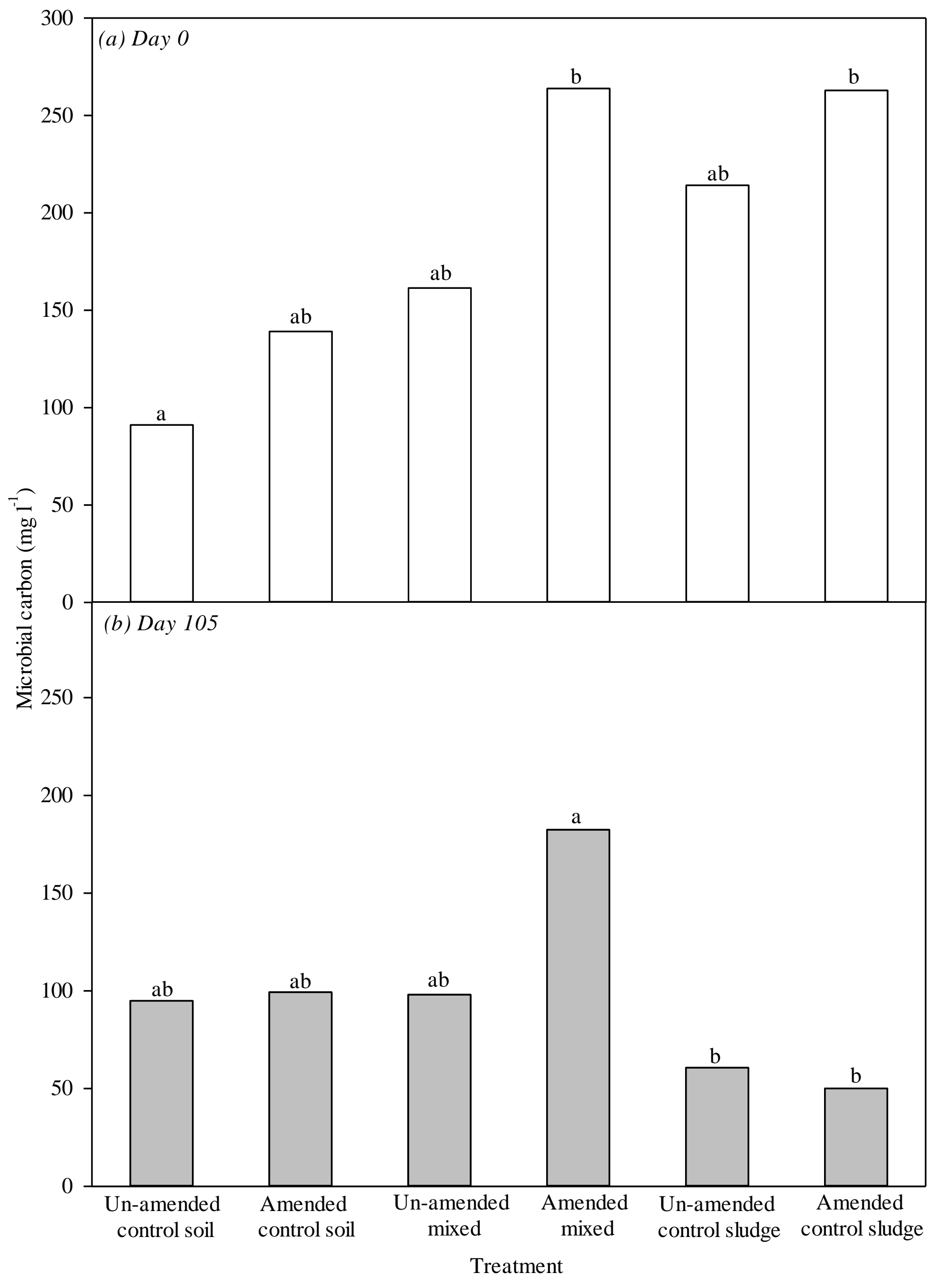

Figure 7.5. Microbial carbon content of soil/sludge microcosms, repeatedly amended with glucose, at (a) Day 0 and (b) Day 105 (n=3, mean). Letters denote homogeneous means at 5% significance level. Pooled standard error of log transformed data: 0.08.

7.5. Discussion

Previous work found that sewage sludge-derived *E. coli* declined with the single addition of glucose. It was postulated that this was due to competition for other essential resources. Upon further examination of the priming effect literature, a subset of work was found that discussed and examined the repeated application of substrate. These studies generally found that repeated addition induced greater priming effects, or increased microbial activity. It was considered that such a phenomenon would be pertinent to the original hot spot theory which instigated this project. Rather than looking at a short burst in microbial activity and its effect on pathogen survival, how would sustained or repeated activity influence the survival of enteric pathogens? However, there was the possibility that the repeated addition of substrate could further sustain enteric pathogens. Considering that under natural conditions the soil environment can provide a continual flux of resources, especially in the presence of plants or substrate amendments, understanding whether repeated substrate addition would be likely to help or hinder enteric pathogens is an important distinction to make. This is especially true in regards to the continual input of rhizo-deposits and in agricultural systems where fertiliser and organic amendments are regularly/repeatedly applied.

Overall, microbial activity was enhanced in microcosms amended with glucose. The increases in respired CO_2 observed after each glucose amendment corresponds to the findings of previous work; where increases in microbial activity was observed after repeated amendments (Sørensen, 1974; Hamer and Marschner, 2005; Chigineva, *et al.* 2009). This generally supports the original premise of the work requiring an increase in microbial activity to instigate competition for resources. The levels of microbial carbon were also enhanced in treatments amended with glucose, as well as within treatments containing sewage sludge.

The first hypothesis stated that the repeated addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli*. This hypothesis was rejected, as the repeated amendments did not cause a significant sustained decline in *E. coli* numbers. Initially, the first addition of glucose caused a

significant decline in *E. coli* concentrations in comparison to its un-amended counterpart. Mirroring the same effect as was observed in the previous experiment (Chapter 6). Subsequent glucose amendments, however, caused the opposite effect, with *E. coli* numbers increasing slightly. These increases were then followed by more gradual declines, than after the addition of the first glucose amendment. This could have occurred due to shifts in the microbial community, from more rapid responders to slower. To prove such a concept, future work could follow a similar experimental protocol whilst also considering the use of a combination of substrates, similar to those seen in Hamer and Marschner, (2005), that would favouring either r strategists or k strategists combined with profiling the microbial community. Furthermore, Griffiths, *et al.* (1999), found that microbial community structure changed consistently as substrate (a synthetic root-exudate analogue) loading increased, with fungi becoming more predominant than bacteria at higher loading rates. Similarly, Baudoin, *et al.* (2003), found that artificial root exudates (glucose, fructose, saccharose, citric acid, lactic acid, succinic acid, alanine, serine and glutamic acid) significantly increased bacterial densities whilst changing the metabolic fingerprint of the respective communities. They also found that the overall genetic structure of the community was consistently modified. Alternately, it is possible that the interval between amendments allowed the *E. coli* to become better established or more acclimatised to their changed environment. If this did occur, it may have made them more capable of utilising subsequent substrate inputs. Or perhaps the successive addition of glucose allowed the sludge microbial community to become more predominant. Finally, soil also appeared to cause greater die-off of *E. coli* numbers in comparison to the sludge control treatments; further suggesting that interactions with the soil microbial community do cause greater die-off of *E. coli*.

In contrast, general coliform concentrations showed a relatively greater variation in survival over time. However, there was an overall decline in general coliforms, with the survival in control sludge and mixed treatments being relatively similar despite the variation. This pattern appeared to be greatly affected by the amendment strategies, whether the treatments were amended with glucose or not. Therefore, the hypothesis postulating that the repeated addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the

persistence of general coliforms, was rejected. Overall, the high variation in the data set is attributed to the nature of the data collection, as several species are included within the general coliform count. This fluctuation may exemplify why such generalised counts are not useful as indicators of die-off, as specific species may or will have different responses to given conditions. Specific enumeration of species would give more accurate information in regards to their survival and their potential risk for transfer and infection. These apparent shifts could also indicate the influence the repeated substrate amendments were having on the overall structure of the community.

The sewage sludge always had a greater respiration rate than the soil. This is possibly due to the differences in composition, whilst sewage sludge will have a high organic carbon content, soil will not. Furthermore, the lack of consistent moisture content could contribute to such differences. This lack of consistent moisture attenuation was discussed in the Chapter 3 and was accepted because it was necessary to keep the microcosms as close to 'reality' as possible. Especially in relation to the length of time the experiments are run. What is important in terms of significant relationships is the dynamics between amended and un-amended counterparts, as well as with spikes/changes over time within treatments (which signify changes in microbial activity).

Whilst the respiratory response to glucose amendment was more immediate after the first and second additions, certain peaks in respiration after the third amendment of glucose were delayed and far larger than each system could logically produce within certain treatments. Therefore these points, indicated in the Section 7.4.4, were considered anomalous and were removed. To ascertain whether these peaks did actually occur and were not a fault within the sampling or analysis protocol, a repeat of the experiment would be required. It should be noted that all equipment and operating protocols were checked repeatedly for issues and none were found. The fact that other samples, as well as standards and quality controls, taken on the same days were acceptable also supports the need to re-run this aspect of the experiment. It would be interesting if these peaks occurred again as this would indicate some form of lag phase with repeated addition of substrate. Finally, the increase in respiration within glucose-amended microcosms following the first application was simultaneously mirrored by an

increase in microbial carbon content across the majority of treatments. Future work could incorporate several microbial carbon readings, to determine the size of the microbial biomass following each pulse of substrate addition

7.5.1. Conclusion

From the above findings, the repeated addition of substrate could, in contrast to earlier hypotheses, promote the persistence of pathogens in pure sewage sludge. It also highlights the importance of considering pathogen survival in an appropriate context, with greater focus required on long-term inputs that are environmentally relevant. To further build on this work, a greater range of treatments including those that are amended once or twice would be of interest. Shorter or longer periods between amendments or use of simpler/complex substrates would also be of interest. These variables, in combination with identification of community turnover through use of molecular techniques, would clarify shifts in microbial composition and how they influence pathogen survival. Furthermore, interactions between sewage sludge/soil/rhizosphere communities and the density of roots would also be interesting to investigate.

8. Discussion

Approximately 10.13 million tons of sewage sludge is produced in Europe annually (RPA, *et al.* 2008). Additionally, the UK produced approximately 1.4 million tons of sewage sludge as of 2008, whilst Ireland produced 42.1 thousand tons as of 2003 (RPA, *et al.* 2008; Water UK, 2010). This sewage sludge is a potentially valuable resource that can enhance both the structure and fertility of soil though due to its hazardous composition and the quantity produced annually, its management is one of the most difficult and expensive issues surrounding the treatment of wastewater (Metcalf and Eddy, 1991). Its re-use as an agricultural fertiliser is perhaps one of the most cost effective and sustainable modes of handling this wastewater by-product (Grey, 2004). However, it can also harbour enteric pathogens. These enteric pathogens can be co-introduced into the soil when sewage sludge is applied as a fertiliser. From here, such pathogens can potentially infect individuals through contact with contaminated water, produce, soil particles or from direct contact (Buchholz, *et al*, 2011; Davis, *et al*, 2005; Hrudey, *et al*, 2003). Therefore it is important to understand the factors that govern the persistence of such pathogens in soil, when co-introduced with sewage sludge, in order to mitigate risk and to further avail of such a valuable resource. This research, then, aimed to elucidate our understanding of the mechanisms which affect the persistence of enteric pathogens in soil, when co-introduced with sewage sludge. It was postulated that the input of organic carbon and nutrients, associated with sewage sludge applied to land, could instigate hot spots of microbial activity leading to a decline in enteric pathogens co-introduced with sewage sludge due to the potential for competition for remaining resources

A 'hot spot' can arise from the accumulation of organic matter, addition of fertiliser, within the rhizosphere in association with root exudates, or due to a disturbance (such as tilling) which would make previously unavailable substrate duly available (Parkin, 1987; Petersen, *et al.* 1996; Griffiths, 1994). It is a zone of localised and increased microbial activity that is above the baseline for that environment prior to disturbance or amendment with substrate. Hot spots are generally small, spatially isolated niches with increased biological activity (Bundt, *et al.* 2001). They can be viewed as synonymous with priming effects, though priming effect studies tend to focus

on the impact of increased microbial activity after the initial addition of substrate and how it affects the degradation of local resources rather than biological interactions (Kuzyakov, *et al.* 2000). For example, Henderson, *et al.* (2010) found that denitrification and respiration increased in soil independently amended with plant residues (red clover, soybean or barley straw) and glucose compared to un-amended soil. Additionally, priming effects have also been observed in the rhizosphere, with live roots significantly controlling SOM decomposition leading to elevated decomposition (Cheng, *et al.* 2003). Furthermore, Fontaine, *et al.* (2003) postulated that competition between microorganisms for energy and nutrient acquisitions was another factor involved in the priming effect. Similarly, Blagodatskaya and Kuzyakov, (2008) theorised that the addition of substrate could stimulate the growth of r strategists, the most active part of the microbial community. They then went on to demonstrate shifts in functional properties of soil microorganisms after substrate addition, with the input of readily available substrate (glucose or root exudates) stimulated r- strategists whilst the addition of less available substrate (plant residues) stimulated slow-growing k-strategists (Blagodatskaya, *et al.* 2009).

Additionally, a large number of studies focus on the amendment of less complex substrates and whilst they are significant in further advancing concepts as well as identifying key factors that should be considered, it can be difficult to apply them to "real world" conditions. Furthermore, the potentially unsavoury connotations associated with the use of treated sewage sludge in agriculture could impact upon the formation of future legislation and could further impinge upon the sustainability of agricultural practices. This negative association has perhaps arisen due a perceived direct and personal threat to health and economic security which has also been perpetuated by the media. Therefore, greater understanding of the potential transmission routes, persistence, as well as environmental and microbiological interactions, is required (Berger, *et al,* 2010). However, it is highly unlikely that we will be able to entirely eradicate all food-borne outbreaks, with greater individual and corporate responsibility being required to mitigate risk (Batt, 1997).

This work attempts to aid in the development of appropriate and realistic policies to improve safety by further supporting and expanding upon the previous

findings and theories already outlined. It illustrates how increases in microbial activity after amendment of soil with sewage sludge, and simple (glucose) and complex substrates (yeast extract) influence the survival patterns of enteric pathogens. With r-strategists potentially favour the simple amendment and K-strategists potentially favouring more complex amendment. It also builds upon this body of work by using a complex amendment that is widely used within agriculture, sewage sludge. This is both socially and economically relevant as the immense quantity of sewage sludge produced annually and its hazardous nature poses a significant environmental, sustainable and public health challenge (Dumontet, *et al.* 2001).

The original concept governing this work postulated that the input of organic carbon and nutrients, associated with sewage sludge applied to land, could instigate hotspots of microbial activity via a localised supply of such resources. Such zones of increased microbial activity could then lead to an increase in competition for local resources, leading to a decline in enteric pathogens co-introduced with sewage sludge due to the potential for competition for remaining resources. Sidhu, *et al*. (2001) further support this theory, suggesting that an increase in the quantity and activity of microorganisms could lead to increased microbial interactions, including mutualistic, antagonistic and neutral modes. However, there is a possibility that such hot spots, created by the addition of sewage sludge to soil, could act as a "poison chalice" of sorts due to the presence of toxic chemicals such as heavy metals (Chaudri, *et al.* 2008). A poisoned chalice in general terminology refers to something that originally appears to be a positive influence, but in fact turns out or becomes a negative one. When using this term in this instance, it refers back to the original concept of the work which postulated that the amendment of sewage sludge to soil would cause an increase in microbial activity due to a greater provision of resources, or the "good" aspect of the poisoned chalice idiom. However, when considering the other physicochemical properties of sewage sludge, most pertinently the toxic or hazardous aspects such as the presence of heavy metals, this then becomes the latter part of the idiom.

It was also theorised that the nature of the interfaces between the soil and sewage sludge may influence such interactions. This is associated with the organisational and physicochemical characteristics which could affect the extent of

exposure and resultant interactions between enteric pathogens and the soil microbial community. For example, Frostegård, *et al*. (1997), examined microbial community dynamics associated with manure hot spots in a sandy soil. They found that microbial biomass doubled within a 2 mm distance of the soil-manure interface after a 3 day period. This also occurred alongside a change in PLFA composition, indicating a change in the phenotypic structure of the microbial community. These changes were attributed to the diffusion of dissolved organic carbon from the manure and did not occur within soil layers further away from the manure amendment.

From this original concept, a further set of questions were developed over the course of the project. Each of the questions built upon the outcome of the previous. These questions were:

1. Does the proportion of sewage sludge to soil affect the persistence of co-introduced bacterial pathogens? (Chapter 4).
2. Does the proportion of sludge to soil affect the persistence of indigenous sewage sludge *E. coli*? (Chapter 5).
3. How does the addition of supplementary substrate, of contrasting composition, to differential phases of sludge and soil mixtures affect the survival of sewage sludge-derived *E.coli*? (Chapter 6).
4. How will the repeated additions of supplementary substrate (glucose), mimicking the recurrent input of carbon that occurs in plant/soil systems, affect the survival of sewage sludge-derived *E. coli* or indigenous sewage sludge general coliforms? (Chapter 7).

The first question was explored in Chapter 4, where two microcosm-based studies with either model strains of *E. coli* or *S*. Dublin added to treatments containing soil or sewage sludge were developed. The *E. coli* and *S*. Dublin was then quantified periodically over a 42 day period. The hypothesis for these experiments stated that there would be a positive correlation between increasing ratios of sludge to soil and the survival of model bacterial pathogens. This hypothesis was rejected for both of the experiments. However, the dynamics of cell death within the set of microcosms containing *E. coli* appeared to be more complex than a simple proportional relationship

to the quantity of sewage sludge present. For example, the treatment with the smallest ratio of soil to sewage sludge appeared to result in the lowest eventual pathogen numbers. This is contrary to the logic of the hypothesis, which postulated that a greater death rate would result with a greater proportion of soil, since an increase in the quantity of soil would mean a proportional increase in the rate and extent of soil microbial interactions and/or encounters with introduced pathogens. Hence it would be expected that the native soil microbes would likely attenuate the introduced pathogens, more-so due to the potential scale of resultant competition. This response found in this study has also been observed before (Jiang, *et al.* 2002), who found that the smallest ratio of manure to soil (1:10) caused a greater decline in *E. coli* O157:H7. This could have occurred due to a balance of factors favouring the soil microbial community, with the addition of the manure bolstering the local soil microbial community. Whilst the 1:10 soil:manure amendment strategy would not have altered the environment enough to favour the local manure microbial community. Conversely, the dynamics of cell death within the set of microcosms containing *S.* Dublin appeared to do the opposite, with the treatment containing only soil causing a greater decline than the treatment containing 75% sewage sludge. This demonstrates that different pathogens respond differently to such circumstances and further illustrates why tracking specific pathogens as opposed to a broad range, for instance in the case of general coliform data, is important in determining persistence and associated risks.

Furthermore, the presence of indigenous *E. coli* within the sewage sludge led to the discussion of whether model pathogens or indigenous sewage sludge pathogens would be appropriate to study in subsequent experiments. As a portion of the sewage sludge contained high quantities of indigenous *E. coli*, the second question in the above series, was posed (Chapter 5). A microcosm-based study was then developed, where varying quantities of soil was added to a consistent quantity of sewage sludge containing indigenous *E. coli*. As previously, the hypothesis for this experiment stated that there would be a greater decline in indigenous sewage sludge *E. coli* with increasing the proportion of soil to sewage sludge. Again, as with the previous experiments (Chapter 4), there were no significant, or consistent, correlations between varying proportions of soil to sludge and the survival of indigenous *E. coli*. Therefore, the hypothesis was again rejected. Furthermore, the indigenous *E. coli* exhibited a more

consistent linear decline after the first week, when compared to the model *E. coli*, which exhibited a more varied response.

Considering that an increasing quantity of soil must mean a proportional increase in the extent of encounters between the native soil microbial community and the introduced microorganisms, this persistent lack of treatment effect is outwardly counter-intuitive. Especially in regards to the findings of Frostegård, *et al*. (1997), who found that microbial biomass doubled within a 2 mm distance of the soil-manure interface after a short period of time. A possible explanation for these results is that the lack of treatment effects could have arisen from *a lack of available substrate or resources* within the sewage sludge. Such a lack of available substrate could, in turn, fail to promote sufficient general microbial activity to instigate the die-off of introduced pathogens. Therefore, the next question in the series (Question 3) was devised to answer this. Two microcosm studies were developed where glucose or yeast extract were added to soil, sewage sludge, or both matrices, with microbial activity and sewage sludge-derived *E. coli* numbers quantified periodically over 42 days (Chapter 6). The provision of two contrasting substrates would offer different resources to the microbial community thus potentially eliciting different microbial responses, leading to different interactions with introduced pathogens. A simple carbohydrate like glucose would only provide carbon, potentially increasing competition for other resources, such as nitrogen, phosphorus and amino acids. Alternately, the amendment of yeast extract would provide a greater variety of resources required by microorganisms thus decreasing the likelihood of competition. However, the provision of such extra resources could also promote the synthesis of 'metabolically expensive' compounds such as antibiotics by the native flora, thereby increasing competitive ability. Furthermore, the amendment of substrate to either soil or sewage sludge or to both matrices also allowed us to test whether the partitioning of such substrate between matrices was an important factor in the survival of introduced pathogens. Two hypotheses were thus developed. The first stated that the addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli*. The second hypothesis stated that the location of labile substrate would significantly affect microbial activity and the persistence of sewage sludge-derived *E. coli*.

It was found that the addition of glucose caused immediate die-off of *E. coli*, corresponding to the rapid utilisation of the substrate by some route. Additionally, the location of the substrate did not significantly affect this response. However, the soil and the sewage sludge microbial communities did potentially play a part in modulating *E. coli* survival, with the presence of soil (and associated soil microbial communities) leading to greater die-off. There was also die-off within the sewage sludge control treatments, further suggesting competition also occurred from within the sludge microbial community. Therefore, the first hypothesis, which stated that the addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli*, was accepted for microcosms amended with glucose. Whilst the hypothesis which stated that the location of labile substrate would significantly affect microbial activity and the persistence of sewage sludge-derived *E. coli,* was rejected for microcosms amended with glucose. Amendment of microcosms with yeast extract resulted in little immediate differential die-off in *E. coli* between treatments. Therefore, the first hypothesis, which stated that the addition of labile substrate to soil/sewage sludge microcosms would significantly increase microbial activity, resulting in a decline in the persistence of sewage sludge-derived *E. coli*, was rejected for microcosms amended with yeast extract. The hypothesis which stated that the location of labile substrate would significantly affect microbial activity and the persistence of sewage sludge-derived *E. coli* was also rejected for microcosms amended with yeast extract. To summarise, the first hypothesis was accepted for glucose amendment, but not for yeast extract amendment implicating a requirement for pure energy (carbon) to instigate greater pathogen die-off. Conversely, the second hypothesis was rejected for both glucose and yeast extract amendments indicating that location of amendment in this instance is not a factor, perhaps because the materials diffuse too quickly to elicit a spatial response.

From these findings it was postulated that the different responses induced when either glucose or yeast extract were used arose due to their chemical composition. Glucose, a pure carbohydrate, only provided a carbon source to the soil and sewage sludge, whereas the yeast extract provided a more complex set of resources, including carbon and nutrients (Harrison and Mora, 2001; Prescott, *et al.* 2005e). This difference

in resource provision could have lead to differences in competition, as well as the production of such molecules as antibiotics, thus impacting upon the survival of introduced pathogens. The provision of only carbon, in the form of glucose, was theorised to have instigated greater competition for other nutrients, with these nutrients acting as a limiting factor in cell growth and propagation. In contrast the provision of a greater range of resources, in the form of yeast extract, did not appear to have instigated such nutrient-limitation competition, nor result in the production of antibiotics. Alternately, the provision of carbon and stimulation of associated microbial carbon content within microcosms may have lead to increased predator numbers in the glucose-amended treatments. This could be related to the greater activity of the microbial community attracting predator species, as well as the greater quantity of microbial carbon supporting the propagation of such predators. To distinguish between these two effects, future works could both monitor the quantity of predator species over time as well as analysing community composition to clarify which species may compete with the introduced enteric pathogens.

These findings are similar to those of Schwarz, *et al.* (2014), who showed a greater decline of *E. coli* and *S. enterica* when anaerobically-digested dewatered biosolids were applied to soils, in comparison to un-amended soils. They also theorised that this greater decline in *E. coli* and *S. enterica* in amended soils could have been caused by enhanced antagonistic activity of the indigenous soil microorganisms, which was attributed to greater availability of nutrients and improved moisture retention. This increased antagonistic behaviour could have occurred in the form of antibiotic production. However, the necessary components of such a molecule would also need to be available and did not appear to occur within the microcosms amended with yeast extract, as no differential *E. coli* die-off between treatments appeared to occur. The use of different substrates, tests for antibiotic production and community structure, assessment of predator species, tracking of nutrient/substrate turnover as well as spatial microbial biomass would further clarify such effects.

With the amendment of glucose eliciting a significant response, and the effects of matrix partitioning discounted in both studies, it was further theorised that repeated addition of glucose would instigate more sustained competition and therefore greater

die-off. It was postulated that the repeated addition of glucose would mimic the continuous flux of nutrients in natural soil systems, for example the presence of plants/roots and the continuous input of root exudates. Additionally, Hamer and Marschner (2005) found that addition of ^{14}C labelled substrates induced an accelerated mineralisation of SOC and increased microbial activity greater than that observed with single amendments. Sørensen (1974) also showed similar findings, with the addition of the OM increased CO_2 evolution and decomposition of labelled SOM. These studies further support the supposition of sustained competition due to sustained microbial activity, which could potentially lead to greater die-off of enteric pathogens. Therefore, the fourth question was developed, which led to the development of a microcosm-based study assessing the survival of sewage sludge-derived *E. coli* and indigenous sewage sludge general coliforms over a period of 105 days, with glucose added on three occasions at bi-weekly intervals (Chapter 7). It was hypothesised that increased and sustained microbial activity, via the repeated application of substrate (glucose), would cause a significant and sustained decline in sewage sludge-derived *E. coli* and/or indigenous sewage sludge coliform bacteria.

It was found that the repeated addition of glucose did not cause a sustained die-off. The initial amendment did cause significant die-off similar to that observed in the previous experiment (Chapter 6). However, subsequent amendments apparently sustained *E. coli* numbers, followed by more gradual declines. This could have occurred due to shifts in the microbial community, changes in microbial activity, or due to the *E. coli* bacteria becoming better adapted/acclimatised to their surroundings. The sustained input of carbon would have lead to an increase in microbial biomass and presumably an increase in corresponding predator numbers. However, there is no apparent effect on introduced pathogens. This could have been due to the pathogens being physically protected from interacting with the soil microbial community, through adhering to soil or sewage sludge particulate matter, or becoming physically protected by passage into smaller pores over time (Wright, *et al.* 1995). The formation of biofilms could also offer the introduced pathogens a form of protection. Biofilms are a structurally complex system which offers a protected form of growth that could allow cells to survive in hostile environments or from predation (Hall-Stoodley, *et al.* 2004; Matz and Kjelleberg, 2005). Similar to the previous work (Chapter 6), the presence of soil caused

greater die-off of *E. coli* numbers in comparison to the sludge control treatments. This again parallels the work undertaken by Schwarz, *et al.* (2014), who found that *E. coli* and *S. enterica* declined to a greater extent in the presence of anaerobically-digested dewatered biosolids applied to soils, in comparison to un-amended soils. Additionally, the repeated provision of substrate, in the form of glucose, could have potentially negated any of the harmful physicochemical characteristics of the sewage sludge. This could have been brought about due to the continual provision of enough energy that would promote the growth rate of the microbial community above the death rate. However, this would require further investigation to clarify such interactions, for example the use of sewage sludge amended with different concentrations and combinations of heavy metals and substrate could be undertaken.

In contrast, general coliform concentrations showed a relatively greater variation over time with greater variation occurring after amendment with glucose or sand. This suggests that the disturbance created by the amendment had greater effect than the glucose itself. From these findings, the original hypothesis stating that the increased and sustained microbial activity, via the repeated application of substrate (glucose), would cause a significant and sustained decline in indigenous sewage sludge sewage sludge coliform bacteria was rejected. The term "general coliforms" refers to a group or community are used as an s of contamination in wastewater, sewage sludge and food produce (Environment Agency, 2002). Therefore, the high variation in the general coliform data was attributed to this community of bacteria. Presumably each individual species within this group would react differently to the repeated amendment of glucose, with different species becoming more predominant or declining over time leading to such variation. To elucidate this, higher-resolution analysis of community structure would be required as opposed to generalised counts. Whilst generalised counts that include a group of bacteria can be useful as an *indicator* of contamination, it does not identify the growth patterns of specific species nor does it aid in identifying which species pose the greatest risk.

8.1. Conclusion

The input of organic carbon and nutrients, associated with sewage sludge applied to land could instigate a hotspot of microbial activity depending upon the provision of such resources within the sewage sludge. Furthermore, depending upon this provision of organic carbon and nutrients, this zone of increased microbial activity could lead to an increase in competition for other resources, further leading to a decline in enteric pathogens co-introduced with sewage sludge. However, it also showed that the sewage sludge used did not cause a hot spot of activity nor a corresponding decline in enteric pathogens. Though this may not be the case for all sewage sludge and should be taken on a case by case basis, depending upon treatment processes used and subsequent physicochemical composition. Additionally, the interface between the soil and sewage sludge does not appear to influence such interactions, possibly due to a high diffusion rate of substrate. However, the definition of interface in this instance is narrow and does not consider other factors, such as the influence or placement of heavy metals. It was also theorised that under long term study the formation of biofilms and the ability of enteric pathogens to "hide" in crevasses could protect enteric pathogens from predation, thus prolonging their persistence. These findings partially support the diagram originally portrayed in the Introduction (Figure 1.1), more specifically the potential for competition and predation leading to pathogen decline with the provision of enough resources. It also discounts the aspect of this diagram pertaining to the potential influence of the interface between soil and sewage sludge matrices.

The greater the quantity of readily available resources that are present within the sewage sludge, the more likely microbial activity will increase to a rate that would instigate competition for resources and thus perhaps lead to pathogen die-off. A high quantity of carbon, with little other nutrients appears to instigate greater competition and subsequent pathogen die-off than when a greater range of nutrients are also provided. The sewage sludge used within this work did not appear to contain enough resources to promote such interactions. This was highlighted by the lack of proportional decline in either model or indigenous sewage sludge pathogens with the application of different proportions of sewage sludge to soil within the first two experimental chapters (Chapters 4 and 5). This would imply that the particular sewage sludge used did not

contain enough sustenance, likely due to the treatment processes used on the sewage sludge, which tend to reduce nutrients/OM content.

This supposition was further supported in the following experiments in Chapter 6, where substrate was added to either or both matrices. The addition of glucose had the most significant affect on initial microbial activity and pathogen survival, though the presence of yeast extract did not cause such an effect. In essence, by adding glucose to soil or sewage sludge an artificial hotspot was created or forced which lead to the decline of *E. coli*. This is highlighted by the faster microbial response to the addition of glucose, characterised by a peak in respiration in comparison to the slower microbial response to the addition of yeast extract. This suggests that simpler substrate is required to cause an immediate hot spot of microbial activity that will subsequently lead to an increase in competition for other resources and a decline in pathogens co-introduced with sewage sludge. From this it can be theorised that is not just the total energy available to the microorganisms within the system that governs the interactions within a hot spot, but the provision of other nutrients also. Enough energy is required to initiate a microbial response but competition is unlikely to occur if resources are available in great enough quantity and whilst biomass may contribute to such interactions, the extent of its influence would presumably be dependent upon the composition of the biomass and the likelihood of competition to occur. Additionally, the length of the time this hot spot would remain in existence is dependent on such resources. Though they tend to last from minutes to days depending on the rate at which such resources are mineralised and at what point limiting factors, such as depletion in nitrogen levels, begin to govern such activity. However, this may not be the case for all sewage sludge, as it can undergo a wide variety of treatment processes that can lead to a wide variety of physicochemical and biological structure. For example, untreated sewage sludge can contain a lower range of protein, nitrogen and phosphorus content than primary treated sewage sludge or activated sewage sludge (Fytili and Zabaniotou, 2008).

It also is apparent from the work that the activity of the microbial community is an important factor, and that when enough nutrients and carbon are present as was the case when yeast extract was amended to soil/sludge microcosms (Chapter 6), *E. coli* are also able to sustain within the soil/sewage sludge microcosms. However, intuitively it

could be theorised that a greater microbial biomass, and thus the greater presence of the microbial community, would lead to greater competition for the introduced enteric pathogens. Furthermore, it could be theorised that the important factors in the decline of enteric pathogens when introduced to the soil environment are the extent of microbial activity, the initial competition for resources and their ability to quickly adapt or gain a foothold when faced with such competition and the change in environmental conditions. However, in the following chapter, Chapter 7, the repeated addition of glucose did not instigate a similar outcome as secondary and tertiary amendments appeared to promote pathogen survival. Suggesting that perhaps the repeated application of sewage sludge (should it contain enough readily available resources) could promote the survival of pathogens possibly through the formation of biofilms or by providing them enough energy to sustain and develop within protected niches within soil/sludge particulate matter.

8.2. Limitations and future work

This work contains a number of limitations. For example it infers microbial activity by assessing respiration but does not provide finer details of the microbial community on a molecular or phenotypic level. Identifying key microbes, such as protozoa, nematodes and bacteria which are likely to compete with enteric pathogens could lead to the development of a biotic culture that could be used to quickly nullify the presence of enteric pathogens in sewage sludge or soil. Additionally, the work only focuses on one form of sewage sludge, from one source and on one type of soil. A greater range of both matrices, with contrasting physicochemical properties, such as with varying proportions of heavy metals, OM, moisture content or nutrients, would further clarify which factors significantly influence microbial activity and interactions. It would also better illustrate how likely the original concept, which postulated that the input of organic carbon and nutrients, associated with sewage sludge applied to land, could instigate hotspots of microbial activity that would lead to a decline in enteric pathogens, would occur. It also focuses on microcosm studies, which necessarily involve a scale considerably smaller than that in agricultural field soil. Furthermore, the effects within macrocosms or field studies could potentially be quite different or more

varied due to the quantity and diversity of microorganisms present. Finally, this work tends to only focus on a small subset of enteric pathogens, most notably, *E. coli*. Much as there were different extraction efficiencies observed between different model pathogens (Appendix 1), it could be hypothesised that different enteric pathogens will persist and/or adapt differently when exposed to similar experimental circumstances that occurred within this work. This is further supported by Moynihan, *et al*. (2015), showed that *S.* Dublin, *L. monocytogenes,* environmentally-persistent *E. coli* and non-toxigenic *E. coli* O157 declined at different rates under different land uses.

However, the experiments discussed here do provide foundations on which future work can build. The next step in this work should involve the assessment of different sewage sludge, with varying composition, on the survival of enteric pathogens. For example, the use of sewage sludge which has undergone varying degrees of treatment is another avenue which could be taken. A less stable version may provide enough sustenance to create the right conditions which would lead to the development of a hot spot and subsequent die-off of any enteric pathogens. Or the amendment of different supplementary nutrients to the sludge could also be studied. The repeated application of yeast extract, or a more complex substrate, and its effect on the survival of sewage sludge-derived *E. coli* would also complement the previous experiments, as it could highlight the role of antibiotic production. Additionally, assessment of the complexity and structure of the microbial communities both within the fertiliser and soil would clarify if microbial interactions are key factors in the survival of enteric pathogens introduced into soil. Furthermore, this work could be developed by considering the impact of plant/rhizosphere interactions on such systems. The continual input of carbon and other root exudates could provide enough resources to promote greater competition through antagonistic interactions, for example, the production of antibiotics. This continual input and consequent dearth of microbial activity could also promote the attraction and propagation of predator species.

References

ABU-ASHOUR, J., JOY, D. M., LEE, H., WHITELEY, H. R., and ZELIN, S. (1004). Transport of microorganisms through soil. *Water, Air and Soil Pollution*. 75(1-2). 141-158.

ACEA, M. J., and ALEXANDER, M. (1988). Growth and survival of bacteria introduced into carbon-amended soil. *Soil Biology and Biochemistry*. 20(5). 703-709.

ACEA, M. J., and CARBALLAS, T. (1988). The influence of cattle slurry on soil microbial population and nitrogen cycle microorganisms. *Biological Wastes.* 23(3). 229-241.

ADAS. (2001). The safe sludge matrix: Guidelines for the application of sewage sludge to agricultural land (3rd ed). *ADAS, Gleadthorpe Research Centre, Mansfield, UK*.

ALDEN, L., DEMOLING, F., and BÅÅTH, E. (2001). Rapid method of determining factors limiting bacterial growth in soil. *Applied and Environmental Microbiology*. 67(4). 1830-1838.

ALLOWAY, B. J., and JACKSON, A. P. (1991). The behaviour of heavy metals in sewage sludge-amended soils. *Science of the Total Environment*. 100. 151-176.

ANDERSON, J. P. E., and DOMSCH, K. H. (1978). A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biology and Biochemistry*. 10. 215-221.

ANDREWS, J.E., BRIMBLECOMBE, P., JICKELLS, T.D., LISS, P.S., and REID, B.J. (2004). Chapter 4: The chemistry of continental solids. In: *An Introduction to Environmental Chemistry* (2nd ed). Wiley-Blackwell Publishing. p103-104.

AVERY, L.M., HILL, P., KILLHAM, K., JONES, D.L. (2004A). *Escherichia coli* O157 survival following the surface and sub-surface application of human pathogen contaminated organic waste to soil. *Soil Biology and Biochemistry*.36. 2101-2103

AVERY, S. M., MOORE, A. and HUTCHISON, M. L. (2004b). Fate of *Escherichia coli* originating from livestock faeces deposited directly onto pasture. *Letters in Applied Microbiology.* 38(5). 355-359.

AWA (Australian Water Association). (2009). What are biosolids? Available at: http://www.biosolids.com.au/what-are-biosolids.php. (Accessed: 15th January 2015).

BAGGE, E., SAHLSTRÖM, L., and ALBIHN, A. (2005). The effect of hygienic treatment on the microbial flora of biowaste at biogas plants. Water Research. 39. 4879-4886.

BARKER, J., and BROWN, M. R. W. (1994). Trojan horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment. *Microbiology*. 140(6). 1253-1259.

BARKER, J., HUMPHREY, T. J., and BROWN, M. W. R. (1999). Survival of *Escherichia coli* O157 in a soil protozoan: Implications for disease. *FEMS Microbiology Letters*. 173(2). 291-295.

BATT, C. (1997). Down on the farm... *Food Microbiology*. 14(3). 199.

BAUDOIN, E., BENIZRIM, E., and GUCKERT, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry*. 35(9). 1183-1192.

BERGER, C. N., SODHA, S. V., SHAW, R. K., GRIFFIN, P. M., PINK, D., HAND, P., and FRANKEL, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology.* 10(9). 2385-2397.

BINKLEY, J., and SIMPSON, J. A. (2003). Chapter 35: Heavy metals in wastewater treatment processes. In: MARA, D. and HORAN, N. (editors); *The Handbook of Water and Wastewater Microbiology*. Academic Press. p602.

BLAGODATSKAYA, E. V., BLAGODATSKY, S. A., ANDERSON, T. H., and KUZYAKOV, Y. (2009). Contrasting effects of glucose, living roots and maize

straw on microbial growth kinetics and substrate availability in soil. *European Journal of Soil Science*. 60. 186-197.

BLAGODATSKAYA, E., and KUZYAKOV, Y. (2008). Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. *Biology and Fertility of Soils.* 45. 115-131.

BRANDL, M. T., ROSENTHAL, B. M., HAXO, A. F., and BERK, S. G. (2005). Enhanced survival of *Salmonella enterica* in vesicles released by a soil-borne *Tetrahymena* species. *Applied Environmental Microbiology*. 71. 1562–1569.

BRAY, H.R., and KURTZ, L.T. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Science*. 59. 39-45.

BRENNAN, F. P., O'FLAHERTY, V., KRAMERS, G., GRANT, J., and RICHARDS, K. (2010). Long-term persistence and leaching of Escherichia coli in temperate maritime soils. *Applied and Environmental Microbiology*. 76(5). 1449-1455.

BRENNAN, F. P., MOYNIHAN, E., GRIFFITHS, B. S., HILLIER, S., OWEN, J., PENDLOWSKI, H., and AVERY, L. M. (2014). Clay mineral type effect on bacterial enteropathogen survival in soil. *Science of the Total Environment.* 468. 302-305.

BROOKES, P. C., LANDMAN, A., PRUDEN, G., and JENKINSON, D. S. (1985). Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biology and Biochemistry*. 17(6). 837-842.

BROOKES, P. C., POWLSON, D. S., and JENKINSON, D. S. (1982). Measurement of microbial biomass phosphorus in soil. *Soil Biology and Biochemistry*. 14(4). 319-329.

BROWN, M. R. W. and BARKER, J. (1999). Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends in Microbiology*. 7(1). 46-50.

BSI (British Standard Institute). (1997). BS 7755-part 4.4.2. *Determination of soil microbial biomass – fumigation extraction methods*. Milton Keynes: BSI, 1997.

BUCHANAN, R. E. (1918). Life phases in a bacterial culture. *The Journal of Infectious Diseases*. 109-125.

BUCHHOLZ, U., BERNARD, H., WERBER, D., BÖHMER, M. M., REMSCHMIDT, C., WILKING, H., DELERÉ, Y., AN DER HEIDEN, M., ADLHOCH, C., DREESMAN, J., EHLERS, J., ETHELBERG, S., FABER, M., FRANK, C., FRICKE, G., GREINER, M., HÖHLESOFIE IVARSSONM M., JARK, U., KIRCHNER, M., KOCH, J., KRAUSE, G., LUBERM P., ROSNER, B., STARK, K., and KÜHNE, M.. (2011). German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine.* 365(19). 1763-1770.

BUNDT, M. F., WIDMER, M., PESARO, J. Z. and BLASER, P. (2001). Preferential flow paths: Biological 'hot spots' in soils. *Soil Biology and Biochemistry*. 33(6). 729-738.

CAMPBELL, N. A., and REECE, J. B. (2005). Unit 5: The evolutionary history of biological diversity; Chapter 27: Prokaryotes. In: *Biology* (7th ed). Pearson Benjamin Cummings. Pp546.

CARSTEN, M., and KJELLEBERG, S. (2005). Off the hook - How bacteria survive protozoan grazing. *TRENDS in Microbiology*. 13(7). 302-307.

CASTRO, H. F., CLASSEN, A. T., AUSTIN, E. E., NORBY, R. J., and SCHADT, W. C. (2010). Soil microbial community responses to multiple experimental climate change drivers. *Applied and Environmental Microbiology*. 76(4). 999-1007.

CEC (Commission of European Communities). (1986). Council Directive 86/278/EEC of 4 July 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

CEC (Commission of European Communities). (1998). Council Directive 91/271/EEC of 21 March 1991 concerning urban waste-water treatment (amended by the 98/15/EC of 27 February 1998).

CEC (Commission of the European Communities). (2006). 232. Proposal of a Directive of the European Parliament and of the Council 2006 establishing a framework for the protection of soil and amending Directive 2004/35/EC. Commission of the European Communities. Brussels.

CEC (Commission of European Communities). (2008). Council Directive 2008/98/EC of waste.

COM (Commission of the European Communities). (2006). 232. Proposal of a Directive of the European Parliament and of the Council 2006 establishing a framework for the protection of soil and amending Directive 2004/35/EC. Commission of the European Communities. Brussels.

CHANDLER, D. S. and CRAVEN, J. A. (1980). Relationship of soil moisture to survival of *Escherichia coli* and *Salmonella typhimurium* in soils. *Australian Journal of Agricultural Research*. 31. 547-555.

CHAUDRI, A., MCGRATH, S., GIBBS, P., CHAMBERS, B., CARLTON-SMITH., BACON, J., CAMPBELL, C., and AITKEN, M. (2008). Population size of indigenous *Rhizobium leguminosarum* biovar *trifolii* in long-term field experiments with sewage sludge cake, metal-amended liquid sludge or metal salts: effects of zinc, copper and cadmium. *Soil Biology and Biochemistry*. 40(7). 1670-1680.

CHENG, W., JOHNSON, D. W., and FU, S. (2003). Rhizosphere effects on decomposition. *Soil Science Society of America Journal.* 67(5). 1418-1427.

CHIBANI-CHENNOUFI, S., BRUTTIN, A., DILLMANN, M-L., and BRÜSSOW, H. (2004). Phage-host interaction: and ecological perspective. *Journal of Bacteriology.* 186(12). 3677-3686.

CHIGINEVA, N. I., ALEKSANDROVA, A. V., and TIUNOV, A. V. (2009). The addition of labile carbon alters litter fungal communities and decreases litter decomposition rates. *Applied Soil Ecology*. 42. 264-270.

CREAMER, R. E., BRENNAN, F., FENTON, O., HEALY, M. G., LALOR, S. T. J., LANIGAN, G. J., REGAN, J. T., and GRIFFITHS, B. S. (2010). Implications of the proposed soil framework directive on agricultural systems in Atlantic Europe - A review. *Soil Use and Management*. 26**.** 198-211.

CRUMP, J. A., LUBY, S. P., and MINTZ, E. D. (2004). The global burden of typhoid fever. *Bulletin of the World Health Organisation*. 82(5). 346-353.

DAHNKE, W.C. (1990). Testing soils for available Nitrogen. In: WESTERMAN, R.L. (editor); *Soil testing and plant analysis*; Book Series 3. Soil Science Society of America. ASA, Madison, WI. p. 120-140.

DAVIS, M. A., CLOUD-HANSEN, K. A., CARPENTER, J., and HODE, C. J. (2005). *Escherichia coli* O157: H7 in environments of culture-positive cattle. *Applied and Environmental Microbiology*. 71(11). 6816-6822.

DEFRA (Department for Environment, Food and Rural Affairs). 2006. Code of Practice for Agricultural Use of Sewage Sludge. 2nd edition. The Stationary Office, London.

DEFRA (Department for Environment, Food and Rural Affairs). (2012a). Wastewater treatment in the United Kingdom – 2012. Implementation of the European Union Urban Waste Water Treatment Directive – 91/271/EEC. The Stationary Office, London. *Crown Copyright*. p18.

DEFRA (Department for Environment, Food and Rural Affairs). (2012b). Wastewater treatment in the United Kingdom – 2012. Implementation of the European Union Urban Waste Water Treatment Directive – 91/271/EEC. *Crown Copyright*. p19.

DE ZOZI, P., BARBIZZI, S., BELLI, M., CICWEI, G., FAJGELJ, A., MOORE, C., Sansone, U., and VAN DER PERK, M. (2005). Terminology in soil sampling (IUPAC Recommendations 2005). *Pure and Applied Chemistry*. 77(5). 827-841.

DILLY, O. (2005). Chapter 6: Microbial energetics in soil. In: BUSCOT, F., and VARMA, A. (editors); *Soil Biology (Vol. 3)*; *Microorganisms in Soils: Roles in Genesis and Function*. Pp123.

DRENOVSKY, R. E., VO, D., GRAHAM, K. J., and SCOW, K. M. (2004). Soil water content and organic carbon availability are major determinants of soil microbial community composition. *Microbial Ecology*. 48(3). 424-430.

DUMONTET, S., SCOPA, A., KERJE, S., and KROVACEK, K. (2001). The importance of pathogenic organisms in sewage and sewage sludge. *Journal of the Air and Waste Management Association.* 51(6). 848-860.

DYTHAM, C. (2006). Choosing and Using Statistics: A Biologist's Guide. Blackwell Publishing. Pp89.

EC (European Commission). (2001a). Evaluation of sludge treatments for pathogen reduction – Final report. Report no. CO 5026/1. European Commission, Luxemburg.

EC (European Commission). (2001b). Disposal and recycling of sewage sludge part 3: Scientific and technical sub-component report. European Commission, Luxemburg.

EC (European Commission). (2007). LIFE ("The Financial Instrument for the Environment") and waste recycling: Innovative waste management options in Europe. European Commission, Luxemburg.

EUROPEAN COMMISSION. 2012. The EU Water Framework Directive - Integrated River Basin Management for Europe. Available: http://www.euwfd.com/html/sewage_treatment.html (Accessed: 20th February 2015). European Commission, Luxemburg.

EC (European Commission). (2015a). Soil: Withdrawal of the proposal for a soil framework directive. Available: http://ec.europa.eu/environment/soil/process_en.htm (Accessed: 20th February 2015). European Commission, Luxemburg.

ELLIS, J. R., and MCCALLA, T. M. (1978). Fate of pathogens in soils receiving animal wastes - A review. *Transactions of the ASAE.* 21. 309-319.

ENVIRONMENT AGENCY, UK. (2002). The microbiology of drinking water. Part 1: Water quality and public health. Pp 13.

ENVIRONMENT AGENCY, UK. (2003). Microbiology of sewage sludge. Part 3: Methods for the isolation and enumeration of *Escherichia coli*, including verocytotoxigenic *Escherichia coli*. Pp 34.

EPA 150.1 (Environmental Protection Agency). (2001). Standard Operating Procedure: pH, Electrometric. US EPA Report Number: 600/4-79-020.

EPA 160.3 (Environmental Protection Agency). (1971). Standard Operating Procedure: Residue, Total (Gravimetric, Dried at 103-105°C). US EPA Report Number: 600/4-79-020.

EPA 300.0 (Environmental Protection Agency). (1984). Standard Operating Procedure: Chloride- IC. US EPA Report Number: 600/4-79-020.

EPA 350.1 (Environmental Protection Agency). (1997). Standard Operating Procedure: Ammonia (as nitrogen) - Colorimetric. US EPA Report Number: 600/4-79-020.

EPA 1687 (Environmental Protection Agency). (1997) Standard Operating Procedure: Total Kjeldahl nitrogen. US EPA Report Number: 821/R-01-004.

EPA 3050B (Environmental Protection Agency). (1996). Standard Operating Procedure: Acid Digestion of Sediments, Sludges and Soils. US EPA Report Number: SW-846 Ch 3.2.

EPA 6010 (Environmental Protection Agency). (1996). Standard Operating Procedure: Inorganics by ICP - Atomic Emission Spectroscopy. US EPA Report Number: SW-846 Ch 3.3.

EPA 7061 (Environmental Protection Agency). (1992). Standard Operating Procedure: Arsenic - AA, Gaseous Hydride. US EPA Report Number: SW-846 Ch 3.3.

EPA 7471 (Environmental Protection Agency). (1994). Standard Operating Procedure: Mercury in Solid or Semisolid Waste. US EPA Report Number: SW-846 Ch 3.3.

EPA 7741 (Environmental Protection Agency). (1994). Standard Operating Procedure: Selenium - AA, Gaseous Hydride. US EPA Report Number: SW-846 Ch 3.3.

ERICKSON, M.C., LIAO, J., MA, L., JIANG, X., and DOYLE, M. P. (2009). Inactivation of *Salmonella* spp. in cow manure composts formulated to different initial C:N ratios. *Bioresource Technology.* 100(23). 5898-5903.

FENLON, D. R., OGDEN, I. D., VINTEN, A., and SVOBODA, I. (2000). The fate of *Escherichia coli* and *E. coli* O157 in cattle slurry after application to land. *Journal of Applied Microbiology*. 88(S1). 149S-156S.

FERNANDES, S. A. P., BETTIOL, W., and CERRI, C. C. (2005). Effect of sewage sludge on microbial biomass, basal respiration, metabolic quotient and soil enzymatic activity. *Applied Soil Ecology*. 30(1). 65-77.

FONTAINE, S., MARIOTTI, A., and ABBADIE, L. (2003). The priming effect of organic matte a question of microbial competition? *Soil Biology and Biochemistry*. 35. 837-843.

FRANZ, E., SEMENOV, A. V., TERMORSHUIZEN, A. J., DE VOS, O. J., BOKHORST, J., and VAN BRUGGEN, A. H. C. (2008). Manure-amended soil characteristics affecting the survival of E. coli O157: H7 in 36 Dutch soils. *Environmental Microbiology*. 10(2). 313-327.

FRANZ, E., VAN HOEK, A. H. A. M., BOUW, E., and AARTS, H. J. M. (2011). Variability of *Escherichia coli* O157 strain survival in manure-amended soil in relation to strain origin, virulence profile and carbon nutrition profile. *Applied and Environmental Microbiology.* 77(22). 8088-8096.

FROSTEGÅRD, Å., PETERSEN, S. O., BÅÅTH, E., and NIELSEN, T. H. (1997). Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analyses. *Applied and Environmental Microbiology*. 63(6). 2224-2231.

FWR. (2012). General Information about the WFD and Water: Sewage treatment. Available: http://www.euwfd.com/html/sewage_treatment.html (Accessed: 20th February 2015).

FYTILI, D., and ZABANIOTOU, A. (2008). Utilization of sewage sludge in EU application of old and new methods – A review. *Renewable and Sustainable Energy Reviews*. 12. 116-140.

GANNON, J.T., MANILAL, V.B., and ALEXANDER, M. (1991). General microbial ecology: relationship between cell surface properties and transport of bacteria through soil. *Applied Environmental Microbiology*. 57(1). 190-193.

GANS, J., WOILINSKY, M., and DUNBAR, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science*. 309. 1387-1390.

GERBA, C. P., ROSE, J. B., and HAAS, C. N. (1996). Sensitive populations: Who is at the greatest risk? *International Journal of Food Microbiology*. 30(1). 113-123.

GERBA, C. P., and SMITH, J. E. (2005). Sources of pathogenic microorganisms and their fate during land application of wastes. *Journal of Environmental Quality*. 34(1). 42-48.

GOURABATHINI, P., BRANDL, M. T., REDDING, K. S., GUNDERSON, J. H., and BERK, S. G. (2008). Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Applied and Environmental Microbiology*. 74(8). 2518-2525.

GRANT, C. L., and PRAMER, D. (1962). Minor element composition of yeast extract. *Journal of Bacteriology*. 84(4). 869-870.

GREENE, S. K., DALY, E. R., TALBOT, E.A., DEMMA, L. J., HOLZBAUER, S., PATEL, N. J., HILL, T. A., WALDERHAUG, M. O., HOEKSTRA, R. M., LYNCH, M. F., and PAINTER, J. A. (2008). Recurrent multistate outbreak of Salmonella Newport associated with tomatoes from contaminated fields, 2005. *Epidemiology and Infection* 136(2). 157-165.

GREY, N. F. (2004). Chapter 2: How man deals with waste. In: *Biology of Wastewater Treatment: Series of Environmental Science and Management* (2nd ed). Imperial college press. Pp 133.

GRIFFITHS, B.S. (1994). Microbial-feeding nematodes and protozoa in soil: Their effects on microbial activity and nitrogen mineralization in decomposition hotspots and the rhizosphere. *Plant and Soil*. 164(1). 25-33.

GRIFFITHS, B. S., RITZ, K., EBBLEWHITE, N., and DOBSON, G. (1999). Soil microbial community structure: Effects of substrate loading rates. *Soil Biology and Biochemistry*. 31. 145-153.

HALL-STOODLEY, L., COSTERTON, J. W. and STOODLET, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*. 2(2). 95-108.

HAMER, U., and MARSCHNER, B. (2005). Priming effects in soils after combined and repeated substrate additions. *Geoderma*. 128. 38-51.

HAMMER, M. J., and HAMMER, M. J. .Jr. (2004a). Chapter 3: Biology. In: *Water and Wastewater Technology*. Pearson Prentice Hall. p 58.

HAMMER, M. J., and HAMMER, M. J. .Jr. (2004b). Chapter 11: Wastewater processing. In: *Water and Wastewater Technology*. Pearson Prentice Hall. p 414.

HAMMER, M. J., and HAMMER, M. J. .Jr. (2004c). Chapter 9: Wastewater flows and charateristics. In: *Water and Wastewater Technology*. Pearson Prentice Hall. p 307.

HAMMER, M. J., and HAMMER, M. J. .Jr. (2004d). Chapter 9: Wastewater flows and charateristics. In: *Water and Wastewater Technology*. Pearson Prentice Hall. p 28-29.

HARPER, J.L., BEGON, M., and TOWNSEND, C. R. (2006). *Ecology: From Individuals to Ecosystems* (4th ed). Blackwell Publishing. p238.

HARRISON, R. M., and DE MORA, S. D. (2001). Introductory chemistry for the environmental sciences (2nd ed). Cambridge Environmental Chemistry Series 7. *Cambridge University press.* p 175.

HENDERSON, S. L., DANDIE, C. E., PATTEN, C. L., ZEBARTH, B. J. BURTON, D. L., TREVORS, J. T., and GOYER, C. (2010). Changes in denitrifier aundance, denitrification gene mRNA levels, nitrous oxide emissions, and denitrification in anoxic soil microcosms amended with glucose and plant residues.*Applied and Environmental Microbiology* . 76(7). 2155-2164.

HOBBIE, J. E. and HOBBIE, E. A. (2013). Microbes in nature are limited by carbon and energy: the starving-survival lifestyle in soil and consequences for estimating microbial rates. *Frontiers in Microbiology*. 4. 1-11.

HORTON, H. R., MORAN, L. A., SCRIMGEOUR, K. G., PERRY, M. D., and RAWM, J. D. (2006). Chapter 1. Introduction to biochemistry. In: *Principles of Biochemistry* (4th ed). Pearson Prentice Hall. p3.

HOWELL, J. M., COYNE, M. S., and CORNELIUS, P. L. (1996). Effect of sediment particle size and temperature on fecal bacteria mortality rates and the fecal coliform/fecal streptococci ratio. *Journal of Environmental Quality* 25. 6. 1216-1220.

HRUDEY, S. E., PAYMENT, P., HUCK, P. M., GILLHAM, R. W., and HURDEY, E. J. (2003). A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Science and Technology.* 47(3). 7-14.

IBEKWE, A. M., PAPIERNIK, S. K., GRIEVE, C. M., and YANG, C. H. (2010). Influence of fumigants on soil microbial diversity and survival of *E. coli* 0157:H7. *Journal of Environmental Science and Health Part B*. 45. 416-426.

INGHAM, E. R. (2000). Soil biology primer. Chapter 1: the soil food web. Available at: http://urbanext.illinois.edu/soil/SoilBiology/soil_food_web.htm (Accessed: 9th December 2014).

INGLEZAKIS, V. J., ZORPAS, A. A., KARAGIANIDES, A., SAMARAS, P., and VOUKSLLI, I. (2012). European Union legislation on sewage sludge management. *Romania, SC European Focus Consulting srl Aristotle University of Thessaloniki*. 475-480.

IMS (Irish Meteorological Service). (2013a). Rainfall. Available: http://www.met.ie/climate/rainfall.asp (Accessed: 20th February, 2015).

IMS (Irish Meteorological Service). (2013b). Air temperature. Available at: http://www.met.ie/climate-ireland/surface-temperature.asp (Accessed: 20th February, 2015).

ISHII, S. and SADOWSKY, M., J. (2008). *Escherichia coli* in the environment: Implications for water quality and human health. *Microbes and Environments*. 23(2). 101-108.

ISLAM, M., MORGAN, J., DOYLE, M. P., PHATAK, S.C., MILLNER, P., and JIANG, X. (2004). Fate of *Salmonella enterica* serovar Typhimurium on carrots and radish grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology.* 70(4). 2497-2502.

ISO. (1997). 14240-1: Soil quality - Part 1: Determination of soil microbial biomass – Substrate induced respiration method. International Standards Organisation.

JÄDERLUND, L., SESSITSCH, A., and ARTHURSON, V. (2010). Persistence of two *Campylobacter jejuni* strains in soil and on spinach plants. *Applied and Environmental Soil Science*. 1182-1189.

JÄRUP, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin*. 68(1). 167-182.

JIANG, X., MORGAN, J., and DOYLE, M. P. (2002). Fate of *Escherichia coli* O157: H7 in manure-amended soil. *Applied and Environmental Microbiology*. 68(5). 2605-2609.

JONES, D. L. (1999). Potential health risks associated with the persistence of *Escherichia coli* O157 in agricultural environments. *Soil use and management.* 15(2). 76-83.

KÄFERSTEIN, F. K., MOTARJEMI, Y., and BETTCHER, D. W. (1997). Foodborne disease control: a transnational challenge. *Emerging Infectious Diseases.* 3(4). 503-510.

KARCH, H., RÜSSMANN, H., SCHMIDT, H., SCHWARZKOPF, A., and HEESEMANN, J. (1995). Long-term shedding and clonal turnover of enterohemorrhagic *Escherichia coli* O157 in diarrheal diseases. *Journal of Clinical Microbiology*. 33(6). 1602-1605.

KEARNEY, T. E., LARKIN, M. J., and LEVETT, P. N. (1994). Metabolic activity of pathogenic bacteria during semicontinuous anaerobic digestion. *Applied and Environmental Microbiology*. 60(10). 3647-3652.

KING, C. H., SHOTTS, E. B., WOOLEY, R. E., and PORTER, K. G. (1988). Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Applied and Environmental Microbiology*. 54(12). 3023-3033.

KOLLING, G., WU, M., and GUERRANT, R. L. (2012). Enteric pathogens through life stages. *Frontiers in Cellular and Infection Microbiology*. 2(114). 1-8.

KOUNO, K., WU, J., and BROOKES, P. C. (2002). Turnover of biomass C and P in soil following incorporation of glucose or ryegrass. *Soil Biology and Biochemistry*. 34(5). 617-622.

KUZYAKOV, Y. (2010). Priming effect: Interactions between living and dead organic matter. *Soil Biology and Biochemistry*. 42. 1363-1371.

KUZYAKOV, Y., and BOL, R. (2006). Sources and mechanisms of priming effect induced in two grassland soils amended with slurry and sugar. *Soil Biology and Biochemistry*. 38. 747-758.

KUZYAKOV, Y., FRIEDEL, J. K., and STAHR, K. (2000). Review of mechanisms and quantification of priming effects. *Soil Biology and Biochemistry*. 32. 1485-1498.

LARTEY, R. (2006). Dynamics of soil flora and fauna in biological control of soil inhabiting plant pathogens. *Plant Pathology Journal.* 5(2). 125-142.

LAUBER, C. L., STRICKLAND, M. S., BRADFORD, M. A., and FIERER, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biology and Biochemistry*. 40(9). 2407-2415.

LIN, C. Y. (1992). The effect of heavy metals on volatile fatty acid degradation in anaerobic digestion. *Water Research.*26. 177-183.

LOGAN, T.J., and HARRISON, B.J. (1995). Physical characteristics of alkaline stabilized sewage sludge (N-vitro soil) and their effects on soil properties. *Journal of Environmental Quality.* 24. 153–164.

MADIGAN, M. T., MARTINKO, J. M., and PARKER, J. (2006a). Chapter 28: Wastewater Treatment, Water Purification, and Waterborne Microbial Diseases. In: *Brock Biology of Microorganisms.* 11^{h} edition. Benjamin-Cummings Publishing Company. Pp 935.

MADIGAN, M. T., MARTINKO, J. M., and PARKER, J. (2006b). Chapter 8: Metabolic Regulation. In: *Brock Biology of Microorganisms.* 11th edition. Benjamin-Cummings Publishing Company.

MAJOWICZ, S. E., MUSTO, J., SCALLAN, E., ANGULO, F. J., KIRK, M., O'BRIEN, S. J., JONES, T. F., FAZIL, A., and HOEKSTRA, R. M. (2010). The global burden of nontyphoidal Salmonella gastroenteritis. *Clinical Infectious Diseases.* 50(6). 882-889.

MALLMANN, W. L., and LITSKY, W. (1951). Survival of selected enteric organisms in various types of soil. *American Journal of Public Health and the Nation's Health*. 41(1). 38-44.

MANTOVI, P., BALDONI, G., and TODERI, G. (2005). Reuse of liquid, dewatered, and composted sewage sludge on agricultural land: Effects of long-term application on soil and crop. *Water Research.* 39. 289-296.Ne

MATZ, C., and KJELLEBERG, S. (2005). Off the hook – How bacteria survive protozoan grazing. *Trends in Microbiology*. 13(7). 302-307.

MAULE, A. (2000). Survival of verocytotoxigemic *Escherichia coli* O157 in soil, water and on surfaces. *Journal of Applied Microbiology Symposium Supplement* .88. 71s-78s.

MCLAUGHLIN, M. J., ZARCINAS, B. A., STEVENS, D. P., and COOK, N. (2000). Soil testing for heavy metals. *Communications in Soil Science and Plant Analysis*. 31(11-14). 1661-1700.

MCLEAN, E.O. (1982). Soil pH and lime requirement. In: PAGE, A.L., *et al.* (editors) *Methods of Soil Analysis - Part 2* (2nd ed). Agronomy Monograph. p.199-223.

MEHLICH, A. (1984). Mehlich-3 soil test extractant: A modification of Mehlich-2 extractant community. *Soil Science and Plant Analysis.* 15. 1409-1416.

METCALF, L., and EDDY, H. P. (1991). Chapter 1: Wastewater engineering: An overview. In: *Wastewater Engineering: Treatment, Disposal and Reuse* (3rd ed). NewYork, USA: McGraw-Hill. Pp1.

MININNI, G., BLANCH, A. R., LUCENA, F., and BERSELLI, S. (2014). EU policy on sewage sludge utilization and perspectives on new approaches of sludge management. *Environmental Science and Pollution Research*. 1-14.

MOYNIHAN, E., RITZ, K., TYRREL, S., RICHARDS, K., and BRENNAN, F. (2012). *Interactions between microbial community structure and pathogen survival in soil.* Doctor of Philosophy, Cranfield University.

MOYNIHAN, E. L., RICHARDSM K. G., BRENNAN, F. P., TYRREL, S. F., and RITZ, K. (2015). Enteropathogen survival in soil from different land-uses is predominantly regulated by microbial community composition. *Applied Soil Ecology.* 89. 76-84.

MUBIRU, D.N., COYNE, M.S., and GROVE, J.H. (2000). Mortality of *Escherichia coli* O157:H7 in two soils with different physical and chemical properties. *Journal of Environmental Quality*. 29. 1821-1825.

MUELLER, R. F., and STEINER, A. (1992). Inhibition of anaerobic digestion by heavy metals. *Water Science and Technology.* 26. 835-846.

NELSON, D.W., and SOMMERS, L. E. (1996). Total carbon, organic carbon and organic matter. InJ.M. Bartels, *et al.* (ed.) *Methods of soil analysis: Part 3 Chemical methods.* (3rd ed.) ASA and SSSA Book Series 5, Madison, WI. p. 961-1010.

NOTTINGHAM, A. T., GRIFFITHS, H., CHAMBERLAIN, P. M., STOTT, A.W., and TANNER, E. V. J. (2009). Soil priming by sugar and leaf-litter substrates: a link to microbial groups. *Applied Soil Ecology*. 42(3). 183-190.

OLIVER, D.M., HAYGARTH, P.M., CLEGG, C.D., and HEATHWAITE, A.L. (2006). Differential *E. coli* die-off patterns associated with agricultural matrices. *Environmental Science and Technology*. 40. 5710-5716.

OXOID LTD. (2014a). Dehydrated culture media: X.L.D agar. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0469&c=UK&lang=EN&org=128&img=CM0469B&sec (Accessed: 20th February 2015).

OXOID LTD. (2014b). Dehydrated culture media: Membrane Lactose Glucuronide agar. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1031&c=UK&lang=EN&org=71&img=CM1031&sec (Accessed: 20th February 2015).

OXOID LTD. (2014c). International product list. Available at: http://www.oxoid.com/uk/blue/prod_list/prod_list.asp?c=uk&lang=EN (Accessed: 10th December 2014).

PARKIN, T. B. (1987). Soil microsites as a source of denitrification variability. *Soil Science Society of America Journal*. 51(5). 1194-1199.

PETERSEN, S. O., NIELSEN, T. M., and HENRIKSEN, K. (1993). Direct measurements of oxygen microprofiles and distribution of phospholipid-P in a two-phase soil-manure system. *Geoderma*. 56. 549-559.

PETERSEN, S. O. NIELSON, T. H., FROSTEGÅRD, Å., and OLESEN, T. (1996). O_2 uptake, C metabolism and denitrification associated with manure hot-spots. *Soil Biology and Biochemistry*. 28(3). 341-349.

PEZZOLI, L., ELSON, R., LITTLE, C. L., YIP, H., FISHER, I., YISHAI, R., ANIS, E., VALINSKY, L., BIGGERSTAFF, M., PATEL, N., MATHER, H., BROWN, D. J., COIA, J. E., VAN PELT, W., NIELSEN, E. M., ETHELBERG, S., DE PINNA, E., HAMPTON, M. D., PETERS, T., and THRELFALL. J. (2008). Packed with *Salmonella*: Investigation of an international outbreak of *Salmonella* Senftenberg infection linked to contamination of pre-packed basil in 2007. *Foodborne Pathogens and Disease*. 5(5). 661-668.

POURCHER, A. M., FRANÇOISE, P. B., VURGINIE, F., AGNIESZKA, G., VASILICA, S., and GÉRARD, M. (2007). Survival of faecal indicators and enteroviruses in soil after land-spreading of municipal sewage sludge. *Applied Soil Ecology*. 35(3). 473-379.

PRESCOTT, L. M., HARLEY, J. P., and KLEIN, D. A. (2005a). Part Eight: Human Diseases Caused By Bacteria. In: *Microbiology* (6th ed). McGraw-Hill. Pp 493.

PRESCOTT, L. M., HARLEY, J. P., and KLEIN, D. A. (2005b). Part Eight: Microbial Diseases, Detection, and Their Control. In: *Microbiology* (6th ed). McGraw-Hill. Pp826

PRESCOTT, L. M., HARLEY, J. P., and KLEIN, D. A. (2005c). The Protozoa. In: *Microbiology* (6th ed). McGraw-Hill. p121-122.

PRESCOTT, L. M., HARLEY, J. P., and KLEIN, D. A. (2005d). Chapter 5: Microbial nutrition. In: *Microbiology* (6th ed). McGraw-Hill. p94.

PRESCOTT, L. M., HARLEY, J. P. and KLEIN, D. A. (2005e). Chapter 5: Microbial nutrition; Section 5.7: culture media. In: *Microbiology Microbiology* (6th ed). McGraw-Hill. p103.

PRETTY, J. N., BRETT, C., GEE, D., HINE, R. E., MASON. C. F., MORISON, J. I. L., RAVEN, H., RAYMENT, M. D., and VAN DER BIJL, G. (2000). An

assessment of the total external costs of UK agriculture. *Agricultural Systems*. 65(2). 113-136.

PROSSER, J. I., BOHANNAN, B. J. M., CURTIS, T. P., ELLIS, R. J., FIRESTONE, M. K., FRECKLETON, R. P., GREEN, J. L., GREEN, L. E., KILLHAM, K., LENNON, J. J., OSBORN, M., SOLAN, M., VAN DER GAST, C. J., and YOUNG, J. P. W. (2007). The role of ecological theory in microbial ecology. *Nature Reviews Microbiology*. 5(5). 384-392.

RAAIJMAKERS, J. M., WELLER, D. M., and THONASHOW, L. S. (1997). Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology*. 63(3). 881-887.

RANGEL., J. M., SPARLING, P. H., CROWE, C., GRIFFIN, P. M., and SWERDLOW, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982- 2002. *Emerging Infectious Diseases.* 11(4). 603-609.

RAYNER, M., and SCARBOROUGH, P. (2005). The burden of food related ill health in the UK. *Journal of Epidemiology and Community Health.* 59(12). 1054-1057.

REDDY, K. R., KHALEEL, R., and OVERCASH, M. R. (1981). Behaviour and transport of microbial pathogens and indicator organisms in soils treated with organic wasted. *Journal of Environmental Quality.*10(3). 255-266.

ROBERTS, J., CUMBERLAND, P., SOCKETT, P., WHEELER, J., RODRIGUES, L., SETHI, D., and RODERICK, P. (2003). The study of infectious intestinal disease in England: Socio-economic impact. *Epidemiology and Infection*. 130. 1-11.

ROCOURT, J., MOY, G., VIERK, K., and SCHLUNDT. J. (2003). The present state of foodborne disease in OECD countries. Geneva, Switzerland, World Health Organisation.

ROESCH, L. F. W., FULTHORPE, R. R., RIVA, A., CASELLA, G., HADWIN, A. K. M., KENT, A. D., DAROUB, S. H., CAMARGO, F. A. O., FARMERIE, W. G., and TRIPLETT, E. W. (2007). Pyrosequencing enumerates and contrasts soil

microbial diversity. *The International Society for Microbial Ecology Journal.* 1. 283-290.

ROSS, D. (1995). Recommended soil tests for determining exchange capacity. In: J.T. Sims and A. Wolf (eitors); *Recommended Soil Testing Procedures for the North-Eastern United States.* Northeastern Regional Bulletin #493. Agricultural Experiment Station, University of Delaware, Newark, DE. p. 62-69.

ROSZAK, D.B., and COLWELL, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews.* 51(3). 365-379.

ROWELL, D. L. (1994). Chapter 3: Organic materials - Alive and dead. *In: Soil science: Methods and Applications*. Longman. p 48.

RPA, MILIEU Ltd, and WRc. (2008). Environmental, economic and social impacts of the use of sewage sludge on land - Part 1: Overview report. Study contract DG ENV.G.4/etu/2008/0076r.

SAHLSTRÖM, L., ASPAN, A., BAGGE, E., DANIELSSON-THAM, M. L., and ALBIHNM A. (2004). Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. *Water Research*. 38(8). 1989-1994.

SANTOS, A. C., ROBERTS, J. A., COOK, A. J. C., SIMONS, R., SHEEHAN, R., LANE, C., ADAK, G. K., CLIFTON-HADLEY, F. A., and RODRIGUES, L. C. (2011). *Salmonella Typhimurium* and *Salmonella* Enteritidis in England: costs to patients, their families, and primary and community health services of the NHS. *Epidemiology and Infection.* 139(5). 742-753.

SCHLOTER, M., DILLY, O., and MUNCH, J.C. (2003). Indicators for evaluating soil quality. *Agriculture Ecosystems and Environment*. 98. 255-262.

SCHULTE, E.E., and HOPKINS, B.G. (1996). Estimation of soil organic matter by weight Loss-On- Ignition. In: MAGDOFF, F.R., TABATABAU, M.A., and HANLON, Jr., E.A. (editors); *Soil Organic Matter: Analysis and Interpretation*. Special publication No. 46. Soil science society; Am. Madison, WI. p. 21-32.

SCHWARZ, K. R., SIDHU, J. P. S., PRITCHARD, D. L., LI, Y., and TOZE, S. (2014). Decay of enteric microorganisms in biosolids-amended soil under wheat (*Triticum aestivum*) cultivation. *Water Research.* 59. 185-197.

SI (1989). United Kingdom Statutory Instruments. The sludge (use in agriculture) regulations, 1989. In: Statutory Instrument, No. 1263. Her Majesty's Stationery office, London.

SI. (1990). United Kingdom Statutory Instruments. The Sludge (Use in Agriculture) Regulations 1990 (Amendment). In: Statutory Instrument, No. 880. Her Majesty's Stationery office, London.

SIDHU, J., GIBBS, R. A., HO. G. E., and UNKOVICH, I. (2001). The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Research*. 35(4). 913-920.

SÖDERSTRÖM, A., ÖSTERBERG, P., LINDQVIST, A., JÖNSSON, B., LINDBERG, A., BLIDE ULANDER, S., WELINDER-OLSSON, C., LÖFDAHL, S., KAIJSER, B., DE JONG, B., KÜHLMANN-BERENZON, S., BOQVIST, S., ERIKSSON, E., SZANTO, E., ANDERSSON, S., ALLESTAM, G., HEDENSTÖM, I., LEDET MULLER, L., and ANDERSSON, Y. (2008). A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathogens and Disease*. 5(3). 339-349.

SOLOMON, E. B., YARON, S., and MATTHEWS, K. R. (2002). Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalisation. *Applied Environmental Microbiology*. 68(1). 397-400.

SØRENSEN, L. H. (1974). Rate of decomposition of organic matter in soil as influenced by repeated air drying-re-wetting and repeated additions of organic material. *Soil Biology and Biochemistry.* 6(5). 287-292.

SPARLING, G.P. (1992). Ratio of microbial biomass carbon to soil organic carbon as a sensitive indicator of changes in soil organic matter. *Soil Research.* 30(2). 195-207.

STATS SOFT Inc. (1984 - 2015). Statistica 12. *Stats Soft Inc*. United States of America.

STEINERT, M., EMÖDY, L., AMANN, R., and HACKER, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Applied and Environmental Microbiology*. 63. 2047-53.

STEVENSON, I. L. (1964). Chapter 6: Biochemistry of soils. In: BEAR, F. E. (editor); *Chemistry of the Soil.* Reinhold Publishing Corporation, New York. Pp242.

STOTZKY, G., and NORMAN, A. G. (1961). Factors limiting microbial activities in soil: I. The level of substrate, nitrogen and phosphorus. *Archice für Mikrobiologie*. 40. 341-369.

TATE, R. L. (1995). Soil Microbiology (1st ed). Wiley, New York. Pp 398.

TAM, C. C., RODRIGUESM, L. C., VIVIANI, L., DODDS, J. P., EVANS, M. R., HUNTER, P. R., GRAY, J. J., LET;EY, L. H., RAIT, G., TOMPKINS, D. S., and O'BRIEN, S. J. (2012). Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. *Gut*. 61, (1). 69-77.

TATUM, E. L., and LEDERBERG, J. (1946). Gene recombination in the bacterium *Escherichia coli*. *Journal of Bacteriology.* 53(3). 673-684.

TAYLOR, H. (2003). Chapter 36: Surface waters. In: MARE, D., and HORAN, N (editors); *The Handbook of Water and Wastewater Microbiology.* Elsevier. p615.

THERMO SCIENTIFIC. (2015a). Dehydrated Culture Media: Sorbitol MacConkey. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0813&org=72&c=UK&lang=EN (Accessed: 20th February 2015).

THERMO SCIENTIFIC. (2014b). Standard plate count agar: CM0463. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0463&c=UK&lang=EN (Accessed: 20th February 2015).

TOPP, E., WELSH, M., TIEN, Y-C., DANG, A., LAZAROVITS, G., CONN, K., and ZHU, H. (2003). Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soils. *FEMS Microbiology Ecology.* 44. 303-308.

TORSVIK, V., GOKSØYR, J., and DAAE, F. L. (1990). High diversity in DNA of soil bacteria. *Applied and Environmental Microbiology*. 56(3).782-787.

TROXLER, J., SVERCEL, M., NATSCH, A., ZALA, M., KEEL, C., MOËNNE-LOCCOZ, Y., and DÉFAGO, G. (2012). Persistence of a biocontrol *Pseudomonas* inoculant as high populations of culturable and non-culturable cells in 200-cm-deep soil profiles. *Soil Biology and Biochemistry.* 44. 122-129.

VANCE, E. D., BROOKES, P. C., and JENKINSON, D. S. (1987). An extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry*. 19(6). 703-707.

VAN ELSAS, J. D., CHIURAZZI, M., MALLON, C. A., ELHOTTOVÁ, D., KRIŠTŮFEK, V., and SALLES, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *PNAS*. 109(4). 1159-1164.

VAN ELSAS, J. D., HILL, P., CHROŇÁKOVÁ, A., GREKOVA, M., TOPLAOVA, Y., ELHOTTOVÁ, D., and KRIŠTŮFEK, V. (2007). Survival of genetically marked *Escherichia coli* 0157:H7 in soil as affected by soil microbial community shifts. *The ISME Journal*. 1. 204-214.

VAN VEEN, J. A., VAN OVERBEEK, L. S., and VAN ELSAS, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews.* 61(2). 121-135.

VAN VEEN, J. A., and KUIKMAN, P. J. (1990). Soil structural aspects of decomposition of organic matte by microorganisms. *Biogeochemistry.* 11(3). 213-233.

VAN VLIET, A. H. M., and KETLEY, J. M. (2001). Pathogenesis of enteric *Campylobacter* infection. *Journal of Applied Microbiology.* 90(6). 45-56.

VINCEVICIENE, V. (2007). Chapter 1: Wastewater regulation. In: QUEVAUVILLER, P., THOMAS, O., AND VAN DER BEKEN, A. (editors); *Wastewater Quality Monitoring and Treatment* (1st ed). John Wiley and Sons, Ltd. Pp1.

VIVANT, A. L., GARMYN, D., MARON, P. A., NOWAK, V., and PIVETEAU, P. (2013). Microbial diversity and structure are drivers of the biological barrier effect against *Listeria monocytogenes* in soil. *PloS One*. 8(10). e76991.

WANG, H., ZHANG, T, WEI, G., WU, L., WU, J., and XU, J. (2014). Survival of Escherichia coli O157: H7 in soils under different land use types. *Environmental Science and Pollution Research*. 21(1). 518-524.

WATER UK. (2010). Recycling of biosolids to agricultural land. Issue 3. January 2010. Water UK, 1 Queen Anne's Gate, London, SW1H 9BT. Available at: www.water.org.uk. (Accessed: 20th February 2015).

WERTHER, J., and OGADA, T. (1999). Sewage sludge combustion. *Progress in Energy and Combustion Science*. 25(1). 55-116.

WEST, A. W., and SPARLING, G. P. (1986). Modifications to the substrate-induced respiration method to permit measurement of microbial biomass in soils of differing water contents. *Journal of Microbiological Methods*. 5. 177-189.

WHITTAM, T. S. (1989). Clonal dynamics of *Escherichia coli* in its natural habitat. *Antonie van Leeuwenhoek*. 55(1). 23-32.

WHO. (2011). Campylobacter - Fact sheet No. 255. Available at: http://www.who.int/mediacentre/factsheets/fs255/en/. (Accessed: 9th December 2014).

WHO. (2013). Diarrhoeal disease - Fact sheet No. 330. Available at: http://www.who.int/mediacentre/factsheets/fs330/en/. (Accessed: 9th December 2014).

WHO. (2015). Food safety: estimation of the global burden of food borne diseases. Available at: http://www.who.int/foodsafety/areas_work/foodborne-diseases/ferg/en/ (Accessed: 13th January 2015).

WIEGERT, R. G., and OWEN, D. F. (1971). Trophic structure, available resources and population density in terrestrial vs. aquatic ecosystems. *Journal of Theoretical Biology*. 30. 69-81.

WINFIELD, M. D., and GROISMAN, E. A. (2003). Role of non-host environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied Environmental Microbiology*. 69(7). 3687-3694.

WRIGHT, D. A., KILLHAM, K., GLOVER, A., and PROSSER, J. I. (1995). Role of pore size location in determining bacterial activity during predation by protozoa in soil. *Applied and Environmental MicrobiologyI.* 61(10). 3537-3543.

YAO, Z., WEI, G., WANG, H., WU, L., WU, J., and XU, J. (2013). Survival of *Escherichia coli* O157: H7 in soils from vegetable fields with different cultivation patterns. *Applied and Environmental Microbiology*. 79(5). 1755-1756.

YOU, Y., RANKIN, S. C., ACETO, H. W., BENSON, C. E., TOTH., J. D., and DOU, Z. (2006). Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology.* 72(9). 5777-5783.

Appendix 1

The following experiments were run to practice procedures and become more familiar with microbiological work. They were also undertaken to test the quality of the methods that were to be implemented in the main body of work, more specifically, the extraction procedure from Troxler, *et al.* (2012). Below is a general/brief description of the procedures and subsequent results. As such they are not intended as independent chapters though they do illustrate a development of knowledge and understanding. Furthermore, no statistical analyses were performed as this work was only intended for basic training purposes. Therefore, only general conclusions are made. Where weaknesses were identified, improvements were made. The final description of the modified protocol by Troxler, *et al.* (2012) can be found in Chapter 3, Section 3, with further specific information found in each subsequent experimental chapter.

1.1. Recovery of *E. coli* lys 9 from live and dead soil

Introduction

This experiment sought to assess the recovery rate of *E. coli* lys 9 from live and dead soil. When inoculating soil, generally a known amount of cells are added. It was theorised that upon immediate extraction, i.e. without incubation for any period of time, the loss of cells between treatments (in this instance live/dead soil) would highlight how many cells are lost due to the change in physicochemical and biological parameters. The loss of cells could be related to a change in the likelihood of biofilm formation on particulate surface or possibly from cell death due to the change in environment from nutrient broth (an optimal environment for growth) to soil (a less optimal environment for growth). This then, led to the question, how would the recovery of *E. coli* lys 9 be affected when it is inoculated into live or dead soil?

Objective

To assess the recovery rate of *E.coli* lys 9 from live and dead soils

Method

A brown earth soil was collected in triplicate from a cattle-grazed pasture at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland (52.3342°N, −6.4575°W). It was then homogenised and sieved to a particle size of 4 mm. The moisture content of the soil was found to be 33.6 % ± 1.2 (mean ± SEM, n=3). The resulting composite sample was then split in two. One half was used for the fresh or live soil treatments. The other half was autoclaved at 121 °C for 30 minutes to destroy any living microorganisms. This was then to be used for the dead soil treatments. The treatments (outlined in Table 1a) were then weighed (5 g ± 0.05 g), in triplicate, into 50 ml test tubes. Aliquots (0.1 ml) of *E. coli* lys 9, grown in LB broth and washed with quarter strength Ringer's solution were subsequently inoculated into each of the relevant treatments, as outlined in Chapter 3.3.3. A dilution series was then created from the culture and used to assess absorbency and cell count, again using quarter strength Ringer's solution (see Chapter 3.3.3). The treatments amended with quarter strength Ringer's (0.1 ml) effectively acted as control treatments, allowing for the assessment of background levels of *E. coli*. The cohort of treatments then underwent the extraction protocol outlined by Troxler, *et al.* (2012). A 10-fold dilution was then formulated from the solution and was then enumerated for *E. coli* lys 9, using SMAC agar, as outlined in Chapter 3.3.4. Controls for ambient air conditions, Ringer's and broth were plated out also. The plates were then incubated at 37 °C for 24 hours.

The recovery rate (%) was then calculated by implementing the following equation:

$$\text{Recovery rate} = (X/Y)*100 \tag{4}$$

Where X is the quantity of *E. coli* lys 9 recovered after inoculation into the treatments, and Y is the quantity of *E. coli* lys 9 initially added to those treatments.

Table 1a. Treatment outline (n = 3) based on fresh weight of soil.

No.	Contents
1	Live soil, inoculated with *E.coli* lys 9
2	Live soil, amended with 1/4 strength Ringer's
3	Dead soil, inoculated with *E.coli* lys 9
4	Dead soil, amended with 1/4 strength Ringer's
5	No soil, inoculated with *E.coli* lys 9

Results

The initial quantity of *E. coli* lys 9 added to relevant treatments was approximately 1.10 x 10^9 CFU ml^{-1}. This was further supported by the estimated value obtained from the regression equation produced from Figure 1a, which estimated 1.04 x 10^9 CFU ml^{-1} (Table 1b). The quantity of *E. coli* lys 9 extracted from each treatment varied (Table 1c), though the SEM for each treatment was also quite high. Furthermore, the recovery rate for the two treatments containing soil inoculated with *E. coli* lys 9 were both below 50%. However, the treatment containing no soil but inoculated with *E. coli* lys 9 showed a recovery rate higher than this.

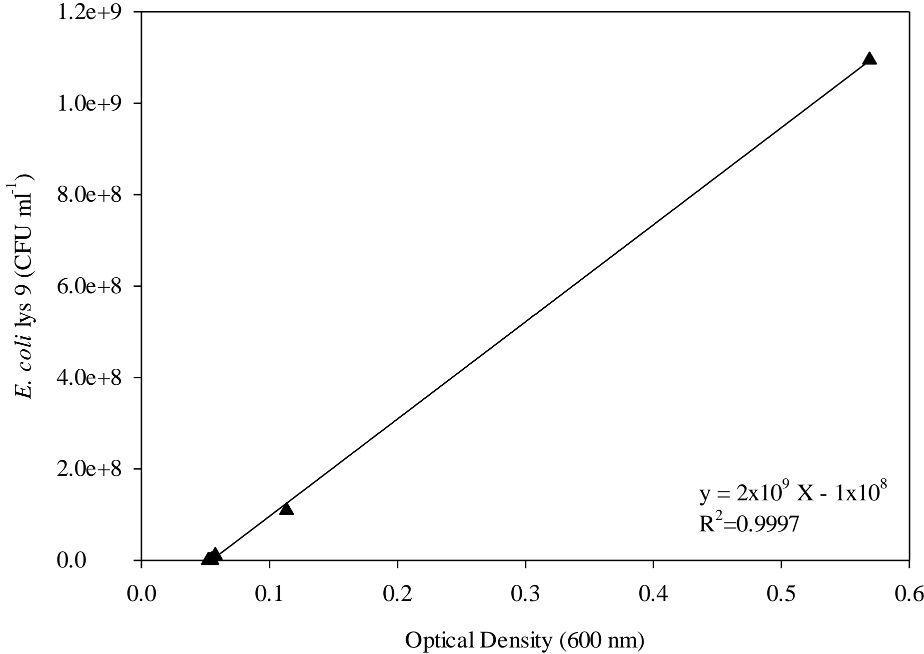

Figure 1a. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* lys 9; — linear regression equation.

Table 1b. Estimated quantity of inoculated *E. coli* lys 9, obtained from the regression equation calculated from the data in Figure 10.1. vs. plate count data. * calculated using regression equation where X equals average absorbency (600 nm)

Pathogen	Average Absorbance (600 nm)	Regression equation	Estimated (CFU ml^{-1}) *	Plate count (CFU ml^{-1})
E.coli lys 9	0.57	$y = 2x10^9 X - 1x10^8$	$1.04x10^9$	$1.10x10^9$

Table 1c. Quantity of *E. coli* lys 9 recovered from each treatment (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment. *Not applicable (NA) as the soil within these treatments was not inoculated prior to extraction

Treatment	*E. Coli* lys 9 (CFU ml^{-1})	Recovery rate (%)
Live soil, inoculated with *E.coli* lys 9	$2 x10^6 \pm 2 x10^5$	16.0
Live soil, amended with 1/4 strength Ringer's	$8 x10^4 \pm 8 x10^4$	NA*
Dead soil, inoculated with *E.coli* lys 9	$3 x10^6 \pm 4 x10^5$	29.7
Dead soil, amended with 1/4 strength Ringer's	$8 x10^4 \pm 6 x10^4$	NA*
No soil, inoculated with *E.coli* lys 9*	$7 x10^6 \pm 5 x10^5$	65.9

Discussion

The high error rate within the recovery data was attributed to human error, or rather to a lack of experience with the protocols in use. It should be highlighted that this was the first attempt made at microbiological work and therefore such inaccuracy is not unexpected.

The presence of live or dead soil appeared to affect the recovery of *E. coli* lys 9, especially as the recovery rate within the treatments without soil were much higher. Additionally, the recovery rates of *E. coli* lys 9 from soil were unexpectedly low. The reason for this was as yet unknown, so it was decided that further testing of the same protocols with a greater range of model pathogens was required.

1.2. Extraction of four model pathogens from fresh soil

Introduction

This experiment built on the last by increasing the number of model pathogens studied. The low recovery of *E. coli* lys 9 was unusually small considering that there was no incubation period between inoculation and extraction. Therefore, it was decided that further testing of the same protocols with a greater range of model pathogens was required (*E. coli* Lys 9, non-toxigenic lux marked *E. coli* O157, *L. monocytogenes* and *S.* Dublin). This would clarify whether such a phenomena could be singularly attributed to *E. coli* lys 9 or if it occurred across all model pathogens to be studied. This then led to the question, how would the recovery of each model pathogen be affected when inoculated into (live) soil?

Objective

To assess the recovery rate of four different types of model pathogens: *E. coli* Lys 9, non-toxigenic lux marked *E. coli* O157, *L. monocytogenes* and *S.* Dublin, when inoculated into soil.

Method

A brown earth soil was collected in triplicate from a cattle-grazed pasture at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland (52.3342°N, −6.4575°W). It was then homogenised and sieved to a particle size of 4 mm. The moisture content of the soil was found to be 32.9 % ± 0.8 (mean ± SEM, n=3). The resulting composite sample was then weighed out (5 g ± 0.05 g), in triplicate, into 50 ml test tubes. A treatment outline is provided in Table 1d. Aliquots (0.1 ml) of model pathogen, *E. coli* Lys 9, non-toxigenic lux marked *E. coli* O157, *L. monocytogenes* and *S.* Dublin, were grown in sterile LB broth and washed with quarter strength Ringer's solution. They were subsequently inoculated into each of the relevant treatments, as described in Chapter 3.3.3. A dilution series was then created from the

culture and used to assess absorbency and cell count, again using quarter strength Ringer's solution (see Chapter 3.3.3). The treatments amended with quarter strength Ringer's (0.1 ml) effectively acted as control treatments, allowing for the assessment of background levels of *E. coli*. The cohort of treatments then underwent the extraction protocol outlined by Troxler, *et al.* (2012). A 10-fold dilution was then formulated from the solution and was then enumerated for *E. coli* lys 9, non-toxigenic lux marked *E. coli* O157, *L. monocytogenes* and *S.* Dublin. SMAC agar was used to enumerate *E. coli* lys 9, SMAC amended with kanamycin for the lux marked *E. coli* O157, XLD for the *S.* Dublin and *Listeria* selective agar (oxford formulation) for the *L. monocytogenes* (Oxoid Ltd, 2014c). Controls for ambient air conditions, Ringer's and broth were also plated out for each agar. The plates were then incubated at 37 °C for 24 hours. Plates containing *L. monocytogenes* and respective controls were left for 48 hours at 35°C. Recovery rate for each model pathogen was then calculated as outlined in Appendix 1, Section 1.1.

Table 1d. Treatment outline (n = 3) based on fresh weight of soil.

No.	Contents
1	*E. coli* lys 9
2	non-toxigenic lux marked *E. coli* O157
3	*L. monocytogenes*
4	*S.* Dublin

Results

The initial quantity of each model pathogen added was approximately three orders of magnitude lower than the estimated value (Table 1e). Though the regression analyses produced appeared to be accurate with R^2 values above 0.95 (Figure 1b; Figure 1c; Figure 1d; Figure 1e). Furthermore the quantity of each model pathogen recovered from the soil again had quite high errors (Table 1f). Additionally, the recovery rate for each model pathogen varied, with *E. coli* Lys 9, *L. monocytogenes* and *S.* Dublin recovery rates falling below 50%. In contrast *E. coli* 0157 had a recovery rate above 100%, this was attributed to some form of contamination.

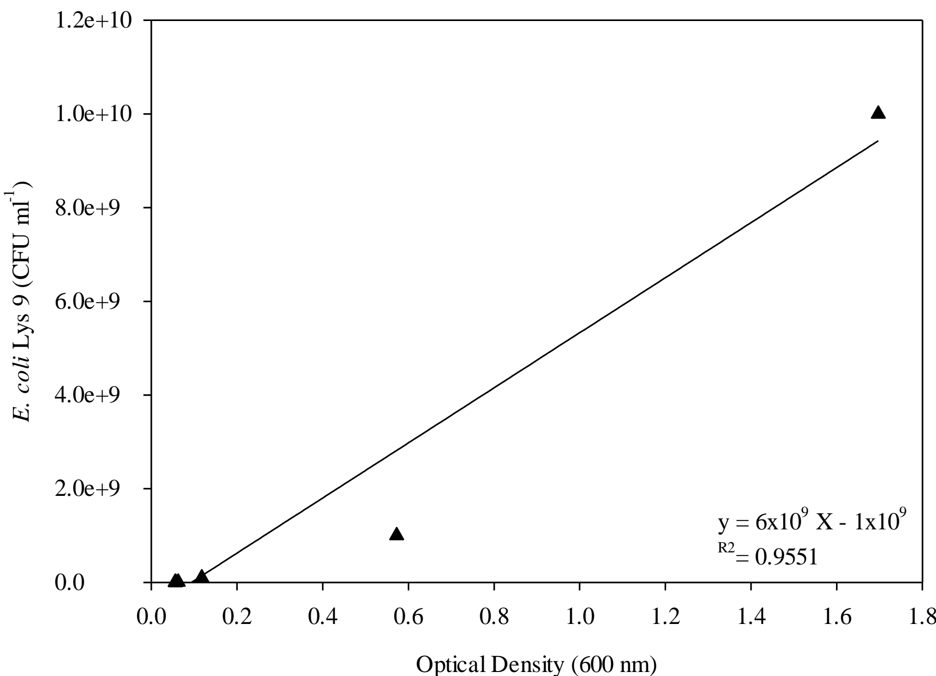

Figure 1b. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* lys 9; — linear regression equation.

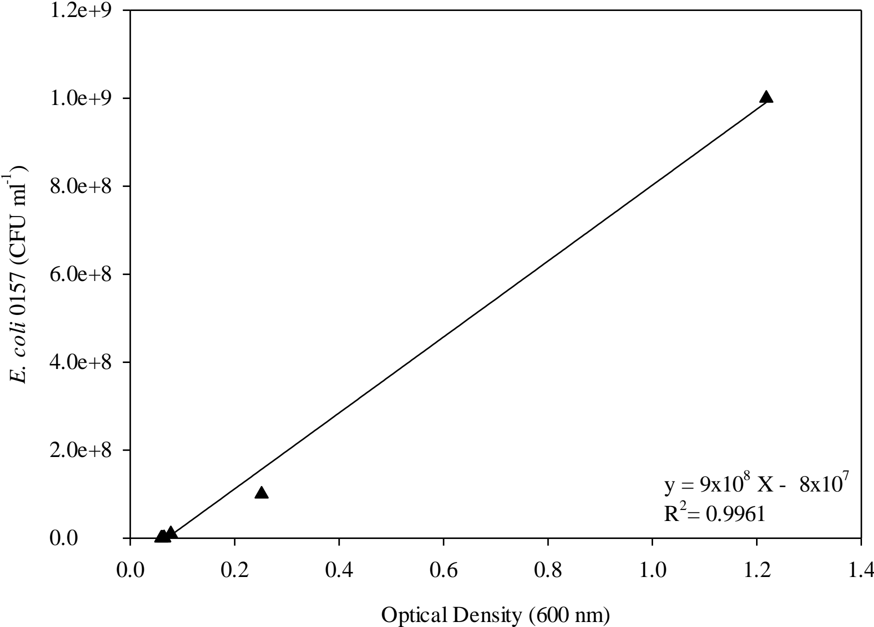

Figure 1c. Optical density of *E. coli* O157 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli* O157; — linear regression equation.

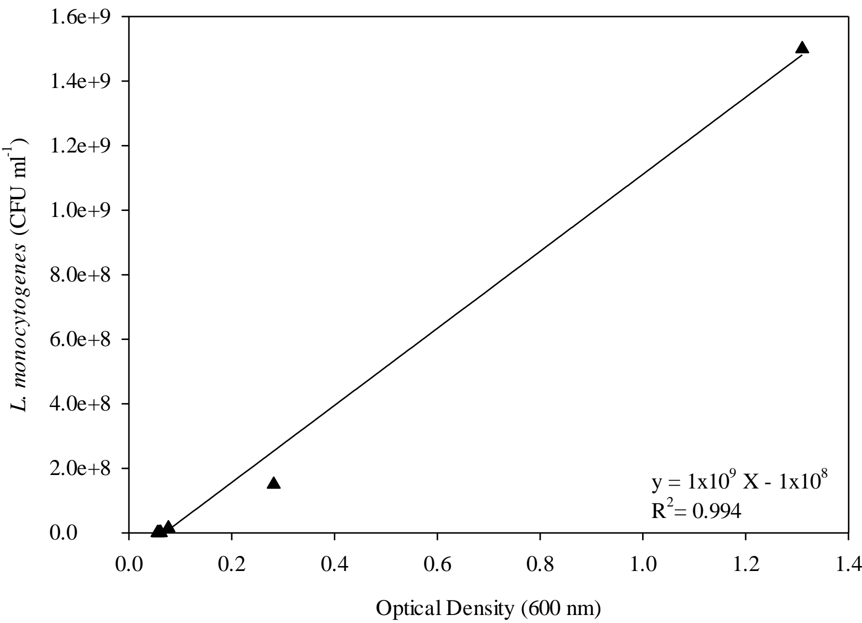

Figure 1d. Optical density of *L. monocytogenes* in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *L. monocytogenes*; — linear regression equation.

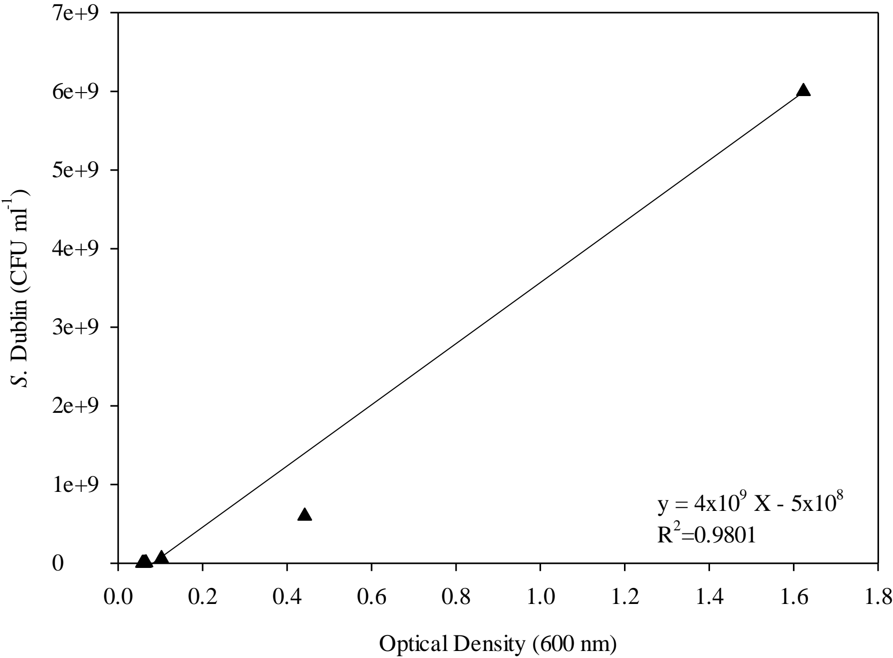

Figure 1e. Optical density of *S*. Dublin in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *S*. Dublin; — linear regression equation.

Table 1e. Estimated quantity of inoculated model pathogens, obtained using the regression equation obtained from the preceding Figures vs. against plate count data. * calculated using regression equation where X equals average absorbency (600 nm)

Pathogen	Average Absorbance (600 nm)	Regression equation	Estimated (CFU ml^{-1}) *	Plate count (CFU ml^{-1})
E.coli lys 9	0.71	$y = 6x10^9 X - 1x10^9$	$3.24x10^9$	$9.50x10^5$
E. coli O157	0.33	$y = 9x10^8 X - 8x10^7$	$2.21x10^8$	$2.47x10^6$
L. monocytogenes	0.35	$y = 1x10^9 X - 1x10^8$	$2.54x10^8$	$9.00x10^5$
S. Dublin	0.59	$y = 4x10^9 X - 5x10^8$	$1.85x10^9$	$2.93x10^6$

Table 1f. Quantity of each model pathogen recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment.

Treatment	Cell count (CFU ml^{-1})	Recovery rate (%)
E. coli lys 9	$1x10^5$ ± $2x10^4$	2.96
E. coli O157	$2x10^5$ ± $3x10^4$	112
L. monocytogenes	$9x10^5$ ± $3x10^4$	35.8
S. Dublin	$3x10^5$ ± $1x10^4$	16.1

Discussion

It would appear that recovery rates vary between model pathogens. However, there were high error rates within the recovery data, as occurred in the previous experiment. This was again attributed to human error. The initial quantity added, when compared to the estimated data was also dissimilar. The reasons for this are unknown. As with the previous experiment, three of the model pathogens (*E. coli* Lys 9, *L. monocytogenes* and *S.* Dublin) used had recovery rates below 50%, which seems unusual considering they had not been incubated within the soil for any period of time. Additionally the recovery rate for *E. coli* 0157, which was above 100%, was attributed to some form of contamination during the experimental process.

1.3. The effect of different proportions of sludge to soil on the recovery rate of *E. coli* lys 9

Introduction

Considering the inaccuracy of the previous work, it was decided that more practice was necessary. As an integral part of this project focuses on the impact of sludge amendments to soil, it was proposed that investigating how increasing proportions of sludge to soil would affect the recovery rates would be appropriate. This lead to the following question, will the increasing proportion of sewage sludge to soil affect the recovery rate of *E. coli* lys 9 from soil?

Objective

To assess the recovery rate of *E. coli* lys 9 in microcosms containing proportions of sewage sludge to soil.

Method

A brown earth soil was collected in triplicate from a cattle-grazed pasture at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland (52.3342°N, −6.4575°W). Sewage sludge was obtained from United Utilities, Ellesmere Port, UK. Both matrices were autoclaved at 121 °C for 30 minutes on two consecutive days, in order to sterilise them. They were then homogenised and sieved to a particle size of 4 mm. The mean moisture content of the soil was found to be 25.6 % ± 0.6 (mean ± SEM, n=3), whilst the mean moisture content of the sewage sludge was found to be 78.4% ± 1.4 (mean ± SEM, n=3). The soil and the sewage sludge were also assessed for background levels of *E. coli* using SMAC agar. The level of E. coli within both matrices was found to be below the limits of detection. The treatments were then weighed (5 g ± 0.05 g), in triplicate, into 50 ml test tubes as outlined in Table 1g. Aliquots (0.1 ml) of *E. coli* lys 9, grown in LB broth and washed with quarter strength Ringer's solution, were subsequently inoculated into each of the treatments, as outlined

in Chapter 3.3.3. The optical density was the same as the previous experiment (Chapter 10.2). The cohort of treatments then underwent the extraction protocol outlined by Troxler, *et al.* (2012). A 10-fold dilution was then formulated from the solution and was then enumerated for *E. coli* lys 9, using SMAC agar, as outlined in Chapter 3.3.4. Controls for ambient air conditions, Ringer's and broth were plated out also. The plates were then incubated at 37 °C for 24 hours. Recovery rate was then calculated as outlined in Appendix 1, Section 1.1.

Table 1g. Treatment outline (n = 3) based on the fresh weight of soil and sewage sludge (± 0.05 g).

No.	Contents	Soil (g)	Sewage sludge (g)
1	Control Soil	5	0
2	25% sludge	3.75	1.25
3	50% sludge	2.5	2.5
4	75% sludge	1.25	3.75
5	Control sludge	0	5

Results

The quantity of *E. coli* lys 9 recovered from the treatment cohort was generally within the 10^6 CFU ml^{-1} range (Table 1h). Again there were quite high errors, within the 10^5 CFU ml^{-1} range. Furthermore, the recovery rate of *E. coli* lys 9 was smallest in the soil, whilst the highest recovery rate was found in the 75% sludge.

Table 1h. Quantity of each *E. coli* lys 9 recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment.

Treatment	*E. coli* lys 9 (CFU ml^{-1})	Recovery rate (%)
Control Soil	$3x10^6 \pm 4x10^5$	11.3
25% sludge	$9x10^6 \pm 6x10^5$	15.6
50% sludge	$7x10^6 \pm 9x10^5$	22.3
75% sludge	$7x10^6 \pm 9x10^5$	24.9
Control sludge	$7x10^6 \pm 8x10^5$	21.2

Discussion

Increasing proportions of sewage sludge to soil did appear to increase the recovery rate of *E. coli* lys 9. However, this was not the case with the control sludge treatment, as its recovery rate of *E. coli* lys 9 was slightly less than both 50% sludge and 75% sludge treatments. The continued low recovery rates and high errors were unusual and it as proposed that the extraction protocol in use may be too extreme, thus leading to cell death and resulting in the unusual data findings.

1.4. The effect of increased quarter strength Ringer's solution, during extraction of *E. coli* lys 9 from soil

Introduction

Within the previous three experiments, there were consistently high errors within the cell count data, as well as quite small recovery rates. The small recovery rates seemed quite unusual, considering the model pathogens used had not been incubated for any length of time within the matrices in use. It was theorised that the protocol used to extract them from the soil and sewage sludge could have been too harsh, thereby destroying the model pathogens during the extraction process, or that not enough solution was being added to extract them. Therefore a modified protocol was implemented, where a greater volume of quarter strength Ringer's was added and a less harsh extraction regime used. This extraction regime included 30 minutes end-over-end and 10 seconds vortexing, both at moderate speeds. This lead to the questions, how will using a modified extraction protocol affect the recovery rate of *E. coli* lys 9 in live and dead soil?

Objective

To assess the recovery rate of *E.coli* lys 9 from live and dead soils, when using a modified extraction protocol.

Method

A brown earth soil was collected in triplicate from a cattle-grazed pasture at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland (52.3342°N, −6.4575°W). It was then homogenised and sieved to a particle size of 4 mm. The moisture content of the soil was found to be 33.6 % ± 1.2 (mean ± SEM, n=3). The resulting composite sample was then weighed out (5 g ± 0.05 g), in triplicate, into 50 ml test tubes. A treatment outline is provided in Table 1i. Aliquots (0.1 ml) of *E. coli* lys 9, grown in LB broth and washed with quarter strength Ringer's solution, were

subsequently inoculated into each of the relevant treatments, as outlined in Chapter 3.3.3. A dilution series was then created from the culture and used to assess absorbency and cell count, again using quarter strength Ringer's solution. The treatments amended with quarter strength Ringer's (0.1 ml) effectively acted as control treatments, allowing for the assessment of background levels of *E. coli*. The cohort of treatments then underwent a modified protocol similar to that described by Troxler, *et al.* (2012). This extraction process is described in Chapter 3.3.3. Quarter strength Ringer's solution (50 ml) was added to each sample, which were then shaken by end-over-end rotation (100 rpm) for 30 minutes. The samples were then vortexed for 10 seconds. A 10-fold dilution was then formulated from the solution and was then enumerated for *E. coli* lys 9, using SMAC agar, as outlined in Chapter 3.3.4. Controls for ambient air conditions, Ringer's and broth were plated out also. The plates were then incubated at 37 °C for 24 hours. Recovery rate was then calculated as outlined in Appendix 1, Section 1.1.

Table 1i. Treatment outline (n = 3) based on fresh weight of soil (± 0.05 g). ***contained 50ml 1/4 strength Ringer's**

No.	Contents
1	Live soil, inoculated with *E.coli* lys 9
2	Live soil, amended with 1/4 strength Ringer's
3	Dead soil, inoculated with *E.coli* lys 9
4	Dead soil, amended with 1/4 strength Ringer's
5	No soil, inoculated with *E.coli* lys 9*

Results

The initial quantity of *E. coli* lys 9 added to each (relevant) treatments was approximately 8.1 x 10^7 CFU ml^{-1}. This was further supported by the estimated value obtained from the regression equation produced from Figure 1f, which estimated 1.04 x 10^7 CFU ml^{-1} (Table 1j). The quantity of *E. coli* lys 9 extracted from each treatment varied (Table 1k). Additionally, the SEM for each treatment was lower than in the previous work, notably in the thousands rather than hundreds of thousands. A more reasonable error rate when considering the cell counts were in the millions to tens of millions. Furthermore, the recovery rate for two of the treatments, containing live soil

and no soil, were more than doubled when compared to the experiment discussed in Appendix 1, Section 1.1. However, the treatment containing dead soil showed similar recovery rates as in this previous work.

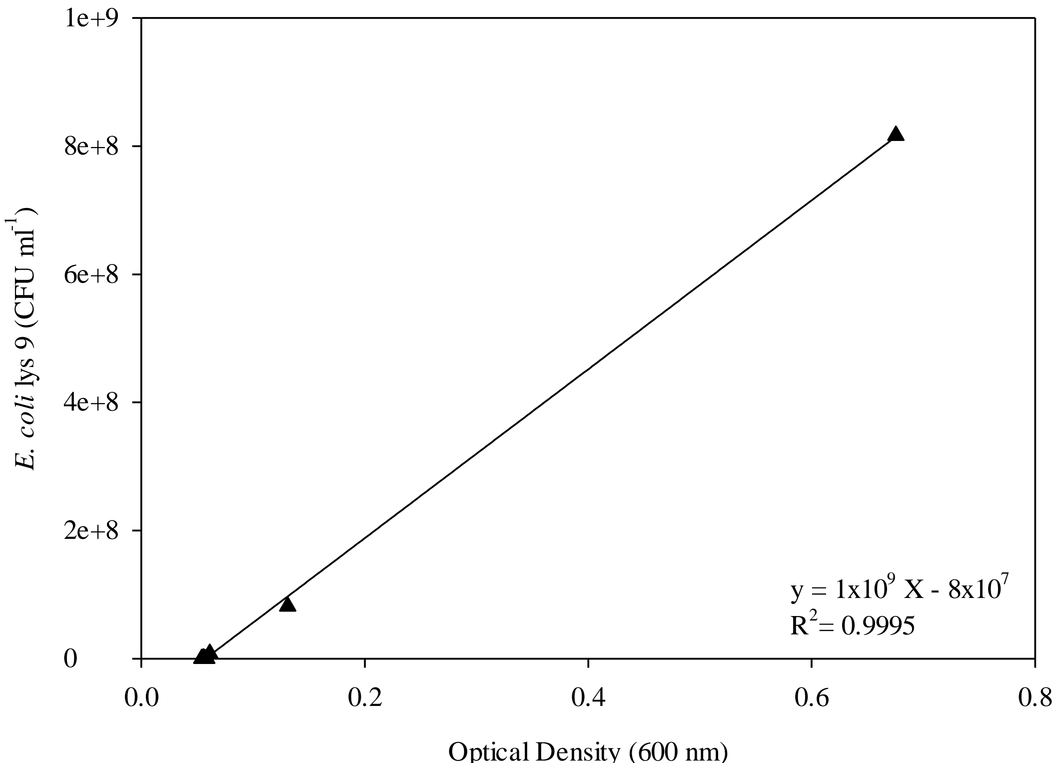

Figure 1f. Optical density of *E. coli* lys 9 in LB broth (serially diluted by a factor of 10) vs. CFU ml^{-1} data ta. Key: ▲ optical density vs. *E. coli* lys 9; — linear regression equation.

Table 1j. Estimated *E. coli* lys 9, obtained from the regression equation calculated from the data in Figure 1.6. against actual plate count data. * calculated using regression equation where X equals average absorbency (600 nm)

Pathogen	Average Absorbance (600 nm)	Regression equation	Estimated (CFU ml^{-1}) *	Plate count (CFU ml^{-1})
E.coli lys 9	0.57	$y = 2x10^8 X - 1x10^7$	$1.04x10^7$	$8.17x10^7$

Table 1k. Quantity of each model pathogen recovered from soil (mean ± SEM, n=3), with corresponding recovery rate calculated using the mean for each treatment.

Treatment	***E. coli* lys 9 (CFU ml^{-1})**	**Recovery rate (%)**
Live soil, inoculated with *E.coli* lys 9	$8x10^6$ ± $5x10^4$	77.7
Live soil, amended with 1/4 strength Ringer's	0 ± 0	NA*
Dead soil, inoculated with *E.coli* lys 9	$3x10^6$ ± $9x10^4$	33.7
Dead soil, amended with 1/4 strength Ringer's	0 ± 0	NA*
No soil, inoculated with *E.coli* lys 9	$7x10^6$ ± $8x10^3$	69.6

Discussion

The use of the modified extraction protocol appeared to improve the recovery rate in both live soil and the treatment containing no soil. As future experiments plan on incorporating live soil, due to the premise of the work involving microbial competition for resources, this modified protocol will be of use in accurately defining pathogen persistence. Error rates were also reduced tenfold, with more experience likely also contributing to greater precision in future work. Again, the presence of live or dead soil appeared to affect the recovery of *E. coli* lys 9, with recovery rates in live soil being more than double that recovered from the dead soil.

1.5. Comparison of enumeration methods.

Introduction

With the refinement of the extraction method, and following the findings within the experiments outlined in Chapter 4, the enumeration technique of pathogens was questioned. Especially in light of the survival of indigenous sewage sludge *E. coli*. The basic plating techniques were appropriate for the model pathogens in use but if we wanted to look at the survival of indigenous sewage sludge *E. coli*, a more pertinent technique would be required. Therefore, the natural levels of *E.* coli within a batch of sewage sludge was analysed using three enumeration methods, basic plating, chromogenic membrane filtration and Most Probably Number (MPN).

Objective

To clarify which enumeration technique is best implemented when studying indigenous sewage sludge pathogens.

Method

Sewage sludge was obtained from United Utilities, Ellesmere Port, UK. It was left unaltered in cold storage for the duration of this experiment at 4°C. Its mean moisture content was 77.4 % ± 0.9 (mean ± SEM, n=3). Samples (60) of pure sewage sludge were then weighed (5 g ± 0.05 g), into sterile 100 ml screw cap containers. Parafilm was then placed over the top, to preserve sterility but also provide aerobic conditions. At each time point (0, 1, 3, 7, 13), a total of 12 samples was removed for analysis, 4 replicates for each enumeration protocol. The samples then underwent a modified extraction protocol similar to the one by Troxler, *et al.* (2012), which can be found in Chapter 3.3.3. They were then enumerated using the previously discussed techniques; the basic plating technique, using SMAC agar (see Chapter 3.3.4. for description of protocol), chromogenic membrane filtration technique (see Chapter 3.3.5. for description of protocol) and MPN (Environment Agency, 2003).

Results

The levels of indigenous sewage sludge *E. coli* within the batch of sewage sludge generally held at $1x10^9$ CFU ml^{-1} for 13 days when enumerated using the basic plating technique (Figure 1g). In contrast, the levels of *E. coli* within the same batch of sewage sludge held at approximately $1.5x10^6$ CFU ml^{-1} when enumerated using the membrane chromogenic technique (Figure 1h). This technique also highlighted the survival of general coliform bacteria, the levels of *E. coli* within this batch of sewage sludge held at $1.5x10^6$ CFU ml^{-1} when enumerated using the MPN technique (Figure 1i).

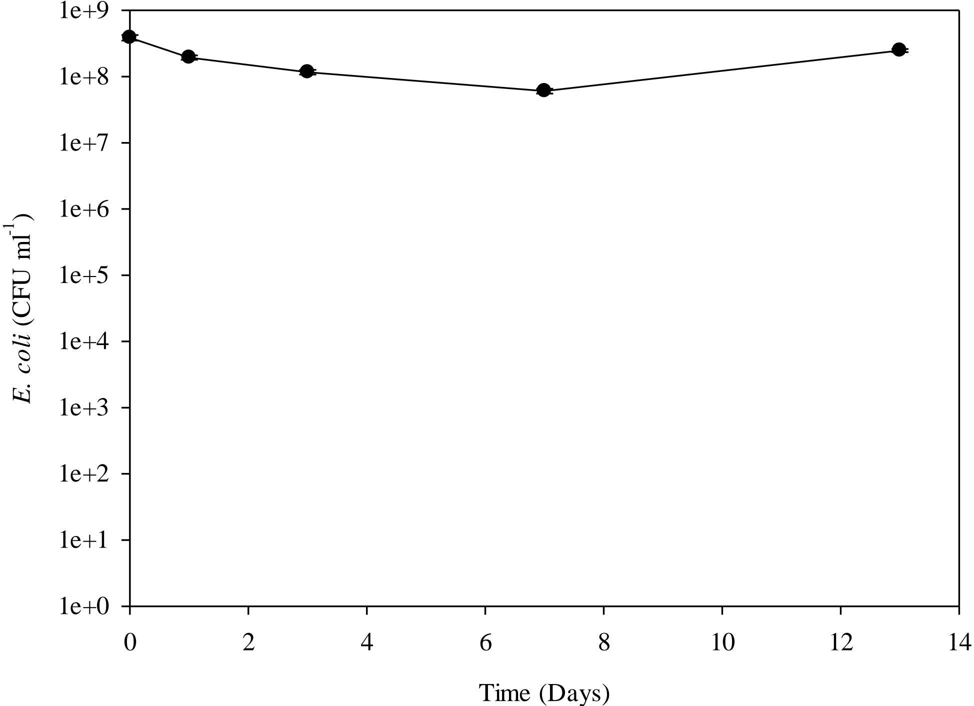

Figure 1g. Survival of indigenous sewage sludge *E. coli* within microcosms comprising of sewage sludge mixtures enumerated using basic plating technique (n = 4, mean ± SEM). Key: —●— *E. coli*. ***SEM falls** within the width of the observed symbols

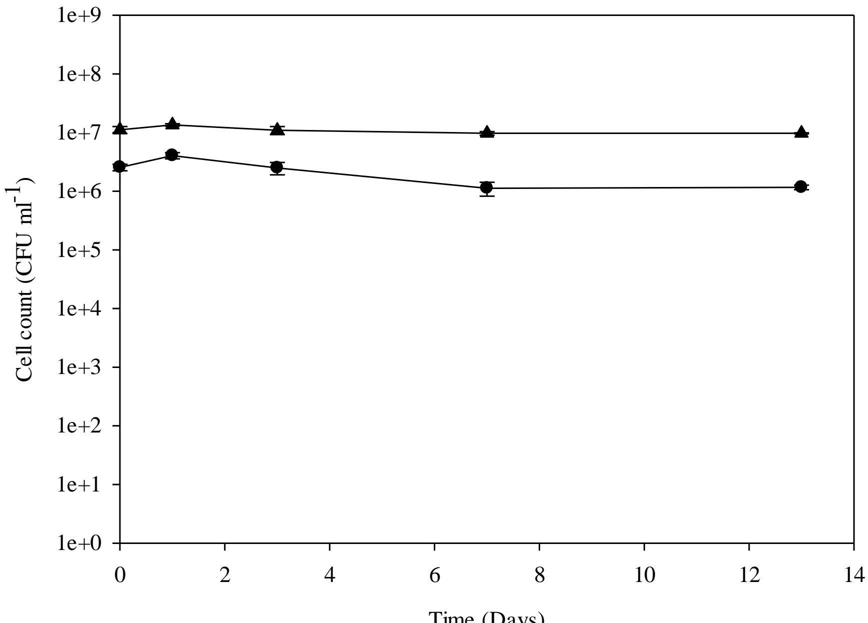

Figure 1h. Survival of indigenous sewage sludge *E. coli* and general coliform bacteria within microcosms comprising of sewage sludge mixtures enumerated using chromogenic membrane filtration technique (n=4, mean ± SEM). Key: —●— *E. coli*; —▲— general coliforms. ***SEM falls** within the width of the observed symbols

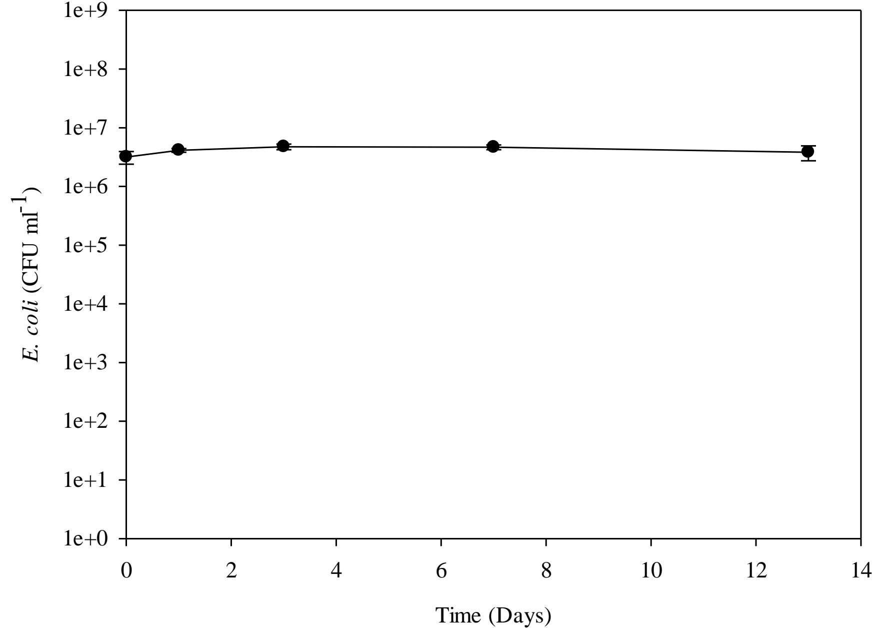

Figure 1i. Survival of indigenous sewage sludge *E. coli* within microcosms comprising of sewage sludge mixtures enumerated using MPN technique (n=4, mean ± SEM). Key: —●— *E. coli*. ***SEM falls** within the width of the observed symbols

Discussion

The use of three enumeration techniques led to the production of marginally similar results. However, the first protocol used, the basic plating onto SMAC agar, showed slightly higher reading than the other two. This could have been because the agar was not specific enough to handle the analysis of sewage sludge. Chromogenic membrane filtration and MPN are both used widely within the wastewater industry to analyse the pathogen load in sewage sludge, as such they are probably better suited for use in future experiments. However, the MPN protocol requires a large quantity of material to be produced for the analysis, which may not be easily manageable during large scale experiment. Therefore, the chromogenic membrane filtration ill most likely be used instead.

Appendix 2

Introduction

Below are the inoculum densities for model pathogens, *E. coli* lys 9 and *S.* Dublin, used in Chapter 4. Comparing optical density to CFU ml^{-1} data of the original inoculum is a fast way of accurately determining cell concentration.

Method

For the full description of the experiment associated with this segment of work, see Chapter 4.

Inoculum preparation and analysis for cell concentration

Aliquots of *E. coli* and *S.* Dublin were removed from frozen storage (-20°C) and inoculated into separate 30 ml plastic vials of sterile LB. They were then incubated at 37°C, with un-spiked LB acting as control treatments for 24 h. Aliquots from these solutions were then added to fresh vials of LB and incubated again for 24 hours at 37°C. The LB was then removed by centrifuging at 4500 rpm for 10 minutes and the supernatant poured off. The remaining cell pellets were then re-suspended in 10 ml of quarter strength Ringer's solution. The centrifuging and re-suspension of cells was repeated in triplicate. After the final run, 20 ml of quarter strength Ringer's was transferred into the plastic vial and the pellets shaken.

Inoculum density was determined via three separate dilution series made up from sub-samples of the *E.coli* and *S.* Dublin cultures (1:10 ratio of quarter strength Ringer's to inoculum). The optical density (OD) values were then read (600nm) for each of the dilution series. Aliquots (0.1 ml) were then taken from five dilutions (10^5, 10^6, 10^7, 10^8 and 10^9) and plated out onto appropriate media, see Chapter 3.3.3. The plates were then incubated for 24 hours at 37°C. Controls for sterility were also incubated overnight at 37°C. The cells were then enumerated counted and a corresponding linear regression equation produced, constructed using OD/CFU data. This was subsequently

used to compare estimated and plated CFUs. Recovery rate was then calculated as outlined in Appendix 1, Section 1.1.

Results

Optical density and cell concentration of inoculum

The initial quantity of *E. coli* added to relevant treatments was approximately 2.27×10^8 CFU ml^{-1} (Table 2a). This was further supported by the estimated value obtained from the regression equation produced from Figure 2a, which estimated 2.09×10^8 CFU ml^{-1} (Table 2a). The regression equation itself was also highly accurate with a R^2 value of 0.9982.

Additionally, the initial quantity of *S.* Dublin added to relevant treatments was approximately 1.03×10^9 CFU ml^{-1} (Table 2a). This was further supported by the estimated value obtained from the regression equation produced from Figure 2b, which estimated 1.34×10^9 CFU ml^{-1} (Table 2a). The regression equation itself was also highly accurate with a R^2 value of 0.9992.

Table 2a. Estimated quantity of inoculated model pathogens, obtained from the regression equation calculated from the preceding figures vs. plate count data. * calculated using regression equation where X equals average absorbency (600 nm)

Pathogen	Average Absorbance (600 nm)	Regression equation	Estimated (CFU ml^{-1}) *	Plate count (CFU ml^{-1})
E.coli	0.48	$y=5 \times 10^8 X - 3 \times 10^7$	2.09×10^8	2.27×10^8
S. Dublin	0.38	$y=3 \times 10^9 X - 2 \times 10^8$	1.34×10^9	1.03×10^9

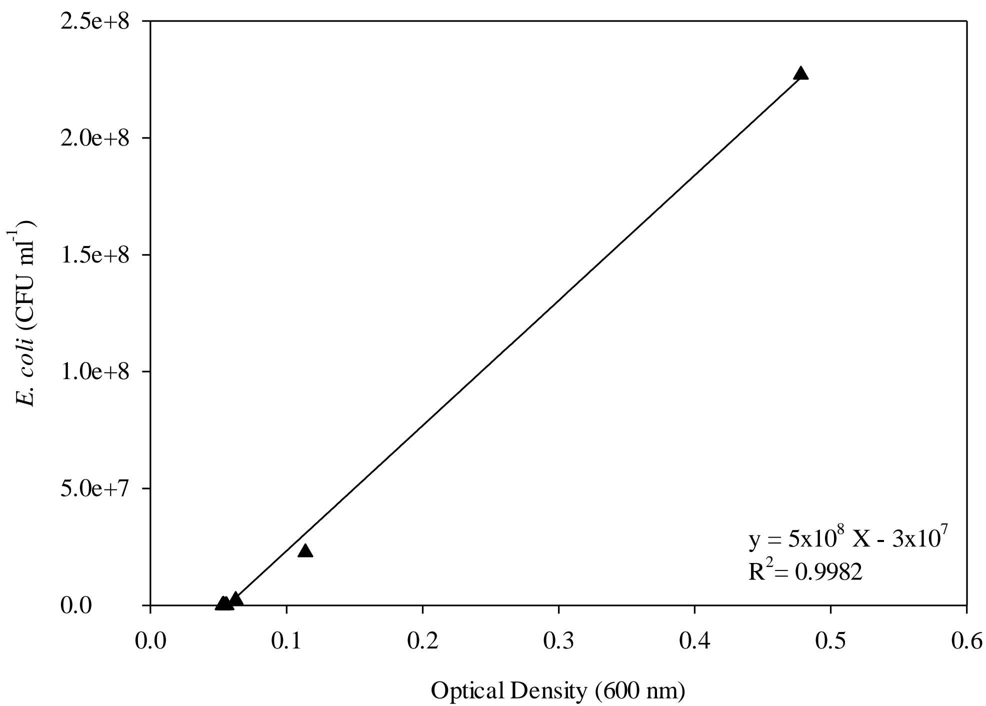

Figure 2a. Optical density of *E. coli* in LB broth (tenfold dilution series) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *E. coli*; ▬ linear regression equation.

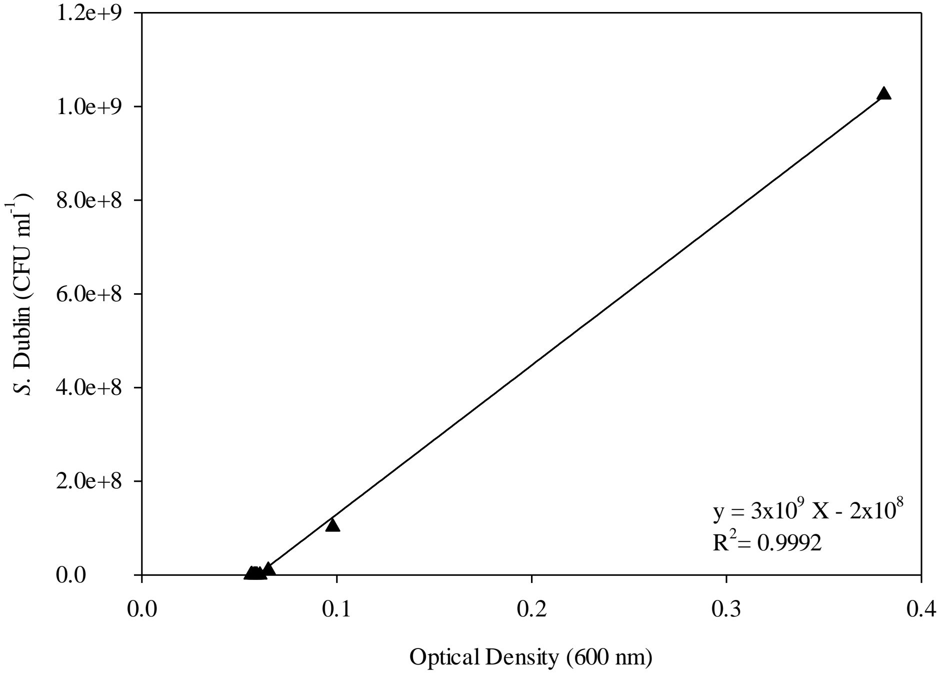

Figure 2b. Optical density of *S.* Dublin in LB broth (tenfold dilution series) vs. CFU ml^{-1} data. Key: ▲ optical density vs. *S.* Dublin; ▬ linear regression equation.

Recovery rate of model pathogenic bacteria

The recovery rate across treatments for *S.* Dublin was not significantly different ($p>0.05$), ranging from 20% to 50% across all treatments (Figure 2a(a)). In contrast the initial extraction rate across treatments for *E. coli* showed a far greater range, from 50% to 200% (Figure 2a(b)). The rates that were above 100% and the high degree of variance within treatments were attributed to the high incidence of indigenous *E. coli* within the sewage sludge.

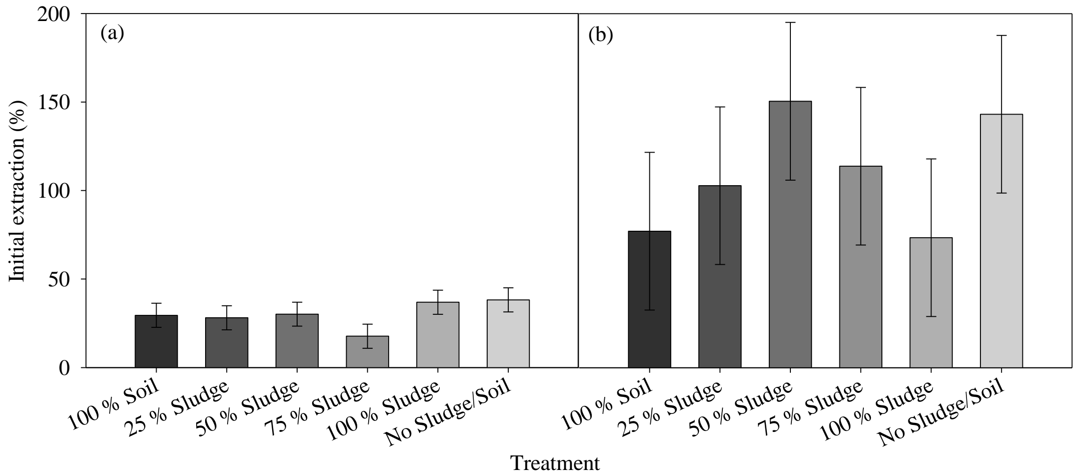

Figure 2c. Recovery rate of (a) *S.* Dublin and (b) *E. coli* in microcosms containing mixtures of sewage sludge and soil (n=3, Mean ± Pooled SE).

Appendix 3

3.1. ANOVA tables for *E. coli* and *S.* Dublin counts within Chapter 4.

Table 3a. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 42) on *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)														
	0	**1**			**3**		**7**	**14**		**29**			**42**		
Treatment	**a**	**a**	**b**	**c**	**a**	**b**	**a**	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**c**
Inoculated control soil	*			***		**	**		****		****			****	
Mixed, 25% sludge	*	***		***	**	**	**	****		****			****		
Mixed, 50% sludge	*	***	***		**		**	****		****			****		
Mixed, 75% sludge	*	***	***		**		**	****		****			****		
Inoculated control sludge	*		***		**		**	****				****			****

Table 3b. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 7) on *S.* Dublin counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)											
	0		**1**			**3**			**7**			
Treatment	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**	**d**
Inoculated control soil	**			****				****		****		
Mixed, 25% sludge	**	**	****	****		****		****			****	
Mixed, 50% sludge	**	**	****			****	****		****			
Mixed, 75% sludge	**	**	****			****	****		****			
Inoculated control sludge		**			****		****					****

Table 3c. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 14 - 42) on *S.* Dublin counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Point (Days)						
	14		28		42		
Treatment	**1**	**2**	**1**	**2**	**1**	**2**	**3**
Inoculated control soil	***			***		****	
Mixed, 25% sludge	***		***	***	****		
Mixed, 50% sludge	***		***	***	****		
Mixed, 75% sludge	***	***	***		****		
Inoculated control sludge		***	***				****

3.2. ANOVA tables for indigenous sewage sludge *E.coli* counts within Chapter 5.

Table 3d. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 0 - 14) on indigenous sewage sludge *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)											
	0		1	3				7			14	
Treatment	**a**	**b**	**a**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**a**	**b**
Mixed, 15% sludge	***	***	*		****			****			****	****
Mixed, 25% sludge	***		*	****	****			****			****	
Mixed, 50% sludge	***	***	*			****			****	****	****	****
Mixed, 75% sludge	***		*	****					****		****	****
Control sludge		***	*				****			****		****

Table 3e. One-way ANOVA Bonferroni tables, showing the effects and interactions of the proportion of sewage sludge to soil per time point (from Days 28- 56) on indigenous sewage sludge *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)							
	28		42			56		
Treatment	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**
Mixed, 15% sludge	****	****	****			****		
Mixed, 25% sludge	****		****			****		
Mixed, 50% sludge	****			****			****	
Mixed, 75% sludge	****	****			****	****	****	
Control sludge		****	****					****

3.3. ANOVA tables for sewage sludge-derived *E. coli* counts, respiration and microbial carbon contents within Chapter 6

Table 3f. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0- 6) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)													
	0		1			3					6			
Treatment	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**
Mixture, both un-amended	***	***		***					***				***	
Mixture, soil amended	***	***	***			***						***		
Mixture, sludge amended		***	***			***						***		
Mixture, both amended	***				***		***				***			
Control sludge, un-amended	***		***	***						***				***
Control sludge, amended	***	***	***					***			***			

Table 3g. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 13- 42) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)								
	13			28			42		
Treatment	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**
Mixture, both un-amended		***			***			****	
Mixture, soil amended	***			***			****		
Mixture, sludge amended	***			***			****		
Mixture, both amended	***			***					****
Control sludge, un-amended			***			***	****	****	
Control sludge, amended	****			****			****		

Table 3h. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0 - 6) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)												
	0		1				3				6		
Treatment	**a**	**b**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**
Mixture, both un-amended	***	***		***	***		***				***	****	***
Mixture, soil amended	***	***	***	***				***	***		***	****	
Mixture, sludge amended	***	***				***	***				***		
Mixture, both amended	***		***					***			***		
Control sludge, un-amended		***			***				***			***	***
Control sludge, amended	***	***	***	***						***			***

Table 3i. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 13 - 42) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)								
	13			28			42		
Treatment	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**
Mixture, both un-amended	***		***	****	****	****		****	****
Mixture, soil amended	***	***		****	****	****	****		
Mixture, sludge amended	***	***		****	****		****	****	
Mixture, both amended			***	****			****		
Control sludge, un-amended		***				****			****
Control sludge, amended	***	***	***		****	****	****	****	

Table 3j. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0 - 3) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)														
	0						1					3			
Treatment	**a**	**b**	**c**	**d**	**e**	**f**	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**
Control soil, un-amended		***						****						****	
Control soil, amended			***						****				****		
Mixture, both un-amended				***						****			****		
Mixture, soil amended	***						****					****			
Mixture, sludge amended	***						****					****			
Mixture, both amended	***						****					****			
Control sludge, un-amended					***		****					****			
Control sludge, amended						***					****				****

Table 3k. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 6 - 13) on respiration (μg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)								
	6					13			
Treatment	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**
Control soil, un-amended			****					****	
Control soil, amended	****								****
Mixture, both un-amended	****					****			
Mixture, soil amended	****	****				****			
Mixture, sludge amended	****	****				****			
Mixture, both amended		****				****			
Control sludge, un-amended				****			****		
Control sludge, amended					****		****		

Table 3l. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 28 - 42) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)								
	28				42				
Treatment	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**e**
Control soil, un-amended				****			****		
Control soil, amended	****						****	****	
Mixture, both un-amended	****				****			****	
Mixture, soil amended	****	****			****	****			
Mixture, sludge amended		****				****			
Mixture, both amended		****			****	****			
Control sludge, un-amended			****						****
Control sludge, amended			****						****

Table 3m. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0 - 6) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)																	
	0						**1**				**3**				**6**			
Treatment	**a**	**b**	**c**	**d**	**e**	**f**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**
Control soil, un-amended					** *			** **						** **				****
Control soil, amended	***						** **					** **	** **		****			
Mixture, both un-amended	***								** **		** **				****	****		
Mixture, soil amended		** *	** *				** **				** **	** **			****	****		
Mixture, sludge amended		** *					** **				** **	** **			****	****		
Mixture, both amended				** *			** **				** **	** **	** **			****	****	
Control sludge, un-amended			** *	** *			** **						** **				****	
Control sludge, amended						** *				** **			** **				****	

Table 3n. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 13 - 42) on respiration (µg CO_2-C g^{-1} h^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)													
	13					28				42				
Treatment	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**	**e**
Control soil, un-amended				****			****					****		
Control soil, amended		****				****	****					****	****	
Mixture, both un-amended	****	****				****				****			****	
Mixture, soil amended	****		****			****				****	****			
Mixture, sludge amended	****	****				****				****	****			
Mixture, both amended	****		****			****		****			****			
Control sludge, un-amended			****						****					****
Control sludge, amended					****			****	****					****

Table 3o. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per time point (from Days 0, 42) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)						
	0				42		
Treatment	**a**	**b**	**c**	**d**	**a**	**b**	**c**
Control soil, un-amended	****						
Control soil, amended	****	****	****		****	****	****
Mixture, both un-amended	****	****				****	
Mixture, soil amended			****	****	****	****	****
Mixture, sludge amended		****	****			****	****
Mixture, both amended			****	****		****	****
Control sludge, un-amended	****	****				****	****
Control sludge, amended				****			****

Table 3p. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per treatment (Treatments: control soil, un-amended – mixture, soil amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Treatment				
	Control soil, un-amended	Control soil, amended	Mixture, both un-amended	Mixture, soil amended	
Time Point (Days)	**a**	**a**	**a**	**a**	**b**
0	*	*	*		**
42	*	*	*	**	

Table 3q. One-way ANOVA Bonferroni tables, showing the effects and interactions of glucose amendment in sewage sludge or soil per treatment (Treatments: mixture, sludge amended – control sludge, amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Treatment					
	Mixture, sludge amended	**Mixture, both amended**		**Control sludge, un-amended**	**Control sludge, amended**	
Time Point (Days)	**a**	**a**	**b**	**a**	**a**	**b**
0	*		**	*		**
42	*	**		*	**	

Table 3r. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0, 42) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)					
	0				**42**	
Treatment	**a**	**b**	**c**	**d**	**a**	**b**
Control soil, un-amended				****	****	
Control soil, amended		****	****		****	****
Mixture, both un-amended	****				****	****
Mixture, soil amended	****				****	
Mixture, sludge amended	****	****			****	
Mixture, both amended	****	****			****	
Control sludge, un-amended	****					****
Control sludge, amended			****			****

Table 3s. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (Treatments: control soil, un-amended – mixture, soil amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Treatment					
	Control soil, un-amended	**Control soil, amended**		**Mixture, both un-amended**	**Mixture, soil amended**	
Time Point (Days)	**a**	**a**	**b**	**a**	**a**	**b**
0	*		****	*		***
42	*	****		*	***	

Table 3t. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (Treatments: mixture, sludge amended – control sludge, amended) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Treatment							
	Mixture, sludge amended		**Mixture, both amended**		**Control sludge, un-amended**		**Control sludge, amended**	
Time Point (Days)	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
0		****		****		**		****
42	****		****		**		****	

3.4. ANOVA tables for sewage sludge-derived *E. coli* counts, respiration and microbial carbon contents within Chapter 7

N.B. Respiration data for this Chapter was only analysed using a two-way Factorial ANOVA.

Table 3u. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 0 - 14) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)												
	0		1			3		7			14		
Treatment	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**c**
Mixture, un-amended	**					****			****		****		
Mixture, amended	**	**			****	****		****			****		
Control sludge, un-amended	**	**	****				****	****				****	
Control sludge, amended		**	****	****			****			****			****

Table 3v. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 15 - 29) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)															
	15			17			21		28				29			
Treatment	a	b	c	a	b	c	a	b	a	b	c	d	a	b	c	d
Mixture, un-amended		****			****		****		****				****			
Mixture, amended			****			****	****			****				****		
Control sludge, un-amended	****			****				****			****				****	
Control sludge, amended	****			****				****				****				****

Table 3w. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 15 - 29) on sewage sludge-derived *E. coli* counts (log CFU gDS^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)													
	31		35		42			49		75		105		
Treatment	a	b	a	b	a	b	c	a	b	a	b	a	b	c
Mixture, un-amended	****		***		****			****		***			****	
Mixture, amended	****		***	***	****			****		***		****		
Control sludge, un-amended		****	***	***		****			****	***	***	****		
Control sludge, amended		****		***			****		****		***			****

Table 3x. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 0 - 14) on general coliform counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)										
	0		1			3		7		14	
Treatment	**a**	**b**	**a**	**b**	**c**	**a**	**b**	**a**	**b**	**a**	**b**
Mixture, un-amended		**	****	****			****	****			****
Mixture, amended	**	**	****			****		****		****	
Control sludge, un-amended	**				****	****			****	****	
Control sludge, amended	**			****	****	****			****	****	

Table 3y. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 17 - 31) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.[*no data for Day 15]

	Time Points (Days)												
	17			21			28		29		31		
Treatment	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**a**	**b**	**a**	**b**	**c**
Mixture, un-amended		****		****				****		****		****	
Mixture, amended	****			****			****		****				****
Control sludge, un-amended	****				****		****		****		****		
Control sludge, amended			****			****	****		****		****		

Table 3z. One-way ANOVA Bonferroni tables, showing the effects and interactions of repeated glucose amendment in sewage sludge or soil per time point (from Days 35 - 105) on sewage sludge-derived *E. coli* counts (log CFU gDS $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)														
	35			42			49				75		105		
Treatment	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**	**d**	**a**	**b**	**a**	**b**	**c**
Mixture, un-amended		****			****			****				****	****		
Mixture, amended			****	****			****				****				****
Control sludge, un-amended	****					****			****		****			****	
Control sludge, amended	****			****						****	****		****		

Table 3aa. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per time point (from Days 0, 105) on microbial carbon content (mg l $^{-1}$). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Time Points (Days)			
	0		105	
Treatment	**a**	**b**	**a**	**b**
Control soil, un-amended		**	**	**
Control soil, amended	**	**	**	**
Mixture, un-amended	**	**	**	**
Mixture, amended	**		**	
Control sludge, un-amended	**	**		**
Control sludge, amended	**			**

Table 3ab. One-way ANOVA Bonferroni tables, showing the effects and interactions of yeast extract amendment in sewage sludge or soil per treatment (all treatments) on microbial carbon content (mg l^{-1}). Letters denote homogeneous means at 5% significance level. Significance terms denoted by **** p<0.001; *** p<0.01; ** p<0.05; * p>0.05.

	Treatment								
	Control soil, un-amended	**Control soil, amended**	**Mixture, un-amended**		**Mixture, amended**	**Control sludge, un-amended**		**Control sludge, amended**	
Time Point (Days)	**a**	**a**	**a**	**b**	**a**	**a**	**b**	**a**	**b**
0	*	*		**	*		**	**	
105	*	*	**		*	**			**