CRANFIELD UNIVERSITY

Sarah Elizabeth Fane

Control of *E. coli* in Biosolids

School of Water, Environment and Energy
Research Degree

PhD
Academic Year: 2013 - 2016

Supervisor: Professor Sean Tyrrel

November 2016

CRANFIELD UNIVERSITY

School of Water, Environment and Energy
Research Degree

PhD

Academic Year 2013 - 2016

Sarah Elizabeth Fane

Control of *E. coli* in Biosolids

Supervisor: Professor Sean Tyrrel

November 2016

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

© Cranfield University 2016. All rights reserved. No part of this publication may be reproduced without the written permission of the copyright owner.

ABSTRACT

Achieving microbial compliance levels in biosolids storage is complicated by the unpredictable increase of *Escherichia coli* (*E. coli*), which serves as an important indicator for pathogen presence risk. Meeting required microbial specifications validates sludge treatment processes and ensures that a safe product is applied to agricultural land. Controlled indicator monitoring provides confidence for farmers, retailers and the food industry, safeguarding the sludge-to-land application route.

Following mechanical dewatering biosolids products are stored before microbial compliance testing permits agricultural application. During storage, concentrations of *E. coli* bacteria can become elevated and prevent the product from meeting the *conventional* or *enhanced* levels of treatment outlined in The Safe Sludge Matrix guidelines. Literature research identified innate characteristics of sludge and ambient environmental parameters of storage which are factors likely to influence *E. coli* behaviour in stored biosolids. The research hypothesis tested whether *E. coli* growth and death in dewatered sewage sludge can be controlled by the modification of physical-chemical factors in the cake storage environment. Parameters including nutrient availability, temperature, moisture content and atmospheric influences were investigated through a series of laboratory-scale experiments. Controlled dewatering and the assessment of modified storage environments using traditional microbial plating and novel flow cytometry analysis have been performed. At an operational scale, pilot trials and up-scaled monitoring of the sludge storage environment have been conducted enabling verification of laboratory results. Understanding the dynamics of cell health within the sludge matrix in relation to nutrient availability has provided a valuable understanding of the mechanisms that may be affecting bacterial growth post-dewatering. The importance of elevated storage temperatures on *E. coli* death rates and results showing the benefits of a controlled atmosphere storage environment provide important considerations for utilities.

Keywords:

Biosolids, Compliance, Safe Sludge Matrix, Storage, Mechanical Dewatering, Temperature, Cell Disruption

ACKNOWLEDGEMENTS

I would firstly like to acknowledge the support of Professor Sean Tyrrel, lead academic supervisor, throughout this PhD project. His steady mentoring has kept motivations high and I could not be more thankful for such a dedicated and approachable supervisor. I am thankful to my original second academic supervisor Dr. Andreas Nocker who was incredibly influential in the development of laboratory techniques and his enthusiasm for flow cytometry provided another dimension of study that has been rewarding to explore. I would like to extend my appreciation to Professor Jim Harris, who took over the role as second supervisor in summer 2015, and who's 'out of the box' thinking has given an entertaining and challenging final year of progress. I would like to thank Professor Elise Cartmell and Dr. Yadira Fernandez-Bajon for their support with the industrial sponsor, Severn Trent Water Plc. and the stakeholder meetings held throughout the project. Thanks must be given to Dr. Monica Rivas Cassado whose statistical consultations developed experimental design and data analysis.

I am immensely grateful to the team of laboratory technicians, colleagues, and to my wonderful placement students in the School of Water, Environment and Agri-food, who have provided a professional, supportive and friendly environment for working. The support provided by Sander Smit at Paleo Terra guided decisions for monitoring probe set up and his technical advice on redox potential has been a valuable source of knowledge and understanding, thank you.

My heart-felt thanks go to Janet and Steve, Michael, and my family who have been boulders of granite upon which to stand during this (rather smelly!) adventure.

To finish, I would like to give special thanks in memory of Mrs Andrea Wilson. Her support as the project's industrial supervisor from 2013- 2015 gave me a platform upon which to build my professional network at Severn Trent Water Plc. and her positive outlook to all challenges was truly inspirational.

TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS iii
LIST OF FIGURES ix
LIST OF TABLES xv
LIST OF EQUATIONS xvi
LIST OF ABBREVIATIONS xvii
1 Introduction 3
1.1 Overview 3
1.2 Aims and Objectives 8
1.3 Thesis Plan 8
1.4 References 13
2 Influence of Innate Sludge Factors and Ambient Environmental Parameters in Biosolids Storage on Indicator Bacteria Survival: A Review 21
2.1 Sludge treatment processes and storage 21
2.2 Legislative controls 24
2.3 Sludge quality concerns 27
2.4 Chemical Microbial Growth Requirements 29
2.5 Physical Environmental Factors 36
2.5.1 Temperature 36
2.5.2 Moisture Content 38
2.5.3 Modified Atmosphere 41
2.6 Summary 45
2.7 References 46
3 Influence of Physical and Chemical Environmental Factors on *E. coli* Survival in Biosolids at a Laboratory Scale 59
3.1 Abstract 59
3.2 Introduction 60
3.3 Methodology 67
3.3.1 Sludge sampling and storage 67
3.3.2 Experimental treatments 67
3.3.3 Analytical methods: 71
3.4 Results 73
3.4.1 Nutrient addition 73
3.4.2 Polyelectrolyte impact on *E. coli* dynamics 74
3.4.3 Temperature and modified atmosphere 77
3.4.4 Moisture content 80
3.5 Discussion 81
3.5.1 *E. coli* growth response to sludge-derived nutrient addition 81
3.5.2 *E. coli* response to polyelectrolyte addition 82
3.5.3 Modified biosolids storage environment 83

3.5.4 Effect of DS% on bacterial dynamics 86
3.6 Conclusions 86
3.7 Acknowledgements 87
3.8 References 87
4 Long Term Characterisation of the Biosolids Storage Environment: Implications for *E. coli* Dynamics 97
4.1 Abstract 97
4.2 Introduction 98
4.3 Methodology 103
4.3.1 Sludge sample source 103
4.3.2 Programme schedule 104
4.3.3 Sludge containment cages 104
4.3.4 Preliminary trials to determine sludge sampling strategy 104
4.3.5 Temperature and redox potential data recording 105
4.3.6 Ambient weather data 106
4.3.7 Sludge treatment site stockpile temperature comparison 107
4.3.8 Full-scale experimental set up 107
4.3.9 Microbial analysis 108
4.3.10 pH monitoring 109
4.3.11 Statistical analysis 110
4.4 Results 110
4.5 Discussion 116
4.5.1 *E. coli* dynamics during storage 116
4.5.2 Effects from temperature on storage environments 118
4.5.3 Influence of moisture content in stored biosolids 120
4.6 Conclusions 122
4.7 Acknowledgements 123
4.8 References 124
5 *E. coli* Death Rates in a Summer and Winter Pilot Trial Testing Modified Biosolids Storage Environments 133
5.1 Abstract 133
5.2 Introduction 134
5.3 Methodology 138
5.3.1 Sludge sample source 138
5.3.2 Pilot trial location 139
5.3.3 Summer trial set up 139
5.3.4 Winter trial set up 140
5.3.5 Data recording of environmental factors 141
5.3.6 Microbial enumeration 143
5.3.7 Statistical analysis 144
5.4 Results 145
5.5 Discussion 152

5.5.1 Dominant environmental parameter affecting *E. coli* death rate 152
5.5.2 Changes in moisture content 154
5.5.3 Influence of treatments to suppress oxygen availability 155
5.6 Conclusions 156
5.7 Acknowledgements 156
5.8 References 157
6 Disruption of Cells in Biosolids Affects *E. coli* Dynamics in Stored Cake 165
6.1 Abstract 165
6.2 Introduction 166
6.3 Methodology 170
6.3.1 Sludge sampling and storage 170
6.3.2 Experimental treatments 171
6.3.3 Analytical methods 173
6.3.4 Statistical analysis 177
6.4 Results 178
6.4.1 Thermal disruption 178
6.4.2 Physical disruption 181
6.4.3 Thermal disruption combined with controlled storage 182
6.5 Discussion 190
6.5.1 Microbial and nutrient response to effects of disruption treatments and growth in subsequent storage 190
6.5.2 Disruption treatment combined with environmental parameter modification of storage environments negatively impact upon *E. coli* dynamics 193
6.5.3 Chemical response to disruption treatment and subsequent storage environments 197
6.6 Conclusions 198
6.7 Acknowledgements 199
6.8 References 199
7 Final Summary 209
7.1 Conclusions of research and implications for practice 209
7.1.1 E. coli die-off in biosolids is significantly increased at storage temperatures greater than 25°C 209
7.1.2 *Sludge-derived nutrient addition to biosolids matrix increases E. coli growth* 210
7.1.3 *Laboratory scale experiments and field trials confirm oxygen availability is not the dominant factor affecting E. coli die-off rates in stored biosolids* 212
7.1.4 Moisture content within the ranges tested has no effect on E. coli survival in stored biosolids 213
7.1.5 Polyelectrolyte addition does not cause E. coli concentrations to increase in dewatered sewage sludge 213

7.2 Contribution to knowledge 214
7.3 Future work 217
7.3.1 Maintaining a stable and elevated stockpile temperature on treatment sites 217
7.3.2 Cell disruption increasing rates of *E. coli* growth and death in an elevated temperature storage environment 217
7.3.3 Oxygen exposure providing a selective advantage for facultative *E. coli* bacterial growth during dewatering processes 218
7.3.4 Indicator organism: Environmental strains of *E. coli* able to survive for prolonged periods at low temperature 218
7.4 References 219
APPENDICES 225
Appendix A *E. coli* variability in biosolids sampled from centrifuge output .. 225

LIST OF FIGURES

Figure 3.1 Impact of sludge-derived nutrient broth addition on *E. coli* concentration monitored over 30 day incubated (22°C) storage period. Key: --◇-- Control (de-ionised water addition), —■—Nutrient addition (nutrient broth added to sludge cake). Error bars represent the standard deviation of 2 repeats with 6 replications in total..... 74

Figure 3.2 Effect of laboratory scale centrifuge dewatering on *E. coli* concentrations with and without polyelectrolyte addition. Key: —◆— Control (no polyelectrolyte addition), ··⊡·· 0.3 g/L FLOPAM addition. Samples stored at 19°C for 14 days. Error bars represent the standard deviation of 3 replicates (n=3) 75

Figure 3.3 FLOPAM polyelectrolyte concentration effect on *E. coli* dynamics in controlled biosolids storage for 14 days after filter press dewatering. Key: —◆— 0.3 g/L FLOPAM, -⊡- 0.5 g/L FLOPAM. Error bars represent the standard deviation of replicates (n=3)..... 76

Figure 3.4 Comparison of FLOPAM and ZETAG polyelectrolyte types on *E. coli* dynamics in controlled biosolids storage for 14 days after filter press dewatering. Key: —◆— 0.3g/L FLOPAM, -⊡- 0.3 g/L ZETAG. Error bars represent the standard deviation of replicates (n=3)..... 77

Figure 3.5 Effect of temperature and modified atmosphere storage on *E. coli* concentrations during 21 day incubation period. Results show colony forming units (CFU) per gram dry solids change in concentration and represent averages taken from three experimental repeats. Figure 3.5A shows open treatment condition, Figure 3.5B: shows gas evacuated storage. Key: —◆— Room temperature (19°C); --■--25°C; --△-30°C; —○—37°C. Error bars represent the standard deviation of experimental repeats (n=3)..... 78

Figure 3.6 Changes in average pH during sample storage under open and modified atmosphere conditions. Key: --◇--open treatment condition, —■—gas evacuated storage. Error bars represent the standard deviation of three experimental repeats (n=3)..... 79

Figure 3.7 Effect of temperature and modified atmosphere storage on levels of total oxides of nitrogen (TON) (mg/kg DS) in biosolids stored for 14 days in aerobic condition (open bags) (A) or gas evacuated condition (sealed bags) (B). Key: ■ 25°C; ⧈30°C; □ 37°C. (No data from 19°C storage condition was collected). Error bars represent the standard deviation of replicates (n=3). 80

Figure 3.8 Effect of modified biosolids DS% on *E. coli* dynamics during incubated storage at 22°C for 21 days. DS% modified within range identified on sludge treatment sites between 2008- 2016 (Severn Trent Water Plc.). Key: —◆— 35% DS, -■- 25% DS, --△-- 20% DS, —○— 17% DS. Error bars represent the standard deviation of replicates (n=3)..... 81

Figure 4.1 Prototype of 150 mm acrylic sampling tube containing 2 x end baffles (250 mm depth) and 3 x 15 mm drainage holes. At an up-scaled size, for the monitoring project, 4 lengths of 0.5 m were joined together with resin to form a 2 m length that would be placed across the whole cage width when filling with sewage sludge cake. 105

Figure 4.2 Redox probe sensor arrangement. 3 x 1 m probes (9 redox sensors recording data) in each of the three test cages (Paleo Terra, Amsterdam, Netherlands). Key: redox sensor location. 106

Figure 4.3 Schematic of sample tube and monitoring probe arrangement within 3 x 1.5 m^3 cages, dimensions are approximate values. Key: sample tube, monitoring probe. 108

Figure 4.4 *E. coli* concentration data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: samples taken from low cage depth, samples taken from middle cage depth, samples taken from top of stored biosolids (~0.1 m below surface). Error bars represent the standard deviation of 3 repeats (n=3). 111

Figure 4.5 Temperature data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured lines show the average temperature of 3 repeats with 3 replicates per repeat. Cage temperature data was recorded at hourly intervals. Key: data recorded from low cage depth, data recorded from middle cage depth, data recorded from top of stored biosolids (~0.25 m below surface). Ambient temperature recorded on-site every 30 minutes. 112

Figure 4.6 Temperature data recorded from sludge treatment site stockpiles on 28/04/2016 (–⊖–) and 18/07/2016 (– ⊟ –). Transect taken from back wall of biosolids storage bay. X-axis indicates distance from back wall along transect. Ambient temperature at site on 28/04/2016 was 6ºC and for 18/07/2016 ambient temperature was recorded as 21.5ºC (data from local weather station (IDERBY108 Weather Underground 2016)). 113

Figure 4.7 pH data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: samples taken from low cage depth, samples taken from middle cage depth, samples taken from top of stored biosolids (~0.1 m below surface). 114

Figure 4.8 Dry solids (%) data and rainfall measurements recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: samples taken from low cage depth, samples taken from middle cage depth, samples taken from top of stored biosolids (~0.1 m

below surface). — Rainfall measurements recorded on-site over 24 hours displayed. 115

Figure 4.9 Redox potential data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured lines show the average redox potential of 3 repeats with 3 replicates per repeat. Cage redox potential data was recorded at hourly intervals. Key: — data recorded from low cage depth, — data recorded from middle cage depth, — data recorded from top of stored biosolids (~0.25 m below surface). Please note redox probes measuring 'top' data were corrupted after first quarter and therefore 'top' data for quarters 2, 3 and 4 are not shown. 116

Figure 5.1 Photographs from summer pilot trial 2014. Treatment condition specified in text boxes on left side of photographs. 140

Figure 5.2 Detailed schematic of conditions in summer and winter pilot trials with repeats. Summer trial included conditions A, B, C and D. Winter trial included conditions A, B, C, E and F..... 141

Figure 5.3 Cross section and aerial view of pilot trial stockpiles (including approximate dimensions) and the sampling points used (x). The bagged treatment was held in a sealed cylindrical bag altering the stockpile shape (sampling valves only present for summer pilot trial). 143

Figure 5.4 *E. coli* concentrations recorded during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: open control, -⊡-barn storage, -△-membrane covered treatment, ⊙ sealed-bag technology. Additional insulated treatments included: -▲-insulated membrane covered treatment and ● insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3). 146

Figure 5.5 Temperature results from summer (28 day) and winter (69 day) pilot trials. Summer results in graph B display data from the sealed-bag treatment which started with a 28 day lag from the other tested treatments. Winter trial tested 2 additional insulated treatments shown separately in winter graph D. Key: open control, -⊡-barn storage, -△-membrane covered treatment, ⊙ sealed-bag technology. Additional insulated treatments included: -▲-insulated membrane covered treatment and ● insulated sealed-bag technology. Ambient temperature data (—+—) was recorded at 30 minute intervals during the pilot trial tests and results here show the average temperature over 24 hours. Error bars represent the standard deviation of 3 repeats (n= 3). 148

Figure 5.6 Results from pH monitoring during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: open control, -⊡-barn storage, -△-membrane covered treatment, ⊙ sealed-bag technology. Additional insulated treatments included: -▲-insulated membrane covered treatment

and ···●··· insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3). 149

Figure 5.7 Moisture content and rainfall results from summer (28 day) and winter (69 day) pilot trials. Summer results in graph B display data from the sealed-bag treatment which started with a 28 day lag from the other tested treatments. Winter trial tested 2 additional insulated treatments shown separately in winter graph D (please note: rainfall data has been repeated in graphs C and D). Key: →×← open control, -⊡-barn storage, -△-membrane covered treatment, ···⊙··· sealed-bag technology. Additional insulated treatments included: -▲-insulated membrane covered treatment and ···●··· insulated sealed-bag technology. Rainfall data is displayed in bars (▬) and results show the total rainfall recorded over 24 hours. Error bars represent the standard deviation of 3 repeats (n= 3). 151

Figure 5.8 Redox potential results recorded during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: →×← open control, -⊡-barn storage, -△-membrane covered treatment, ···⊙··· sealed-bag technology. Additional insulated treatments included: -▲-insulated membrane covered treatment and ···●··· insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3). 152

Figure 6.1 Set up for experiments testing the effects of thermal disruption followed by storage under controlled temperature conditions and modified atmosphere treatments. Diagram represents the number of samples held under one of three temperature conditions tested. 173

Figure 6.2 Representative FCM cytogram showing distribution of microbial clusters in samples of prepared sewage sludge cake. Signals from intact cells are within the P1 (green) gated region (Gatza *et al* 2013) and signals from damaged bacteria (Propidium Iodide stained cells) are shown in gate P2 (red shaded region) (Folardori *et al* 2007, Foladori *et al* 2010, Harry *et al* 2016). Other signals are caused by organic and inorganic background (Harry *et al* 2016). 176

Figure 6.3 Proportions of intact and damaged cells in samples after thermal disruption treatments. Samples thermally disrupted at 62°C for 20, 80 and 120 minutes or autoclaved at 121°C for 15 minutes at 1.03 Bar (± 0.03 bar). Second autoclave sample included 0.5 g undisrupted sludge addition to reintroduced sludge derived *E. coli* after autoclaving sample. Control condition represents an undisrupted sample. Key: ■ % Intact, ■ % Damaged. 178

Figure 6.4 *E. coli* concentration during controlled storage at 22°C for 10 days after thermal disruption treatment at 62°C for (A) 20 minutes (—◆—), (B) 80 minutes (—◆—) and (C) 120 minutes (—◆—). All plots include *E. coli* concentrations recorded from undisrupted control condition (–◇–). Error bars represent the standard deviation of three repeats (n=3). 179

Figure 6.5 *E. coli* concentration during controlled storage at 22°C for 10 days after samples thermally disrupted through autoclaving for 15 minutes at 121°C (pressure = 1.03 Bar ± 0.03). Key: (A) Autoclaved sample, (B) Autoclaved sample inoculated with 0.5 g undisrupted sludge sample to reintroduce sludge derived *E. coli*. Both conditions plotted against undisrupted control conditions ( ). Error bars represent the standard deviation of 3 repeats (n=3). 180

Figure 6.6 Proportions of intact and damaged cells in samples after physical disruption treatment and in undisrupted control condition. Key: ■ % Intact, ■ % Damaged. 181

Figure 6.7 Dissolved Organic Carbon concentration in control (undisrupted) and samples exposed to physical disruption. Error bars represent the standard deviation of three replicates (n= 3). 181

Figure 6.8 Effect of physical disruption treatment on *E. coli* bacteria concentration plotted against control (undisrupted) sample. Key: undisrupted control, physically disrupted sample. Error bars represent the standard deviation of 3 replicates (n=3). 182

Figure 6.9 Proportions of intact and damaged cells in samples after thermal disruption treatment at 62°C for 80 minutes and undisrupted control condition. Key: ■ % Intact, ■ % Damaged. 182

Figure 6.10 Dissolved Organic Carbon concentration in samples exposed to thermal disruption treatment at 62°C for 80 minutes and undisrupted control condition. Error bars represent the standard deviation of three replicates (n= 3). 183

Figure 6.11 Total Oxides of Nitrogen concentration in samples exposed to thermal disruption treatment at 62°C for 80 minutes and in undisrupted control condition. Error bars represent the standard deviation of three replicates (n= 3). 183

Figure 6.12 Storage temperature and storage condition impact on *E. coli* growth and death over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3). 184

Figure 6.13 Storage temperature and storage condition impact on *E. coli* growth and death over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3). 186

Figure 6.14 Storage temperature and storage condition impact on sample pH over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage,

nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3)..... 187

Figure 6.15 Storage temperature and storage condition impact on sample pH over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: -⊙- open treatment condition, —□— gas evacuated storage, ···▲··· nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3)..... 188

Figure 6.16 Storage temperature and storage condition impact on Total Oxides of Nitrogen (TON) in samples over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C storage. Key: -⊙- open treatment condition, —□— gas evacuated storage, ···▲··· nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3)..... 189

Figure 6.17 Storage temperature and storage condition impact on Total Oxides of Nitrogen (TON) in samples over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: -⊙- open treatment condition, —□— gas evacuated storage, ···▲··· nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3). 189

Figure A-1 *E. coli* (•) variation in the mesophilic digested biosolids from the centrifuge outlet at the sample source wastewater treatment site for experiments conducted between June 2013 and July 2016. 225

LIST OF TABLES

Table 1.1 PhD thesis structure and chapter interaction 11

Table 2.1 Safe Sludge Matrix guidelines for the acceptable level of treatment for any sewage sludge based product before land application (ADAS 2001) . 25

Table 2.2 Elements required for microbial growth with specific sources and cell functions defined (Sigma Aldrich 2016, Todar 2012) 30

Table 3.1 International microbial compliance levels required/ proposed for treated biosolids prior to agricultural application 61

Table 3.2 Polyelectrolyte addition experiments; dewatering operations and polyelectrolyte dosing 69

Table 3.3 Modified sewage sludge cake DS% after sludge drying to 35% and de-ionised water addition 71

Table 7.1 Contributions to knowledge presented by the research work 215

LIST OF EQUATIONS

Equation Redox Potential (4-1) 106

Equation Redox Potential (5-1) 142

LIST OF ABBREVIATIONS

AD	Anaerobic Digestion
ANOVA	Analysis of variance
ANCOVA	Analysis of co-variance
ATP	Adenosine triphosphate
BAS	Biosolids Assurance Scheme
BRC	British Retail Consortium
CFU	Colony Forming Unit
DFSM	Dry Feed Surface Material
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
DS	Dry Solids
EPA	Environment Protection Agency
EPS	Extracellular Polymeric Substances
FCM	Flow Cytometry Measurement
HACCP	Hazard Analysis and Critical Control Point
HDPE	High-density polyethylene
ICC	Intact Cell Concentration
IWA	International Water Association
LSD	Least Significant Difference
MAC	Maximum Allowable Concentration
MAD	Mesophilic Anaerobic Digestion
MAP	Modified Atmosphere Packaging
MLGA	Membrane Lactose Glucuronide Agar
MPN	Most Probable Number
MRD	Maximum Recovery Diluent
NERC	Natural Environment Research Council
PAM	Polyacrylamide based polyelectrolyte
(c)PCR	(Competitive) Polymerase Chain Reaction
(q)PCR	(Quantitative) Polymerase Chain Reaction
PE	Population Equivalent
PI	Propidium Iodide
RNA	Ribonucleic acid
rpm	Revolutions per minute
SA:Vol	Surface area to volume ratio
SCM	Standard Culturing Methods
SSM	Safe Sludge Matrix

TON	Total Oxides of Nitrogen
TVC	Total Viable Count
WEF	Water and Environment Federation
VBNC	Viable But Non Culturable
VOSC	Volatile Organic Sulphur Compound

Chapter 1

Introduction

1 Introduction

1.1 Overview

Biosolids, derived from wastewater treatment, provide a sustainable source of organic fertiliser for agriculture land (Deeks *et al*., 2013). The cycling of biosolids as a soil conditioner in Europe and the USA. are governed by industrial agreements and regulations which stipulate levels of specific indicator bacteria in the treated product. These controls protect public health and provide an assurance framework for farmers, retailers and the food industry. The agreements and regulations safeguard the sludge to land route and give confidence that agricultural land application is safe. Due to economical and practical restraints, it is not possible to test for all potential pathogens present in the biosolids. Microbiological safety is therefore always measured against concentrations of specified indicator organisms. *E. coli* bacteria is considered an organism whose detection provides a surrogate measure for the risk of pathogens present within a sample (Edberg *et al*., 2000; Ashbolt *et al*., 2001; Stevens *et al*., 2003; Haller *et al*., 2009; Sidhu and Toze, 2009). Industrial agreements and international regulations such as the Safe Sludge Matrix (SSM) (ADAS, 2001) and the United States Environmental Protection Agency Part 503 rule (US EPA, 2012) stipulate maximum acceptable concentrations for specified treatment processes. For example, the Environment Agency in England commits biosolids producers to two acceptable treatment levels. *Conventional* treatments will have achieved a 2 Log reduction in *E. coli* between the digester inlet and the final cake product after 14 days of storage (representing 99% pathogen destruction) (ADAS, 2001; Environment Agency, 2003). Alternatively a higher valued product would meet *enhanced* treatment requirements achieving a 6 Log reduction (99.9999% pathogens destroyed) and a product free from *Salmonella* (ADAS, 2001; Environment Agency, 2003).

Increasing levels of *E. coli* bacteria post-dewatering and the maintenance of elevated indicator concentrations in stored sludge cake has raised concern amongst biosolids producers (Monteleone *et al*., 2004; Higgins *et al*., 2006; Higgins *et al*., 2007a; Higgins *et al*., 2007b; Dentel *et al*., 2008; Chen *et al*., 2011).

Currently compliance failures are difficult to predict and have been attributed to dewatering processes such as centrifuge shear force (Qi *et al*., 2004, 2007; Chen *et al*., 2011), non-culturable cell reactivation (Higgins *et al* 2007b) and subsequent storage environments propagating and preserving *E. coli* levels (Higgins *et al* 2007a).

Biosolids storage is intended to further reduce pathogen concentrations before land application. Storage usually comprises of open cake bays segregated by concrete baffles, often holding >200 tonnes treated biosolids material in each bay. Characteristics of the storage environment are uncertain and therefore the key environmental factors responsible for indicator growth and death in stored biosolids, have not been clearly identified (Dentel *et al*., 2008).

Environmental conditions of biosolids storage will be determined, in part, by the mechanical dewatering process. This operation causes substantial change to the sludge matrix and alters the intrinsic environmental characteristics (Higgins *et al*., 2007a; Chen *et al*., 2011). Mechanical dewatering is designed to have the greatest impact on sludge moisture levels, with the aid of polyelectrolyte products to enhance flocculation, dry solids typically alter from 4 to 25% over the dewatering process (Severn Trent Water Plc. Operational Data 2016).

The significant change in percentage dry solids is likely to increase oxygen exposure as pore spaces within the sludge matrix become air voids and the mechanical forces active during dewatering agitate the material. Shift from an anaerobic to aerobic state is likely to affect cell function and cause levels of, previously dominant, anaerobic bacteria to reduce. *E. coli* organisms are facultative anaerobes, able to function in both anaerobic and aerobic conditions and therefore resistant to oxygen deprivation (Metcalf *et al* 2003; Henkel *et al* 2014). In up-stream sludge processes, such as anaerobic digestion, levels of pathogenic indicators reduce as conditions are optimised for the growth of methanogenic bacteria to produce biogas (Weiland 2010). Typical digester operating conditions are mesophilic with temperatures stabilised between 30-42°C (Seadi *et al* 2013). In addition to temperature, Alberts *et al* (2010) report that competition for substrate resources in anaerobic digestion (AD) is high and the

anaerobic nature of the digester environment can substantially affect cell activity, regulating respiration and consequential energy production. A combination of anaerobic conditions and additional inimical factors present in AD, such as high temperature and substrate competition, may cause growth inhibition and therefore typically a 3 Log reduction in *E. coli* bacteria is observed during anaerobic digestion treatment (Severn Trent Water Plc. Operational Data 2016). Published data has shown mesophilic anaerobic digestion to reduce *E. coli* concentrations by 1.35 to 3.36 Log (UKWIR, 1999). The transition to a modified aerobic environment after dewatering may favour the growth of intact bacterial cells and enable previously unculturable cells to reactivate and grow (Higgins *et al* 2007a).

In addition to oxygen availability, cell growth is dependent on temperature. Mechanical dewatering processes will cause temperature variations in the sludge matrix as mesophilic digested liquid sludge is dewatered to form a solid cake product. When the liquid fraction of the sludge matrix is removed during dewatering a substantial decrease from previously mesophilic temperatures is likely to occur. A fall in temperature to milder conditions (~10°C) has been shown to be advantageous for the growth and survival of indicators (Paruch *et al* 2015), such a low temperature would not be favourable for other bacteria present in the sludge such as methanogens. As biosolids form an analogous environment with soils and manure, studies on bacterial dynamics within these environments may contribute to understanding indicator growth and survival. In livestock manures researchers have found evidence suggesting that temperature is the principal factor determining pathogen survival (Nicholson, *et al*., 2000). Semenov *et al*., (2007) demonstrated the importance of temperature on *E. coli* and *Salmonella* bacteria in small microcosms of manure stored at different temperatures ranging between 7 and 33°C. *E. coli* concentrations fell to undetectable levels within 7 days of storage at 33°C demonstrating the likely effect of mesophilic digestion processes on pathogenic bacteria. Under storage conditions at 7°C, Semenov *et al* (2007) found that *E. coli* took approximately 159 days to reach undetectable levels presenting a possible similarity with biosolids storage conditions and the prolonged survival of indicators after mechanical dewatering. Temperature

profiles of stored biosolids have not been extensively recorded making it difficult to attribute storage temperatures with prolonged *E. coli* survival. To address this, research is needed to monitor the temperature variations in stored biosolids and correlate changes with levels of indicator bacteria. Temperature of cake pads is a characteristic that could be feasibly modified on treatment sites and therefore a suitable study parameter for the research work.

Temperature is an important parameter for the rate of cell function while nutrient availability ultimately determines the rate of cell growth and the peak concentrations possible. In the natural environment microorganisms are optimized for feast and famine conditions as nutrients fluctuate in a cell's immediate environment (Bosdriesz *et al* 2015). In anaerobic sludge digestion, for example, *E. coli* will experience competitive stress for substrates as reactor conditions favour the growth of methanogenic bacteria (Town *et al* 2014). Immediately after dewatering however, records of faecal coliforms show increases of 4 orders of magnitude compared to pre-dewatered samples (Higgins *et al* 2007a) with additional increases during cake storage. *E. coli* indicator fluctuations within the sludge treatment system give evidence of how changing environmental parameters, including substrate availability, may modify cell population dynamics. As dewatering operations increase aerobic conditions and reduce sludge matrix temperatures (as described above) shear forces acting upon the sludge matrix during mechanical dewatering may be a possible source of nutrient release and dispersal through the biosolids material (Qi *et al* 2004; Qi, Dentel and Herson 2007; Chen *et al* 2011). A notable effect of dewatering is possible cell disruption from physical stresses leading to membrane damage and consequential cell lysis. Sun *et al* (2015) investigated dewatering processes on cyanobacteria-containing sludges and attributed cell lysis to flocculation turbulence and pressure from mechanical operations on flocs resulting in cell damage. Recent research has suggested cellular excretion materials, as a consequence of cell lysis, may be used as nutrients for the remaining population. Noor (2015) identified lysis of VBNC *E. coli* cells in the early stationary phase as a response to heat stress. The triggered cell lysis provided a possible cellular survival output, contributing to the removal of damaged cells with resultant

aggregates serving as nutrients to remaining living cells. Confirmation of this is evident in work completed by Murate *et al* (2012) who examined the release of cytoplasmic materials into culture medium by studying the activity of β-galactosidase (cytoplasmic enzyme). Results from culture medium fractions, after incubation, suggested that cytoplasmic materials are released into culture medium as a consequence of cell lysis and are therefore likely to act as a nutrient supplement sustaining the remaining cell population. Further study is required to clarify whether nutrients from lysed cells indeed support remaining cell population survival (Murate *et al* 2012).

In the natural environment there are limited amounts of nutrients for microorganisms causing sporadic growth or dormancy in cells (Murate *et al* 2012). Biosolids storage occurs in open cake bays exposed to fluctuating atmospheric weather conditions which will likely influence *E. coli* growth behaviours. Varying annual precipitation amounts may cause sludge dry solid percentages to alter from post-dewatered levels which can modify the sludge morphology. Pesaro *et al* (2004) found that this can impact upon cell health as localised desiccation and moisture saturation may influence cell respiration rates and substrate availability. In addition, exposure to fluctuating weather conditions substantially influences sludge cake temperatures and cell heating, altering cell metabolic rate and growth (Caspeta *et al* 2009). The effects of moisture content are an important consideration for the understanding of bacterial growth dynamics within stored sewage sludge cake.

Whilst established research hypothesises on contributory factors to *E. coli* growth and death in biosolids there yet remains uncertainty in explaining this phenomena. Limited trials to control indicator presence in the sludge product have been completed and are often restricted to up-stream modifications in anaerobic digestion processes (Orzi *et al* 2015, Mondal *et al* 2015, Rosenblum *et al* 2014, Smith *et al* 2005, Le *et al* 2002). The identification of influential environmental precursors in the biosolids matrix and their relative effects on *E. coli* indicator dynamics will enable the characterisation of biosolids storage environments and

aid the development of storage modifications to inhibit indicator survival and achieve stipulated compliance levels.

1.2 Aims and Objectives

The aim is to identify factors affecting the dynamics of *E. coli* growth and death in stored sewage sludge cake and make an assessment of possible solutions to control indicator growth. The research hypothesis tested is *E. coli* growth and death in dewatered sewage sludge can be controlled by the modification of physical-chemical factors in the cake storage environment.

The key project objectives are to:

1. Assess the scientific literature on *E. coli* growth and survival in biosolids and analogous environments with a particular focus on biological and physiochemical environmental parameters.
2. Evaluate the effect of environmental parameters on *E. coli* die-off in laboratory-scale biosolids storage environments.
3. Characterise the biosolids storage environment and assess whether environmental parameter modification at an operational scale may successfully reduce indicator concentrations.
4. Identify the mechanisms that may contribute to the dynamics of *E. coli* cell concentrations in dewatered sludge samples.

1.3 Thesis Plan

Each thesis section has been structured in the form of published, submitted or in preparation journal papers, which together constitute the PhD thesis. All thesis sections have been written by the first author, Sarah Elizabeth Fane and supervisor's comments provided by Professor. Sean Tyrrel, Professor Jim Harris, Dr. Andreas Nocker and Professor Elise Cartmell. Industrial supervision from Severn Trent Water Plc. was provided by Mrs. Andrea Wilson and Dr. Yadira Bajon Fernandez. Laboratory work was undertaken by Sarah Elizabeth Fane, with exception of Chapters 3, 5 and 6 where there was laboratory support, in part, from an additional author or an external laboratory service under the direction of Sarah Fane. All data analyses has been completed by Sarah Fane.

The connection between thesis sections is highlighted in Table 1.1. Chapter 2 assesses the scientific literature on *E. coli* growth and survival in biosolids and analogous environments with a particular focus on biological and physiochemical environmental parameters. Chapter 2 is written up as a review article with the aim of submission to Critical Reviews in Environmental Science and Technology (CREST).

Chapter 3 forms a series of laboratory studies testing the effect of environmental parameters on *E. coli* die-off in biosolids storage environments. Results from experiments included in Chapter 3 were, in part, presented and included in conference proceedings of the European Biosolids and Organic Resources Conference (November 2014) and WEF/IWA Residuals and Biosolids Conference (June 2015). This chapter investigates innate cake characteristics in controlled laboratory-scale dewatering operations and continues with the analysis of ambient storage parameters assessing how modifications can impact on *E. coli* survival. Chapter 3 is written up in preparation for submission to the BioResource Technology journal.

Chapter 4 characterises the biosolids storage environment at an operational scale and investigates relationships between physical and chemical environmental factors in the storage environment with *E. coli* indicator behaviour. The novel work of this chapter surrounds the long term up-scaled monitoring of stored biosolids that reveals characteristics previously unknown. The manuscript was developed with the aim of submission to the BioResource Technology journal.

Chapter 5 investigates whether environmental parameter modification during biosolids storage can successfully reduce indicator concentrations. This chapter, focusing on up-scaled pilot work, highlights important considerations for sludge treatment operators and verifies laboratory scale trials explored in Chapter 3. Chapter 5 was, in part, presented at the SludgeTech conference (June 2015) and at the IWA Holistic Sludge Management conference (June 2016). Chapter submission is targeted at the Water and Environment Journal.

Chapter 6 studies the mechanisms of biosolids cell activity. The section contributes to the characteristics of cell cycling within biosolids dewatering and subsequent storage that may affect the dynamics of total cell concentrations. Novel flow cytometry measurement techniques were applied to identify damaged bacterial concentrations. Chapter 5 was presented, in part, at the SludgeTech 2016 conference and submission to BioResource Technology is targeted. A key output of this section has been to develop understanding on cell damage and the supplementary nutrients released for the survival of the remaining bacteria population.

A final discussion section forms Chapter 7 and gives an overall synthesis of the PhD research outputs. Research conclusions and future work are evaluated with the contributions to knowledge that the work presents.

A summary of the PhD thesis chapter structure is detailed in Table 1.1.

Table 1.1 PhD thesis structure and chapter interaction

Thesis Chapter	Objective	Hypothesis	Title	Chapter Interaction
1	Introduce project research topic and thesis scope		Introduction	
2	Establish the state of current knowledge on factors influencing *E. coli* growth and survival in biosolids and identify research gaps from scientific literature		Influence of innate sludge factors and ambient environmental parameters in biosolids storage on indicator bacteria survival: A review	• Current state of knowledge established and used to inform experimental design in Chapter 3. • Survey indicates no up-scaled, long-term monitoring of environmental parameters in biosolids storage exists, research gap addressed in Chapter 4
3	Quantify the effect of environmental parameters on *E. coli* die-off in biosolids storage environments at laboratory-scale	1. Growth and survival of *E. coli* bacteria are enhanced by higher sludge nutrient concentrations 2. Polyelectrolyte addition will increase levels of *E. coli* growth in the sludge matrix 3. *E. coli* die-off in stored biosolids will be accelerated under elevated storage temperatures in combination with modified atmosphere conditions 4. Within the operational range of biosolids moisture contents, a higher level of moisture will cause *E. coli* numbers to increase.	Influence of physical and chemical environmental factors on *E. coli* survival in biosolids at a laboratory scale	• Results used in the design of up-scaled pilot trials to verify results at operational scale (Chapter 5).

4	Identify the characteristics of the biosolids storage environment through a long term, up-scaled, environmental monitoring programme	1. Temperature of stored biosolids is a significant factor controlling *E. coli* concentrations. 2. pH changes in biosolids stockpiles is a parameter significantly influencing *E. coli* dynamics. 3. Dry solids % prevailing within the biosolids material has a significant effect on *E. coli* survival. 4. Redox potential conditions of stored biosolids are a significant factor controlling *E. coli* concentrations.	Long term characterisation of the biosolids storage environment: implications for *E. coli* dynamics	• Environmental monitoring data addresses research gap identified in Chapter 2 and emphasises the importance of sludge storage temperatures for the control of *E. coli,* linking in with results from Chapters 3 and 5. • Results provide knowledge for experimental design considerations in Chapter 5
5	Identify whether modification of parameters in biosolids storage environments, at an operational scale, is a successful method to reduce bacterial concentrations	1. *E. coli* die-off will increase under manipulated storage environments where stockpile temperature is elevated. *2.* *E. coli* die-off will increase in conditions where exposure to oxygen is reduced.	*E. coli* death rates in a summer and winter pilot trial testing modified biosolids storage environments	• Pilot trial data confirms laboratory results obtained in Chapter 3 • Builds on datasets from Chapter 4 and advances design considerations for manipulated storage environments in Chapter 6
6	Identify the characteristics of cell cycling within biosolids dewatering and subsequent storage contributing to the dynamics of total cell concentrations	1. Cell disruption provides a substrate source able to support the growth of *E. coli* bacteria in sewage sludge cake stored under favourable conditions. 2. Following cell disruption, enhanced *E. coli* die-off is achieved in controlled biosolids storage environments with higher temperatures and a gas evacuated or modified atmosphere.	Disruption of cells in biosolids affects *E. coli* dynamics in stored cake	• Current knowledge on dewatering process and cell disruption gathered in Chapter 2 used to inform experimental methods in Chapter 6 • Learning from Chapter 3 results on temperature and modified atmosphere built into final experiment of Chapter 6
7	Evaluation of PhD results and review of key learning outcomes	-	Final summary	Synopsis of PhD research and synthesis of whole thesis

1.4 References

ADAS, (2001) THE SAFE SLUDGE MATRIX: Guidelines for the Application of Sewage Sludge to Agricultural Land. Gleadthorpe Research Centre.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Ashbolt, N., Grabow, W., Snozzi, M., (2001) Indicators of microbial water quality, Water Quality: Guidelines, Standards and Health. London.

Bodriesz, E., Molenaar, D., Teusink, B. and Bruggeman, F. J. (2015) How fast-growing bacteria robustly tune their ribosome concentration to approximate growth-rate maximisation. *The FEBS Journal* Vol. 282 pp. 2024-2044

Caspeta, L., Flores, N., Pérez, N. O., Bolivar, F., Ramirez, O. T. (2009) The effect of heating rate on *Escherichia coli* metabolism, physiological stress, transcriptional response, and production of temperature-induced recombinant protein: A scale-down study. *Biotechnology and Bioengineering* Vol.102 No. 2 pp.468-482

Chen, Y.C., Higgins, M.J., Beightol, S.M., Murthy, S.N., Toffey, W.E., (2011) Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Res.* Vol. 45, pp. 2616–2626.

Deeks, L.K., Chaney, K., Murray, C., Sakrabani, R., Gedara, S., Le, M.S., Tyrrel, S., Pawlett, M., Read, R., Smith, G.H., (2013) A new sludge-derived organo-mineral fertilizer gives similar crop yields as conventional fertilizers. *Agron. Sustain. Dev*. Vol. 33, pp. 539–549.

Dentel, S.K., Qi, Y., Herson, D.S., (2008) Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration and assessment. *Water Sci. Technol*. Vol. 57, pp. 189–193.

Edberg, S.C., Rice, E.W., Karlin, R.J., Allen, M.J., (2000) *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol*. Vol. 88, pp. 106S– 116S.

Environment Agency, (2003) The Microbiology of Sewage Sludge-Part 1- An overview of the treatment and use in agriculature of sewage sludge in relation to its impact on the environment and public health. Bristol.

Haller, L., Pote, J., Loizeau, J.-L., Wildi, W., (2009) Distribution and Survival of Faecal Indicator Bacteria in the Sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Ecol. Indic*. Vol. 9, pp. 540– 547.

Henkel, S.G., Ter Beek, A., Steinsiek, S., Stagge, S., Bettenbrock, K., Teixeira de Mattos, M.J., Sauter, T., Sawodny, O., Ederer, M., (2014). Basic Regulatory Principles of *Escherichia coli's* Electron Transport Chain for Varying Oxygen Conditions. *PLOS one* Vol. 9. No. 9 e107640

Higgins, M.J., Chen, Y., Murthy, S.N., Hendrickson, D., Schafer, P., Farrell, J., (2006) WERF Phase 2: The Impact of Digestion and Dewatering on Reactivation and Regrowth of Viable but Non-Culturable Indicator Bacteria, in: WEFTEC 06. *Water Environment Foundation*, pp. 3146–3161.

Higgins, M.J., Chen, Y.C., Murthy, S.N., Hendrickson, D., (2007a) Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering, in: Procedings on Moving Forward Wastewater Biosolids Sustainability: Technical, Managerial and Public Synergy. International Water Association, Moncton, Canada, pp. 204– 419.

Higgins, M.J., Chen, Y.C., Murthy, S.N., Hendrickson, D., Farrel, J., Schafer, P., (2007b) Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Res*. Vol. 41, pp. 665–673.

Le, M.S., Mayhew, M., Back, P. (2002) Effectiveness of Secondary Digesters as a Pathogen Controller in Winter *CIWEM Journal* Vol. 16 pp. 292- 295

Metcalf & Eddy., Tchobanoglous, G., Burton, F. L & Stensel, H. D (2003). Wastewater Engineering: Treatment and Reuse (4th Ed.). Boston: McGraw-Hill

Mondal, T., Rouch, D. A., Thurbon, N., Smith, S.R., Deighton, M. A. (2015) Factors affecting decay of *Salmonella* Birkenhead and coliphage MS2 during mesophilic anaerobic digestion and air drying of sewage sludge *Journal of Water and Health* Vol. 13 No. 2 pp. 459-472

Monteleone, M.C., Furness, D., Jefferson, B., Cartmell, E., (2004) Fate of *E. coli* across mechanical dewatering processes. *Environ. Technol*. Vol. 25, pp. 825–831.

Murate, M., Noor, R., Nagamitsu, H., Tanaka, S and Yamada, M. (2012) Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*. *Genes to Cells* Vol. 17 pp. 234-247

Nicholson, F. A., Hutchison, M. C., Smith, K. A., Keevil, C. W., Chambers, B. J., & Moore, A. (2000). A study on Farm Manure Application to Agricultural Land and an Assessment of the Risks of Pathogens Transfer into the Food Chain. *Final Report to the Ministry of agriculture, Fisheries and Food*. London.

Noor, R (2015) Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. *SpringerPlus* Vol. 4 pp. 599

Orzi, V., Scaglia, B., Lonati, S., Riva, C., Boccasle, G., Alborali, G.L., Adani, F. (2015) The role of biological processes in reducting both odor impact and pathogen content during mesophilic anaerobic digestion *Science of the Total Environment* Vol. 526 pp. 116-126

Paruch, A. M (2015) Effects of temperature, storage time and pH on survival of *Escherichia coli* in source-separated yellowwater. *Water and Environment Journal* Vol. 29 pp. 98-104

Pesaro, M., Nicollier, G., Zeyer, J and Widmer, F. (2004) Impact of soil drying-rewetting stress on microbial communities and activities and on degredation of two crop protection products. *Applied and Environmental Microbiology*. Vol. 70 No. 5 pp. 2577-2587

Qi, Y. N., Gillow, S., Herson, D. S., & Dentel, S. K. (2004). Reactivation and/ or growth of fecal coliform bacteria during centrifugal dewatering of anaerobically digested biosolids. *Water Science adn Technology,* Vol. 50 No. 9, pp. 115- 120.

Qi, Y., Dentel, S. K., & Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. *41*, pp. 571- 580.

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Seadi, T., Rutz, D., Janssen, R., Drosg, B. (2013) *The Biogas Handbook: Science, Production and Applications.* Elsevier Inc. Switzerland

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M., (2007) Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol*. Vol. 60, pp. 419–428.

Sidhu, J.P.S., Toze, S.G., (2009) Human pathogens and their indicators in biosolids: A literature review. *Environ. Int*. Vol. 35, pp. 187–201.

Stevens, M., Ashbolt, N., Cunliffe, D., (2003). Review of Coliforms as Microbial Indicators of Drinking Water Quality. Recommendations to Change the Use of Coliforms as Microbial Indicators of Drinking Water Quality. Canberra.

Sun, F., Hu, W., Pei, H., Li, X., Xu, X., Ma, C. (2015) Evaluation on the dewatering process of cyanobacteria-containing $AICI_3$ and PACI drinking water sludge. *Separation and Purification Technology* Vol. 150 pp. 52- 62

Town, J.R., Links, M. G., Fonstad, T. A., Dumonceaux, T. J. (2014) Molecular characterisation of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. *BioResource Technology* Vol. 151 pp. 249- 257

UK Water Industry Research (UKWIR), (1999) *E. coli* in UK Mesophilic anaerobically digested sludge. London.

US EPA (United States Environmental Protection Agency), (2012) A Plain English Guide to the EPA Part 503 Biosolids Rule [WWW Document]. URL http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm [accessed 5.1.15]

Weiland, P (2010) Biogas Production: Current state and perspectives. *Appl Microbiol Biotechnol* Vol. 85 pp. 849-860

Chapter 2

Influence of Innate Sludge Factors and Ambient Environmental Parameters in Biosolids Storage on Indicator Bacterial Survival: A Review

Statement of author contributions:

S. Fane: Principal investigator and writer

Professor S. Tyrrel: Principal supervisor supporting development of structure and evaluation of final chapter write up

2 Influence of Innate Sludge Factors and Ambient Environmental Parameters in Biosolids Storage on Indicator Bacteria Survival: A Review

Fane, S. and Tyrrel, S

Cranfield Water Science Institute, Cranfield University, Cranfield, MK43 0AL

The objective of this review survey is to establish the state of current knowledge on factors influencing *Escherichia coli (E. coli)* growth and survival in biosolids and identify research gaps from scientific literature. The review will give background to sludge treatment and the legislation governing biosolids application to agricultural land. An evaluation into the environmental parameters of sludge storage and analogous environments will be used to inform understanding on principal factors that are likely to affect the dynamics of *E. coli* bacterial behaviour in biosolids storage.

2.1 Sludge treatment processes and storage

Sludge production forms a large fraction of wastewater treatment and suitable disposal routes are receiving increased attention as a consequence of more stringent criteria (DEFRA 2013). Sludge may be disposed on landfill or incinerated, however the most practicable environmental option recommended by EU and UK governments is land application (Water UK 2010). To ensure that a safe product is applied to agricultural land sludge treatment processes must be validated for treatment efficacy (European Union 2000).

During treatment a typical unit of sludge will undergo mesophilic anaerobic digestion (AD) and mechanical dewatering, typically through a centrifuge. Following this the resultant sludge cake, or biosolids product, is stored for a minimum period of 14 days (DEFRA 2006) before compliance level microbial testing. Anaerobic digestion is a bacteria process carried out in the absence of oxygen (Wang *et al* 2008). The treatment is used as a method to stabilise and reduce the mass of biosolids (Metcalf and Eddy 2003) with the primary benefit of

high biogas yield for energy recovery (Wang *et al* 2008). Digestion typically operates under mesophilic, anaerobic conditions (MAD) at 30- 40°C. In the UK approximately 60% of sludge produced is stabilised by MAD (Smith *et al* 2005). The Code of Practice for the agricultural use of sewage sludge (DEFRA 2006) directs a mean retention period of at least 12 days primary digestion in temperature range 35°C±3°C. A survey by UKWIR (1999) of primary MAD facilities found *Escherichia coli* (*E. coli)* log reduction ranged from 1.35 to 3.36 emphasising the importance of up-stream sludge treatment processes in the control of pathogen indicator bacteria. Much research has been completed on the optimisation of anaerobic digestion and profiling reactor performance for bacterial pathogen inactivation and responses to environmental parameter controls. Factors to consider in the optimisation of digestion processes for indicator removal are solids retention time (Forster-Carneiro *et al* 2010, Chen *et al* 2012), reactor temperature (Ziemba and Peccia 2011) and efficient stabilisation of the biowaste (Smith *et al* 2005).

Once digested, the stabilised liquid sludge is dewatered to reduce the sludge water content. This limits capital, transportation and operational expenditure (Mowla *et al* 2013). Dewatering is defined by Mowla *et al* (2013) as the application of pressure to force out the water molecules from sludge. Centrifuges and belt presses are commonly employed for this operation on municipal wastewater sludges which can reach a dry solids (DS) content of 50% (Mowla *et al* 2013). In the UK centrifuge dewatering with polyelectrolyte flocculant is typically employed, reaching a target DS% of 25 (Severn Trent Water Plc. Operational Data 2008-2016).

Polyelectrolytes play an important role in sludge conditioning to improve dewaterability. Bolto and Gregory (2007) suggest that organic polymers in the form of polyelectrolytes condition the sludge to form flocs through charge neutralisation and polymer bridging. In wastewater sludge, impurity particles are negatively charged and therefore cationic polymers are the most effective flocculants. Reduced surface charge of particles limits electrical repulsion between them, aiding flocculation (Bolto and Gregory 2007). Polymer bridging

occurs when segments of polymer chains are able to attach to the unoccupied surface on a particle causing bridging flocculation. In addition to flocculation mechanisms, kinetic characteristics are a fundamental aspect to consider when polymeric flocculant is dosed into a suspension of particles (Bolto and Gregory 2007). High shear forces present in centrifuge dewatering make polyelectrolyte addition essential as coagulation and flocculation of solids particles improves settlebility (Mowla *et al* 2013). Mowla *et al* (2013) highlights three limiting factors in sludge dewatering. These include low settleability of solids particles, high compressibility of sludge solids and high affinity of extracellular polymeric substances (EPS) for trapping water molecules. Particle size and particle size distribution determine settleability of solid particles and the porosity and permeability of the solid cake product (Mowla *et al* 2013). A successful sludge dewatering process, defined by Mowla *et al* (2013), will produce a permeable and ridged lattice structured cake product which can remain porous and incompressible under high positive pressure, maintaining the size of micropassages for water expression.

The cake product undergoes a storage treatment before land application. This provides a final barrier preventing the transmission of wastewater contaminants to agricultural land. Biosolids storage is employed with microbial compliance monitoring to verify effective treatment and removal of indicator bacteria (*E. coli*). The Code of Practice for the agricultural use of sewage sludge (DEFRA 2006) stipulates that sludge, subject to primary mesophilic anaerobic digestion, must be stored for a minimum period of 14 days. The primary purpose of this final treatment is to significantly reduce the potential health hazard posed by the risk of pathogens in sewage sludge (DEFRA 2006). Regulations require that where sludge is used on agricultural land, usage will be registered by sludge producers (ADAS 2001). In addition, records of sampling and analysis of sludges and the quantities of sludge produced and supplied for use in agriculture must be recorded by each source works so that a permanent running record of operations exist.

2.2 Legislative controls

Pathogen indicator bacteria monitoring in sewage sludge treatment has been a relatively recent addition to Directives governing sewage sludge use in agriculture. The original DEFRA Code of Practice document was issued in 1989 and focused on analysis after processing to ascertain the concentrations of metals in the sludge product intended to be used on agricultural land, before delivery to the user (DEFRA 1989). Key elements of concern in the 1989 document were Chromium, Zinc, Copper, Nickel, Cadmium, Lead and Mercury. Although scientific literature published as early as 1977 gave caution of organic waste application on agricultural crop land due to the possible presence of viral and bacterial pathogens (Elliott and Ellis 1977) no mention of pathogen transfer risk was included in this edition of the Code of Practice document. In 1998 agricultural application of sewage sludge became a more prevalent disposal route as EU Commission member states were directed to ensure that by 31st December 1998 the disposal of sludge to surface waters was phased out (Council Directive 91/271/EEC). This change increased the risk of higher pathogen-loading rates on crop land should inadequate sludge treatment be left unmonitored. As a precautionary industrial action to the amended disposal route ADAS, Water UK (representing 14 UK Water and Sewage Operators) and the British Retail Consortium (BRC) issued guidance for the application of sewage sludge to agricultural land (ADAS 2001). The guidelines, formally known as the Safe Sludge Matrix (SSM), consist of a table of crop types with clear direction on the minimum acceptable level of treatment of any sewage sludge (biosolids) based product which may be applied to that crop (ADAS 2001). The provisions of the SSM agreement were incorporated into the Sludge (Use in Agriculture) Regulations, 2001 under the Environment Protection Act (L.N. 212). The Matrix ensures the highest possible standards of food safety and provides a framework giving farmers, retailers and the food industry confidence that sludge reuse on agricultural land is safe (ADAS 2001). The agreement allows the beneficial properties of sewage sludge to continue to be used as a valuable source of nutrients and organic matter. Table 2.1 quantifies the levels of treatment stipulated in the SSM.

Table 2.1 Safe Sludge Matrix guidelines for the acceptable level of treatment for any sewage sludge based product before land application (ADAS 2001)

Biosolids Treatment Category	***E. coli***
Conventionally Treated	2 Log $_{10}$ reduction / 99% pathogens destroyed
Enhanced Treated	6 Log $_{10}$ reduction / 99.9999% pathogens destroyed

For conventionally treated sludge the Environment Agency additionally stipulates a Maximum Allowable Concentration (MAC) with a threshold value of 10^5 *E. coli* CFU (colony forming unit) per gram dry solid (Environment Agency 2003).

At an international level, a similar standard to the SSM is the Part 503 rule set by the Environmental Protection Agency (EPA) in the USA. Two levels of sludge treatment are permitted, a Class A and a Class B standard. The Class A standard has a higher level of sludge quality and requires the density of faecal coliforms to be <1000 Most Probably Number (MPN) per gram total solids (dry weight basis) (US EPA, 2012). In addition to faecal coliform control, Class A must additionally achieve a density of <3 MPN per 4 grams of total dry solids of *Salmonella*. The Class B level of treatment is lower and reflects more similarly the conventional treatment category stipulated in the SSM (ADAS 2001). The Class B standard specifies that sludge treatment meets <2 x 10^6 MPN faecal coliforms per gram of total solids or <2 x 10^6 CFU faecal coliforms per gram of total solids at the time of use or disposal based on a geometric mean of 7 samples (US EPA, 2012).

A more recent scheme to increase food industry reassurance is the Biosolids Assurance Scheme (BAS) which has been under development with Water UK since 2013. BAS aims to promote public acceptance, protection of the environment and endorse sustainable practices. It provides food chain stakeholders with a single transparent assurance scheme standard, subject to independent inspection and certification (Water UK 2013). Objectives of BAS are to achieve operational consistency and transparency whilst combining legislative and non-legislative requirements with best practices such as sludge storage risk assessments and Hazard Analysis and Critical Control Point (HACCP) process

controls. A full draft of the BAS Scheme Standard was published in September 2015 (Assured Biosolids Ltd. 2015) and is underpinned by a separate Hazard Assessment document based on the potential hazards in source material and the end product. The assessment identifies management control measures which are referred to in the Scheme Standard.

Regulations on the storage environment focus upon surface and ground water contamination with no advisory comments on the best practice to support bacterial die-off. The key conditions stipulated by the Environmental Agency on sludge storage are listed below (Environment Agency 2012):

1. 10 metres from any watercourse
2. 50 metres from any spring or well, or from any borehole not used to supply water for homes or producing food
3. 250 metres from any borehole used to supply water for homes or producing food

Treatment sites can store up to 1,250 tonnes of sewage sludge at any one time and the environmental regulator must be informed of any storage facility in operation (DEFRA 2013). The regulation on sludge containment does not encourage consistent or parameter-controlled storage and as a consequence facilities have often been developed to meet low capital expenditure targets. A typical sludge cake bay holds between 200-250 tonnes of sewage sludge and is constructed with a concrete base where run off is channeled into surface drains. Concrete side walls segregate individual bays and no top cover is present. Considering the level of environmental parameter control and monitoring that takes place in up-stream sludge treatment processes the storage of this valuable organic waste by-product appears to be overlooked and inconsistent with highly controlled wastewater treatment processes.

As the attractiveness of sewage sludge use in agriculture increases due to synthetic fertiliser sources becoming more expensive, a market for sludge is opening up (London Economics 2010). While biosolids have long been given away for free, it is now more widely accepted that farmers will pay to receive this

alternative fertiliser. A price of £1-2 per tonne is typical with utility companies providing the service of spreading product onto receiving land. Although this price does not necessarily offset the processing costs it is conceivable that when all the value contained in sludge is extracted (chemicals, power production, inert matter) a positive price and a market for sludge treatment and disposal can emerge (London Economics 2010). Future direction such as this will drive competition in sludge quality and the preservation of intrinsic sludge value. A possible factor that may limit sludge commercialisation is uncertainty surrounding the acceptance of sludge recycling to agriculture. Due to this, operators are encouraged to maintain tight control and mitigate against the risk of this major disposal route being closed off. It therefore seems plausible that for sludge markets to open up, a consistent and predictable sludge quality is necessary to safeguard the sludge-to-land route.

2.3 Sludge quality concerns

In wastewater treatment microorganism survival is increased if cells are associated with solid particles (Sidhu and Toze 2009). The separation of wastewater solids into the sludge-stream means higher pathogen concentrations and survival rates are likely in the sludge product. Although anaerobic digestion provides stabilisation and additional benefits of reduced biological activity in sludge (Chen *et al* 2011), research has identified high concentrations of indicator bacteria in anaerobically digested biosolids, immediately after mechanical dewatering (Monteleone *et al* 2004, Higgins *et al* 2006, Higgins *et al* 2007a, Higgins *et al* 2007b, Dentel *et al* 2008, Qi *et al* 2008, Chen *et al* 2011). A revival of *E. coli* bacteria may indicate the presence of pathogens and therefore a significant risk of non-compliance, causing concerns for wastewater treatment operators (Springings and Le 2011). Although resurgent *E. coli* levels are frequently observed in biosolids storage this is not the case at all treatment facilities. Examples of indicator increases range from -0.4 Log to +6.4 Log units after centrifuge dewatering (Qi *et al* 2007) emphasising the highly variable effects amongst treatment plants. This unpredictable indicator bacterial behaviour in treatment and storage causes much concern for utility operators and limits the

quality and value of sludge products. Cooper *et al* (2010) observed *E. coli* concentrations in cake samples from 8 UK wastewater treatment sites and recorded an average increase of 0.5 Log units for digested and centrifuge dewatered sludge. Of interest were findings on belt pressing which showed a smaller, but nevertheless significant, increase of 0.2 Log (Cooper *et al* 2010). Dewatering and digestion treatment type appear to have a significant impact on *E. coli* growth (Monteleone *et al* 2004, Higgins *et al* 2007a). A study comparing single phase thermophilic digestion with mesophilic digestion processes showed that although effluent from thermophilic digesters had $<10^2$ CFU/g DS (a 6 Log reduction from the raw influent), immediately after centrifuge dewatering the density of faecal coliforms was 10^6 CFU/g DS, an increase of four orders of magnitude. For mesophilic plants *E. coli* increase by approximately one order of magnitude in the dewatered cake using standard culturing methods (Higgins *et al* 2007b). A continuation of this study also looked at *E. coli* densities in the stored biosolids produced after thermophilic and mesophilic digested sludge was centrifuge dewatered and stored at 35°C. For both treatments *E. coli* concentrations increased within the first three days to between 10^8 and 10^9 cells/g DS. Higgins *et al* (2007b) argues that these results suggest the growth conditions are favourable after dewatering. Centrifuge dewatering may result in the destabilisation of the microbial ecology and provide conditions within the cake that encourage growth of faecal coliforms and *E. coli*. Higgins *et al* (2007b) highlights that continued storage of the biosolids is a method that may allow the desired goals for indicator organism compliance to be met. Little research has been completed, however, on how the biosolids storage environment may influence the control of indicator behaviour. The key factors responsible for indicator growth and survival in stored dewatered biosoilds and their relative importance have not yet been clearly identified (Dentel *et al* 2008). The immediate concentration elevation post-dewatering and the prolonged survival during subsequent biosolids storage, suggests characteristics of the storage environment may be the underlying reason (Higgins *et al* 2007a).

2.4 Chemical Microbial Growth Requirements

In addition to favourable environmental conditions, microorganisms require key elements to survive and grow. In biosolids products, cells must be able to find all the substances required for energy generation and cellular biosynthesis (Todar 2012). These nutrients form a cells basic elemental composition and are found in the form of water, inorganic ions, small molecules and macromolecules (Todar 2012). Table 2.2 shows 10 nutritional elements a cell will need for general physiological function (Sigma Aldrich 2016, Todar 2012).

Table 2.2 Elements required for microbial growth with specific sources and cell functions defined (Sigma Aldrich 2016, Todar 2012)

Elemental Requirement for Growth	Natural Source	Cellular Function
Carbon	Carbon Dioxide (CO_2), HCO_3^-, Organic Compounds	Cellular material
Hydrogen	Water (H_2O), Organic Compounds, H_2	Organic compounds within cell and cell water
Oxygen	Water (H_2O), oxygen gas (O_2), Organic Compounds	Constituent of cell material and cell water O_2 is an electron acceptor in aerobic respiration
Nitrogen	Ammonia (NH_3), Nitrate (NO_3^-), Organic Compounds (amino acids, nitrogen gas (N_2)	Constituent of amino acids, nucleic acids, nucleotides and coenzymes
Phosphorous	Phosphate (PO_4^{3-}), Inorganic Phosphates (PO_4),	Constituent of nucleic acids, nucleotides, phospholipids, Lipopolysaccharides (LPS), teichoic acids
Sulphur	Hydrogen Sulphide (H_2S), Sulphate (SO_4^{2-}), Organic Sulphur Compounds (cysteine)	Constituent of cysteine, methionine, glutathione and several coenzymes
Potassium	Potassium Salts (K^+)	Main cellular cation Cofactor for certain enzymatic reactions
Magnesium	Magnesium Salts (Mg^{2+})	Inorganic cellular cation Cofactor for certain enzymatic reactions
Calcium	Calcium Salts (Ca^{2+})	Inorganic cellular cation Cofactor for certain enzymes Component of endospores
Iron	Iron Salts (Fe^{3+}), Organic Iron Complexes	Component of cytochromes and certain non-heme iron proteins Cofactor for some enzymatic reactions

In the natural environment, for example soil, microorganisms are optimized for feast and famine conditions as nutrients fluctuate in a cell's immediate environment (Bosdriesz *et al*., 2015). *E. coli* can reproduce rapidly and are not inhibited easily by varying chemical conditions, a characteristic of a good faecal indicator (Edberg *et al* 2000, Stevens *et al* 2003, Haller *et al* 2009, Alberts *et al* 2010). The sludge treatment system provides some clues as to the conditions that may influence nutrient availability and subsequential bacterial survival.

An important benefit of anaerobic digestion in sludge treatment is the reduction of pathogen indicator bacteria alongside sludge stabilisation and biogas production. Substrate competition is, in part, responsible for pathogen reductions in AD (Orzi *et al* 2015) as *E. coli* experience competitive stress for nutrients in an environment favouring the growth of methanogenic bacteria (Town *et al* 2014). Biological competition for nutrients is primarily influenced by digester loading rates. Rosenblum *et al* (2014) found a direct relationship between increase loading rates and higher levels of indicator bacteria. This relationship provides evidence that substrate levels, influenced by solids retention time and subsequent nutrient limitation, play a substantial role in faecal indicator quantities (Chen *et al* 2012, Rosenblum *et al* 2014). Smith *et al* (2005) and Scaglia *et al* (2014) argue the importance of organic matter stabilisation, substrate limitation and microbial competition between pathogens and indigenous microflora in controlling the inactivation rate of pathogens under mesophilic conditions. Under optimal conditions, organic matter is extensively degraded in AD, producing a high stability in the sludge effluent. High levels of biological stability after digestion suggests microbial activity is low, a likely result of substrate limitation (Orzi *et al* 2010). Following digestion, liquid sludge is combined with polyelectrolyte as a flocculation aid before mechanical dewatering. The polyelectrolyte addition determines the final solids concentration of the biosolids product. Monteleone *et al* (2004) suggests that polyelectrolyte effects are an important consideration when explaining % *E. coli* increase in biosolids products. Qi *et al* (2007) studied the effects of polymer addition on faecal coliforms in digested biosolids at polymer: total solid ratios of 1:100 and 1:10 before and after 24 hours of incubation at 37°C. After incubation results showed a higher faecal coliform

concentration (2 orders of magnitude increase) with greater polymer addition (1:10 concentration). From this data Qi *et al* (2007) argue that polyelectrolyte addition does have a significant influence on final coliform concentrations in dewatered biosolids. The physical effects of flocculation or the degradability of the polyelectrolyte itself (Chang *et al* 2001) could be attributed to this coliform increase. As the 1:10 concentration shows significantly higher levels of coliform bacteria the researchers suggest a substrate effect is likely (Qi *et al* 2007). Cationic polyacrylamide (PAM) polymers are commonly used in wastewater treatment and contain the residual monomer acrylamide. Acrylamide is known to be a toxic compound (Junqua *et al* 2015) and it therefore seems unlikely that polymer degradation would lead to increased faecal coliform growth due to a substrate effect. In addition PAMs have a high molecular weight and are therefore unable to pass biological membranes, limiting any potential biodegradation (Caulfield *et al* 2002). If biodegradation were to occur the action of extracellular enzymes would be necessary (Guezennec *et al* 2015). In contrast to this however, researchers have observed that PAMs in contact with certain microorganisms can be subject to biodegradation. Wen *et al* (2010) reported the isolation of 2 strains of bacteria from activated sludge and oil-contaminated soil (*Bacillus ceraus* and *Bacillus flexu*). Both strains were able to grow on a medium containing PAM as a sole carbon and nitrogen source (Wen *et al* 2010). After 72 hours of cultivation in a mineral salt medium supplemented with 60 mg/ L of PAM at 30°C, more than 70% of the PAM was consumed (Wen *et al* 2010). More work is still needed on polyelectrolyte addition in sludges and the degradation processes of PAMs to confirm whether it does provide a nutrient source for indicator bacteria.

The physical effects of flocculation from polymer addition may be a supporting factor of bacterial survival. In mechanical dewatering, polymers condition the sludge to form a floc, inevitably aggregating bacteria and sludge organic matter. Research has shown that large amounts of bioavailable protein and polysaccharide exist in centrifuge dewatered cakes (Higgins *et al* 2006). These elements will be incorporated within floc matrices and could therefore provide a readily available substrate source promoting bacterial growth. The formation of a

floc may also provide protection for bacteria cells from other environmental stressors, prolonging cell survival. A strong argument put against this by researchers however, are the effects of centrifuge shearing which is thought to cause floc disruption and bacterial dispersal, elevating indicator concentrations in dewatered biosolids (Monteleone *et al* 2004, Qi *et al* 2007,Qi *et al* 2008, Chen *et al* 2011). To observe the effects of shearing on faecal coliform growth Chen *et al* (2011) conducted a laboratory scale simulation of both belt filter press and centrifuge dewatering. The shearing test replicated a belt filter press with 0 passes and then pressed cakes were subject to 5 and 10 conveying passes to represent the internal conveying scroll of a decanter centrifuge at 2 levels of shearing force. The results indicate that with more shear during dewatering, a greater increase in faecal coliform density occurred during subsequent storage. With 10 passes faecal coliform density increased from original values by 5 Log units after 2 days of storage (Chen *et al* 2011). To explain this phenomenon Chen *et al* (2011) suggests that soluble proteins and other organics are released during shearing and serve as substrates for microbial growth. Evidence for this argument was observed in cake samples spiked with 1 mL of a glucose/ bacto-peptone mixture. When compared with control treatments (water addition only) after 24 hours faecal coliforms had increase by 2 orders of magnitude in spiked samples. These results indicate that substrate is a limiting factor in biosolids and that the provision of substrates stimulates microbial growth (Chen *et al* 2011).

As a counter argument to this Higgins *et al* (2007b) highlights potential problems with the explanation of shearing causing increased indicator densities in dewatered cake. For regrowth to occur a significant time is needed to increase counts by several orders of magnitude, *E. coli* doubling time is 20 minutes under optimal conditions (unlikely to be found in sludge treatment environments). The typical retention time a unit of sludge will be held in a centrifuge is 20 minutes and therefore the large increase in bacteria density immediately after centrifuge dewatering cannot be explained by regrowth alone. In addition, the release and floc break up in a centrifuge seems unlikely as the process is conditioned with polyelectrolyte flocculation aids to support aggregation and the formation of the cake product (Higgins *et al* 2007b). Instead, Higgins *et al* (2007b) suggest that

bacteria enter a viable but non culturable (VBNC) state during digestion. The VBNC state prevents bacteria enumeration using standard plating techniques, giving a value of only culturable bacteria after digestion. During dewatering the bacteria may be reactivated or resuscitated, entering a culturable state and giving a greater number of counts in standard enumeration methods. Bacteria will enter a VBNC state after exposure to environmental stress such as nutrient deprivation or high temperature which are conditions that can be present in digestion (Higgins *et al* 2007b). Research methods compared (competitive*)* polymerse chain reaction (c)PCR (targeting *E. coli*) and standard culturing methods (SCM) from thermophilic and mesophilic digestion plants. For both thermophilic and mesophilic samples results support the non-culturable theory. The *E. coli* densities measured with cPCR before and after dewatering were not statistically different however values measured with SCM indicated less *E. coli* bacteria were present after digestion, suggesting that a proportion of the bacteria measured were in a non-culturable state (Higgins *et al* 2007b). The cPCR results from mesophilic digestion samples were at least one order of magnitude higher in *E. coli* density for digester effluent than SCM results. Following dewatering, cPCR and SCM *E. coli* results were equivalent. Higgins *et al* (2007b) attributes this difference to a reactivation of bacteria during dewatering. Although Higgins *et al* (2007b) does dispute the argument of shear force effects in the centrifuge dispersing bacterial flocs he does argue that one of the reactivation mechanisms may be due to the release of growth factors as a result of shear. The release of growth factors such as auto inducers may support the resuscitation of bacteria into a culturable state (Higgins *et al* 2007b).

It may be possible that the shear experienced in centrifuge dewatering is able to disrupt cells. Chen *et al* (2005a) identified two factors that negatively impacted methanogenic activity when studying volatile organic sulphur compound (VOSC) production in anaerobically digested biosolids. A comparison between high and medium solids centrifuges found a 3.7 times reduction in methane production rate from high solids centrifuge samples. This difference was attributed to the possible effect of shearing on methanogens associated with the high solids centrifuge. Researchers suggested that a higher level of shearing would lead to greater cell

lysis inhibiting methanogenesis (Chen *et al* 2005a). The shear and destruction of bacterial cells may provide additional bioavailable protein which supports the theory of nutrient release in centrifuge dewatering operations to support bacterial populations in the fresh cake. Physical stressors acting upon cells during dewatering may lead to membrane damage and consequential cell lysis. Sun *et al* (2015) investigated dewatering processes on cyanobacteria-containing sludge and attributed cell lysis to flocculation turbulence and pressure from mechanical operations on flocs. Recent research has suggested cellular excretion materials, as a consequence of cell lysis, may be used as nutrients for the remaining microbial population. Noor (2015) identified lysis of viable but non-culturable (VBNC) *E. coli* cells in the early stationary phase as a response to heat stress. The triggered cell lysis provided a possible cellular survival output, contributing to the removal of damaged cells with resultant aggregates serving as nutrients for remaining viable cells. Although Higgins *et al* (2007b) suggests it is the reactivation of VBNC cells that causes indicator increase it may be feasible that a proportion of the VBNC cells lyse and provide cellular nutrients for the remaining culturable and reactivated bacteria in the dewatered biosolids. Confirmation of this is evident in work completed by Murate *et al*., (2012) who examined the release of cytoplasmic materials into culture medium by studying the activity of β-galactosidase (cytoplasmic enzyme). Results from culture medium fractions, after incubation, suggested that cytoplasmic materials are released into culture medium as a consequence of cell lysis and may therefore act as a nutrient supplement for the survival of the remaining population. Although stored biosolids are considered nutrient limiting with regards to readily bioavailable nutrients, studies above suggest the sludge material after mechanical dewatering processes may have an increased amount of bioavailable nutrients able to support cell growth. As *E. coli* are able to replicate within 20 minutes under favourable growth conditions (Todar 2012) the indicator bacteria are likely to have a competitive advantage, utilising available nutrients and proliferating within the post-dewatering biosolids environment. Further study is required to clarify whether nutrients from lysed cells support remaining cell population survival (Murate *et al*., 2012) particularly in wastewater sludges.

2.5 Physical Environmental Factors

For microbial growth, a cell will not only require key nutritional elements but also suitable environmental conditions. In a laboratory setting, Knijnenburg *et al* (2009) observed that steady state chemostat cultures enable accurate control, measurement and manipulation of microbial cultures and individual environmental parameters such as temperature. For cells held in a sludge matrix, the biosolids storage environment will not offer such favourable growth conditions. As a consequence survival is dependent upon a cell's ability to adapt in changing environmental conditions. The variation in temperature throughout sludge treatment processes and storage will influence cell activity. Knowledge of indicator bacteria concentrations in MAD identifies inhibitory environmental factors contributing to bacterial reductions which may help understand what storage conditions are relevant to their control.

2.5.1 Temperature

Temperature has been identified as a critical parameter controlling cell death rates and determining the biochemical conditions of AD (Scaglia *et al* 2014). Le *et al* (2002) identified temperature as a key factor for pathogen deactivation in operational-scale secondary digesters. Tanks operating at a maximum temperature of 28°C and 21°C showed *E. coli* reductions of 1.7 and 1.0 Log respectively. Lang and Smith (2008) highlight that the optimum temperature for growth and survival of enteric organisms is within the range of 30-40°C (ICMSF, 1980) and therefore mesophilic temperatures during MAD do not exert a critical thermal stress on the decay of *E. coli* or *Salmonella*. Temperature regulates processes including substrate limitation and microbial competition which, as described above, have an influence on pathogen reduction and can therefore indirectly cause pathogen inactivation (Smith *et al* 2005; Rosenblum *et al* 2014). Post-digestion, the temperature of sludge will gradually reduce particularly after dewatering and during the biosolids storage phase which nominally occurs in uncontrolled, open bays. Iranpour *et al* (2005) completed temperature monitoring in the post-digestion train during pilot tests on a battery of 6 thermophilic digesters. Tests showed a large drop in the biosolids temperature; digester outlet

52.6°C, centrifuge outlet 48.2°C, silo at truck loading facility 41°C, farm storage 40.3°C. The reoccurrence of faecal coliforms in post-digestion biosolids was attributed to this temperature reduction and researchers argue that maintaining a minimum temperature of 50°C (representative of the up-stream thermophilic digestion process) may prevent growth of faecal coliforms. Further tests with insulated silos and electrical heat-tracing confirmed that faecal coliform levels were able to be controlled by preventing the reduction in biosolids temperature (Iranpour *et al* 2005). A comparative on-site study was completed by Sprigings and Le (2011) and focused on determining the temperature representative of the biosolids storage conditions. Cake pad temperature monitoring was undertaken from the centre of the cake pile and taken alongside ambient temperature measurements at two treatment sites over a period of 48 hours. Results showed the immediate cake temperature after centrifuge dewatering was approximately 30°C (±2.5°C) with the cake retaining this higher temperature for approximately 12 hours, possibly an effect of residual heat from the digestion process. Over the following 48 hours the temperature profile gradually reduced and for both treatment sites fell below the ambient temperature recorded (Sprigings and Le 2011). Sprigings and Le (2011) suggest that the role of residual heat in the first 12 to 24 hours is an important factor in aiding the proliferation of *E. coli* with peak concentrations measured at 10^7 CFU g/DS. Arguably this temperature dataset is not fully representative of the biosolids storage environment as treatment requires a 14 day storage period after digestion (DEFRA 2006). As data shows the temperature value to fall below the ambient temperature after 48 hours (Sprigings and Le 2011) it may be that cooler temperatures in the following days of storage are preserving the growth and survival of bacteria within the biosolids storage environment. A long term monitoring programme of biosolids storage environments with temperature recordings has not been undertaken in previous research.

Studies on microbial dynamics in livestock manure and soils, which have greater associated datasets and form an analogous environment to biosolids may enhance understanding on the key parameters influencing indicator behaviour in biosolids. In a study by Semenov *et al* (2007) testing the effects of temperatures

ranging from 7 to 33°C on *Salmonella* and *E. coli* 0157:H7 survival in cow manure microcosms, survival of both pathogens declined significantly with increasing mean temperatures. The authors hypothesised that the reduced survival at higher temperatures may be a consequence of greater stress and energy expenditure for a particular organism (Semenov *et al* 2007). Therefore, the reducing storage temperatures identified by Sprigings and Le (2011) and Iranpour *et al* (2005) in biosolids storage are likely to lessen the stress on cell functions and limit excessive energy expenditure for indicator bacteria held within the sludge matrix. Consequently, the steady growth and prolonged survival of *E. coli* bacteria is likely in uncontrolled biosolids storage environments. In addition, microorganisms antagonistic to enteropathogens in manure are more competitive at temperatures between 16 and 33°C, possibly due to increased temperature initially causing faster growth (Semenov *et al*., 2007). Reduced antagonistic activity and competition from indigenous microorganisms is likely at lower temperatures (Cools *et al* 2001). In samples of manure Semenov *et al* (2007) demonstrated that at temperatures of 7°C *E. coli* 0157:H7 reached the detection limit of 1 Log CFU g^{-1} DS after 159 days, highlighting prolonged survival times at colder temperatures. The argument for increasing biosolids storage temperature is supported by evidence from Plachá *et al* (2001) who showed that lower temperatures during winter months cause prolonged cell survival in pig slurry. Temperature appears to have a significant influence on the growth and survival of indicators within biosolids and analogous environments. Further work to understand the temperature conditions favouring *E. coli* die-off will contribute to the control of biosolids quality and support the predictability in achieving compliance targets.

2.5.2 Moisture Content

An interesting phenomenon noted in Sprigings and Le (2011) is the effect moisture may have on retaining heat in the biosolids matrix. A comparison of sites A and B showed differences in the DS% of the dewatered sludge cake (Site A: 25% DS, Site B: 31% DS) and corresponded with site A maintaining a higher sludge cake temperature. A lower DS% will help the fresh cake retain heat from

the AD process for a longer period, delaying cake cooling to ambient temperatures that are likely to support prolonged cell survival (Plachá *et al* 2001, Cools *et al* 2001, Semenov *et al* 2007). The moisture content of biosolids is often targeted at 25% (Sprigings and Le 2011, Severn Trent Water Plc. Operational Data 2008- 2016) and achieved through the use of polyelectrolyte addition and mechanical dewatering operations on sludge treatment sites, as described above. Currently biosolids storage post-dewatering, is conducted in open bays exposed to fluctuating weather conditions. Controlling the water content of sludge not only has operational benefits but may also be an influential factor for the control of indicator organisms (Berry and Miller 2005, Zaleski *et al* 2005a, Rouch *et al* 2011). In a study on cattle feedlot soil moisture content impacts on *E. coli* 0157, researchers found that at the lowest water contents (0.11 g H_2O g^{-1} Dry Feed Surface Material (DFSM)) microbial activity was not detectable and *E. coli* 0157:H7 viability was lost (Berry and Miller 2005). Where feedlot soils had higher water contents, between 0.43/0.67 g H_2O g^{-1} DFSM, *E. coli* 0157:H7 populations persisted at high levels and in some cases multiplied under these conditions. These data confirm that moisture content can improve survival and allow the growth of pathogenic bacteria. In agreement with these findings is work by Zaleski *et al* (2005b) who studied the effects of solar drying in concrete lined drying beds on anaerobically digested biosolids. Over a period of 22 weeks the relationship between the number of faecal coliforms and percent dry solids was tracked. Results showed that as the percent dry solids increased the numbers of faecal coliforms reduced and vice versa. Although often above the dry solids range typically observed in more temperate climates (25-30% DS (Sprigings and Le 2011)) the study did show the change in percent dry solids fluctuating in response to rainfall events. Between weeks 15 and 19 of the study the dry solids fell to approximately 20% and with this an increase of 1.5 orders of magnitude in faecal coliform levels was observed. Subsequently this higher concentration decreased as the biosolids dried to 80% solids in the final study weeks (Zaleski *et al* 2005b). Re-wetting of biosolids by rainfall events was examined by Rouch *et al* (2011) in air-dry storage of anaerobically digested biosolids. Rouch *et al* (2011) found an inverse relationship to the DS contents of the biosolids with regards to the survival

of *E. coli*, enterococci and coliphages. Due to the growth of dormant or small residual populations of bacterial in the biosolids, bacterial regrowth could occur as cells become active upon rewetting (Rouch *et al* 2011).

In contrast to this, laboratory-scale research conducted by Lang and Smith (2007) suggests that in dry, biosolids-amended soil bacteria are protected within particles of sludge cake as drying restricts predatory activity. Results showed a marked increase in *E. coli* survival in air-dried samples of sludge-amended soil. The results highlight that ecological processes contributing to the decay of *E. coli* in sludge-amended soil are active under moist conditions but suppressed in dry soil (Lang and Smith 2007). Although this is contradictory to views of other researchers (Pietronave *et al* 2002, Wang *et al* 2004, Zaleski *et al* 2005b, Berry and Miller 2005, Rouch *et al* 2011) the research provides indication that the survival times of enteric bacteria in biosolids amended soil may be shorted in moist, and extended in dry conditions (Lang and Smith 2007). Further evidence supporting this argument is highlighted by Jiang *et al* (2002) who studied *E. coli* 0157:H7 cell survival in manure-amended autoclaved soil. The study revealed that *E. coli* 0157:H7 could survive for extended periods of time in manure-amended soil even under very dry conditions of <1% moisture content.

The effect of moisture content on bacterial behaviour in biosolids and analogous environments is varied. It has been highlighted that bacterial survival is possible across a range of moist and dry conditions (Jiang *et al* 2002, Zaleski *et al* 2005a, Lang and Smith 2007, Rouch *et al* 2011). The sludge environment is a challenging matrix, likely to be influenced by a great number of factors which may impede experiments studying the effects of environmental parameters on bacterial growth. For example, the source material of sludge will be influential with regards to nutrient availability and the indigenous organisms present in the sampled material which will influence the dynamics of the studied microorganisms. In addition the experimental design, particularly in up-scaled trials will be affected by ambient conditions such as seasonality and temperature. Nevertheless, as indicator survival could be sufficient for stored biosolids to fail microbial compliance assessments understanding of bacterial dynamics within

operational dry solids ranges observed on treatment sites is necessary, particularly in temperate environments.

2.5.3 Modified Atmosphere

Although dewatering operations will alter sludge moisture content and invariably cause temperature changes, one of the key modifications to occur as the liquid fraction is separated from the sludge solids is a change from anaerobic to aerobic conditions. In digestion the combination of an oxygen limiting environment and other pernicious reactor conditions will have a controlling effect on pathogen concentrations. In particular, oxygen availability has a substantial effect on cell respiration and consequential energy production, regulating most cell activities (Alberts *et al* 2010). Although *E. coli* bacteria are facultative anaerobes, and therefore resilient to oxygen deprivation (Hutchison *et al* 2000, Henkel *et al* 2014), the anaerobic conditions coupled with additional inimical factors present in AD, such as temperature and substrate limitation, may cause growth inhibition, and death. The rapid resurgence of *E. coli* post dewatering suggests processes of MAD may result in cell death, dormancy and loss of culturability (Higgins *et al* 2006, Higgins *et al* 2007b, Chen *et al* 2011). Mechanical dewatering causes substantial change of environmental conditions in the sludge product (Higgins *et al* 2007a, Chen *et al* 2011). In particular the effects of oxygen differentiation in the process need to be explored. Qi *et al* (2008) investigated the impact of total solids on faecal coliform growth and found that centrifugation led to higher solids concentrations, which coincided with higher faecal coliform counts. Study results highlighted that the intensity of centrifugation was not related to the extent of faecal coliform regrowth, and researchers suggest that the concentration of solids or the liquid to solid ratio governs regrowth. This finding is in contrast to other authors (Monteleone *et al* 2004, Qi *et al* 2007, Chen *et al* 2011) who attribute regrowth to effects of centrifuge shearing. In agreement with Qi *et al* (2008) is a study conducted by Erdal *et al* (2003) who found more reactivation and regrowth in high solids cakes when compared to low solids cakes. One reasonable explanation for the increased level of faecal coliforms in higher solids cakes, aside from arguments on moisture content (see Section 2.5.2), is the increased

exposure to oxygen as centrifuge dewatering produces smaller flocs with an overall larger surface area (Chen *et al* 2007). Often continuous flow decanter centrifuges are employed in sludge treatment and contain an internal scroll conveyor that can amplify oxygen exposure (Chen *et al* 2011). As anaerobic digestion creates an environment dominated by obligate anaerobes the oxygen introduced during dewatering could have lethal effects on these populations of bacteria. *E. coli* are fast growing, facultative anaerobes that are likely to experience a selective advantage with increased oxygen availability (Chen *et al* 2007). This may contribute to the higher level of indicator growth observed in the initial phase of storage. Oxygen can disrupt methanogens which form a large fraction of the anaerobically digested sludge (Chen *et al* 2007, Chen *et al* 2011). A reduction in the methanogen population may reduce substrate competition and, if lysed, methanogen cells may provide additional nutrients for the surviving bacteria (Murate *et al* 2012). Chen *et al* (2011) suggests that this selective advantage may quickly disappear as the storage conditions of biosolids return to an anaerobic state. Although the oxygen exposure may be short lived, the biosolids storage environment can still harbour high concentrations of indicator bacteria (Higgins *et al* 2007a, Sprigings and Le 2011) which present non-compliance risks. It may be possible that the increased growth resulting from factors associated with oxygen exposure may enable previously non-viable populations of *E. coli* bacteria to establish (Higgins *et al* 2007a) and dominate, preserving elevated indicator concentrations. Controlling oxygen exposure during dewatering and subsequent storage may present a strategy to stabilise *E. coli* concentrations at post-digestion levels, ensuring compliance targets are predictably met.

A common agricultural practice in which oxygen exclusion restricts bacterial growth is silage production. Ensiling is a crop preservation method based on natural lactic acid fermentation of residual biodegradable carbon under anaerobic conditions (Gollop *et al* 2005, Herrmann *et al* 2011). Compacting and sealing the forage to exclude air enables important physico-chemical and microbial changes to occur in storage (Dunière *et al* 2013). In silage, the growth and survival of pathogens depends on the degree of anaerobiosis and several studies have

shown that *E. coli* 0157:H7 does not survive a good fermentation process (Avery *et al* 2005, Bach *et al* 2002, Byrne *et al* 2002). Poor silage management has been shown to be a factor in *E. coli* 0157:H7 survival (Fenlon and Wilson 2000). Studies on the growth of *E. coli* 0157 in poorly fermented laboratory silage showed an increase from initial numbers of 10^3 *E. coli* 0157 CFU g^{-1} to numbers in excess of 10^6 CFU g^{-1} reflecting the ability of this organism to multiply rapidly in air spoiled silage (Fenlon and Wilson 2000). A comparable study completed by Chen *et al* (2005b) studied the fate of *E. coli* during ensiling of wheat and corn. Observations showed that for wheat, samples inoculated with *E. coli* and sealed after a delay of 24 hours had less lactic acid and more acetic acid than other treatments. Researchers suggest that this may have been the cause of more prolonged activity in *E. coli* bacteria contained within these treatments as insufficient anaerobiosis can delay the lactic acid fermentation process, slowing pH decrease and increasing the survival of *E. coli* (Chen *et al* 2005b). Chen *et al* (2005b) highlights that high concentrations of *E. coli* were observed in decaying parts of silage, particularly in areas most susceptible to air penetration.

The pH evolution plays an important role in silage preservation. Dunière *et al* (2011) observed *E. coli* strains surviving and growing only in silage showing a pH increase after samples were opened and had experienced 144 hours of aerobic exposure. Once exposed to oxygen, aerobic microorganisms can develop and increase the pH levels in the remaining forage (Dunière *et al* 2011). It is therefore reasonable to conclude that oxygen depletion in silage preservation is not directly affecting *E. coli* growth and survival, but that the secondary effects of oxygen depletion, nominally lactic acid fermentation and subsequential pH reduction, cause the decreased microbial activity.

A ‘controlled atmosphere’ process that is directly linked to microbial inhibition is Modified Atmosphere Packaging (MAP) in food preservation. The principle of MAP is the replacement of air in the package with a different fixed gas mixture (Sivertsvik *et al* 2002). The three main gases used in MAP are Nitrogen (N_2), Oxygen (O_2) and Carbon Dioxide (CO_2) (Floros and Matsos 2005). Nitrogen is an inert and tasteless gas without any antimicrobial activity, Floros and Matsos

(2005) suggest that nitrogen's primary use in MAP is to displace oxygen. Oxygen inhibits anaerobic microorganism growth but can promote aerobic microbe populations. Due to this, is it often avoided in MAP but can be a necessary additive for certain food stuffs such as fruit and red meat that require a minimum oxygen concentration to maintain the basic process of aerobic respiration (Floros and Matsos 2005). Carbon dioxide is the only gas used in MAP that exhibits antimicrobial qualities and can have a bacteriostatic effect, slowing down respiration (Floros and Matsos 2005). As oxygen exposure to the dewatered sludge matrix may be a precursor to *E. coli* growth, understanding principles and successes of MAP may be beneficial for sludge management applications.

For many years MAP has mainly consisted of the bulk gas N_2 combined with more or less CO_2 (Heinrich *et al* 2016). In a study on strains of *E. coli* on gelatin-agar media and ham, gas mixes of CO_2- N_2 (20:80) showed the capacity to reduce bacterial growth of *E. coli* to below -0.5 log CFU g^{-1} (Heinrich *et al* 2016). An additional innovation in MAP food packaging has been the use of vacuum treatment (Floros and Matsos 2005). The process of using a vacuum is considered as active atmosphere modification and is commonly used in canning or bottling processes. The main target of vacuum packing is to reduce the residual oxygen in the package which will reduce oxidative chemical reactions and aerobic growth (Floros and Matsos 2005). A study comparing the effects of vacuum and modified atmosphere on seabream fillets was conducted by Garrido *et al* (2016). Vacuumed fillets were tested alongside those packaged in a gas mixture of 40% CO_2, 30% N_2 and 30% O_2 and all samples were stored at 3°C (±1°C) for 14 days. Total Viable Counts (TVC) showed that by day 4 of storage control samples had exceeded 7 Log CFU g^{-1} (the limit established by the International Commission on Microbiological Specifications for Foods (ICMSF 2002)), while vacuum treatments and samples stored in the modified gas mixture did not reach this limit until day 11. This result shows that both types of MAP packaging resulted in a longer shelf life of the seabream fillets tested (Garrido *et al* 2016). In agreement with these results are those by Sivertsvik *et al* (2002) who found that vacuum packaging also inhibited the growth of TVC bacteria in salmon fillets. Researchers attribute the absence of O_2 in vacuumed packaging and the partial dilution of O_2

and/or the presence of CO_2 in modified gas mixture treatments to reduced microorganism growth and increased microbial stability (Garrido *et al* 2016). Vacuum packaging and reduced concentrations of oxygen exposure appear to be most influential in preserving the shelf life of food products and diminishing the growth of bacteria. It may therefore be possible that similar practices are able to reduce indicator growth or enhance die-off rates in biosolids storage environments where oxygen availability in the initial phases of storage has been attributed to higher concentrations of pathogenic indicator bacteria.

2.6 Summary

Research has identified high concentrations of indicator bacteria in anaerobically digested biosolids, immediately after mechanical dewatering (Monteleone *et al* 2004, Higgins *et al* 2006, Higgins *et al* 2007a, Dentel *et al* 2008, Chen *et al* 2011). This review aimed to establish the current state of knowledge on factors influencing *E. coli* growth and survival in stored biosolids, drawing on understanding from up-stream treatment processes and analogues storage environments that successfully limit the proliferation of *E. coli* bacteria. Key factors that have been identified from the scientific literature are the effects of mechanical dewatering processes, transforming the environmental conditions of the sludge matrix whilst possibly releasing growth inducers. Nutrient availability for cell survival and growth is a significant factor to be considered alongside the physical environmental conditions that will be acting on cells held within stored biosolids. The rigorous level of treatment control that precedes biosolids storage is arguably superseded by a highly uncontrolled storage treatment in which external factors including temperature and moisture content may preserve elevated indicator concentrations. Examples of modified storage practices such as ensiling and MAP in the agricultural and food industries might give indication of methods to inhibit bacterial growth and survival, particularly when considered with external environmental parameter modifications. As assurance of biosolids quality is increasingly sought by agriculturalists, retailers and the public, regulations and safety assurance schemes are becoming more stringent. At a time when the water industry regulator of England and Wales, Ofwat, sets forward

a new direction towards the opening of sludge markets in 2020 it is critical that the ambiguity concerning pathogen indicator concentrations is dispelled and sludge-to-land disposal routes are safeguarded.

2.7 References

ADAS, (2001) THE SAFE SLUDGE MATRIX: Guidelines for the Application of Sewage Sludge to Agricultural Land. Gleadthorpe Research Centre.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Assured Biosolids Ltd (2015) UK Water Industry 'Biosolids Assurance Scheme' The Scheme Standard (Draft Only published September 2015) Huntingdon, Cambridgeshire, UK

Avery, S., Walters, L. and Hutchinson, M. (2005) Fate of *Escherichia coli* 0157 and detection of *stx* phage during fermentation of maize, an animal feedstuff. *Letters in Applied Microbiology* Vol. 40, pp. 99-105

Bach, S. J., McAllister, T. A., Baah, J., Yanke, L. J., Veira, D. M., Gannon, V. P. J., Holley, R. A. (2002) Persistence of *Escherichia coli* 0157:H7 in barley silage: effect of a bacterial inoculant *Journal of Applied Microbiology* Vol. 93, pp. 288-294

Berry, E. D. and Miller, D. N. (2005) Cattle Feedlot Soil Moisture and Manure Content: II Impact on *Escherichia coli* 0157 *Journal of Environmental Quality* Vol. 34 pp. 656- 663

Bodriesz, E., Molenaar, D., Teusink, B. and Bruggeman, F. J. (2015) How fast-growing bacteria robustly tune their ribosome concentration to approximate growth-rate maximisation. *The FEBS Journal* Vol. 282 pp. 2024-2044

Bolto, B. and Gregory, J. (2007) Organic polyelectrolytes in water treatment *Water Research* Vol. 41 pp. 2301- 2324

Byrne, C. M., O'Kiely, P., Bolton, D. J., Sheridan, J. J., McDowell, D. A., Blair, L. S. (2002) Fate of *Escherichia coli* 0157:H7 during silage fermentation *Journal of Food Protection* Vol. 65, pp. 1854- 1860

Chang, L.L., Raudenbush, D.L., Dentel, S. K., (2001) Aerobic and anaerobic biodegradability of a flocculant polymer *Water Science and Technology* Vol. 44 No. 2-3, pp. 461- 468

Caulfield, M.J., Qiao, G.G., Solomon, D. H. (2002) Some aspects of the properties and degredation of polyacrylamides *Chem Rev* Vol. 102 pp. 3067- 3084

Chen, Y., Higgins, M. J., Maas, N. A., Murthy, S. N., Toffey, W. E., Foster, D. J., (2005a) Roles of methanogens on volatile organic sulfur compound production in anaerobically digested wastewater biosolids *Water Science and Technology* Vol 52 No. 1-2 pp. 67- 72

Chen, Y., Sela, S., Gamburg, M., Pinto, R., Weinberg, Z. G. (2005b) Fate of *Escherichia coli* during ensiling of wheat and corn *Applied and Environmental Microbiology* Vol. 71, No. 9, pp. 5163- 5170

Chen, Y., Higgins, M. J, Beightol, S. M, Araujo, G. G., Murthy, S. N., Barben, E. J., Toffey, W. E. (2007) Odour Generation and Pathogen Indicator Regrowth after Dewatering: Are They Related? In: Proceedings of the Mid-Atlantic Biosolids Assocation Conference

Chen, Y. C., Higgins, M. J., Beightol, S. M., Murthy, S. N., & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research, 45*, 2616- 2626.

Chen, Y., Fu, B., Wang, Y., Jiang, Q., Liu, H. (2012) Reactor performance ad bacterial pathogen removal in response to sludge retention time in a mesophilic anaerobic digester treating sewage sludge *Bioresource Technology* Vol. 106 pp. 20-26

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., (2001) Survival of *E. coli* and *Enterococcus spp*. derived from pig slurry in soils of different texture. *Appl. Soil Ecol.* Vol. 17, pp. 53–62.

Cooper, J., Cass, J., Rogers, M., Smith, S.R. (2010) Effects of sludge dewatering and digestion processes on *Escherichia coli* enumeration *Water and Environment Journal* Vol. 24 pp. 303- 308

DEFRA (1989) The Sludge (Use in Agriculture) Regulations 1989: Schedule 1 Testingof Sludge http://www.legislation.gov.uk/uksi/1989/1263/schedule/1/made [Accessed on 31/08/2016]

DEFRA (2006) Code of Practice for Agricultural Use of Sewage Sludge sludgehttp://adlib.everysite.co.uk/adlib/defra/content.aspx?id=000IL3890W.16NTC17O1X82UF [accessed on 31/08/2016]

DEFRA (2013) Managing sewage sludge, slurry and silage https://www.gov.uk/guidance/managing-sewage-sludge-slurry-and-silage [accessed on: 30/08/2016]

Dentel, S. K., Qi, Y., & Herson, D. S. (2008). Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration, and assessment. *Water Science and Technology,* Vol. *57, No.* 2

Dunière, L. Gleizal, A., Chaucheyras- Durand, F., Chevallier, I., Thévenot-Sergentet, D. (2011) Fate of *Escherichia coli* 026 in corn silage experimentally contaminated at ensiling, at silo opening, or after aerobic exposure, and protective effect of various bacterial inoculants *Applied and Environmental Microbiology* Vol. 77, No. 24, pp. 8696- 8704

Edberg, S. C., Rice, E. W., Karlin, R. J., & Allen, M. J. (2000). *Escherichia coli:* the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology,* Vol. *88 No.* S1, pp. 106S- 116S.

Elliott, L. F. and Ellis, J. R. (1977) Bacterial and viral pathogens associated with land application of organic wastes *Journal of Environmental Quality* Vol. 6 No. 3 pp. 245- 251

Environment Agency, (2003) The Microbiology of Sewage Sludge-Part 1- An overview of the treatment and use in agriculature of sewage sludge in relation to its impact on the environment and public health. Bristol.

Environment Agency (2012) Waste exemption: S3 storing sludge https://www.gov.uk/guidance/waste-exemption-s3-storing-sludge [Accessed on 01/09/2016]

Erdal, Z. K., Mendenhall, T. C., Neely, S. K., Wagoner, D. L., Quigley, C. (2003) Implementing improvements in a North Carolina residuals management program. In: Proceedings of the WEF/AWWA Joint Residuals and Biosolids Management Conference. Water Environment Federation, Alexandria, VA.

European Union, (2000) Working Document on Sludge, 3rd Draft. ENV.E.3/LM. Brussels.

European Commission (1991) Council Directive 91/271/EEC concerning urban waste-water treatment http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:31991L0271 [accessed on 31/08/2016]

Fenlon, D. R. and Wilson, J. (2000) Growth of *Escherichia coli* 0157 in poorly fermented laboratory silage: a possible environmental dimension in the epidemology of *E. coli* 0157 *Letters in Applied Microbiology* Vol. 30, pp. 118-121

Floros, J. D and Matsos, K. I. (2005) Introduction to modified atmosphere packaging. In Han, J. H. (Ed.), *Innovations in Food Packaging* (pp. 159- 184). Elsvier Ltd.

Forster-Carneiro, T., Riau, V., Pérez, M. (2010) Mesophilic anaerobic digestion of sewage sludge to obtain class B biosolids: Microbiological methods development *Biomass and Bioenergy* Vol. 34 pp. 1805-1812

Garrido, M. D., Hernández, M. D., Espinosa, M. C., López, M. B. (2016) Enhanced quality characteristics of refrigerated Seabream (Sparus aurata) fillets packed under different systems (modified atmosphere Vs. vacuum) *Journal of Aquatic Food Product Technology* Vol. 25, No. 2, pp. 156-168

Gollop, N., Zakin, V. and Weinberg, Z. G. (2005) Antibacterial activity of lactic acid bacteria included in inoculants for silage and in silages treated with these inoculants *Journal of Applied Microbiology* Vol. 98, pp. 662- 666

Guezennec, A. G., Michel, C., Bru, K., Touze, S., Desroche, N., Mnif, I., Motelica-Heino, M. (2015) Transfer and degredation of polyacrylamide- based flocculants in hydrosystems: a review *Environ Sci Pollut Res* Vol. 22 pp. 6390- 6406

Haller, L., Pote, J., Loizeau, J.-L., & Wildi, W. (2009). Distribution and Survival of Faecal Indicator Bacteria in the Sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Ecological Indicators*, Vol. 9 pp. 540- 547.

Heinrich, V., Zunabovic, M., Nehm, L., Bergmair, J., Kneifel, W. (2016) Influence of argon modified atmosphere packaging on the growth potential of strains of *Listeria monocytogenes* and *Escherichia coli Food Control* Vol. 59, pp. 513- 523

Herrmann, C., Heiermann, M., Idler, C., (2011) Effects of ensiling, silage additives and storage period on methane formation of biogas crops. *Bioresour. Technol.* Vol. 102 pp. 5153–5161

Henkel, S.G., Ter Beek, A., Steinsiek, S., Stagge, S., Bettenbrock, K., Teixeira de Mattos, M.J., Sauter, T., Sawodny, O., Ederer, M., (2014) Basic Regulatory Principles of *Escherichia coli's* Electron Transport Chain for Varying Oxygen Conditions. *PLOS one* Vol. 9 No. 9 e107640

Higgins, M. J., Chen, Y. C., Murthy, S. N., & Hendrickson, D. (2007a). Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering. *Procedings on moving forward wastewater biosolids sustainability: Technical, managerial and public synergy*, pp. 204-19.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Henderickson, D., Farrel, J., & Schafer, P. (2007b). Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Research*, Vol. 41 pp. 665-673.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Hendrickson, D., Schafer, P., & Farrell, J. (2006). WERF Phase 2: The Impact of Digestion and Dewatering on

Reactivation and Regrowth of Viable but Non-Culturable Indicator Bacteria. WEFTEC.

Hutchison, M.L., Nicholson, F.A., Smith, K.A., Keevil, C.W., Chambers, B.J., Moore, A., (2000) Study on Farm Manure Applications To Agricultural Land and an Assessment of the Risks of Pathogen Transfer Into the Food Chain FS2526.

ICMSF, International Commission on Microbiological Specifications for Foods (1980) International Commission on Microbiological Specifications for Foods (Ed.), Microbial Ecology of Foods, Vol 1: Factors Affecting Life and Death of Microorganisms. Academic Press, New York, USA

ICMSF (2002) Microorganisms in Foods 7: Microbiological testing in food safety management. New York, NY: Kluwer Academic/ Plenum Publisher.

Iranpour, R., Palacios, R., Cox, H.H.J., Abkain, V. (2005) Solving fecal coliform growth/ reactivation in biosolids during full-scale post-digestion processes *Water Science and Technology* Vol 52, No. 1-2, pp. 283- 288

Jiang, X., Morgan, J. and Doyle, M. P. (2002) Fate of *Escherichia coli* 0157:H7 in manure amended soil *Applied Environmental Microbiology* Vol. 68, pp.2605-2609

Junqua, G., Spinelli, S., Gonzalez, C. (2015) Occurrence and fate of acrylamide in water-recycling systems and sludge in aggregate industries *Environ Sci Pollut Res Vol.* 22 pp. 6452- 6460

Knijnenburg, T.A., Daran, J-M. G., Van den Broek, M.A., Daran-Lapujade,P.A.S., de Winde, J.H., Pronk, J.T., Reinders, M.J.T., Wessels, L.F.A. (2009) Combinatorial effects of environmental parameters on transcriptional regulation in Saccharomyces cerevisiae: A quantitative analysis of a compendium of chemostat-based transcriptome data *BioMed Central Geonomics* Vol. 10 No. 53

Lang, N. L. and Smith, S. R. (2007) Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions *Journal of Applied Microbiology* Vol. 103, pp. 2122- 2131

Lang, N.L. and Smith, S.R., (2008) Time and temperature inactivation kinetics of enteric bacteria relevant to sewage sludge treatment processes for agricultural use. *Water Res*. Vol. 42, pp. 2229–2241.

Le, M.S., Mayhew, M.E., Back, P.A., (2002) Effectiveness of Secondary Digesters as a Pathogen Controller in Winter. *J. CIWEM* Vol. 16, pp. 292–295.

London Economics (2010) Competition in upstream sewage and sludge markets (PROC/01/0166) Final Report, Ofwat, London

Metcalf & Eddy., Tchobanoglous, G., Burton, F. L & Stensel, H. D (2003). Wastewater Engineering: Treatment and Reuse (4th Ed.). Boston: McGraw-Hill

Monteleone, M. C., Furness, D., Jefferson, B., & Cartmell, E. (2004). Fate of *E. coli* Across Mechanical Dewatering Processes. *Environmental Technology*, Vol. 25 No.7 pp. 825-831.

Mowla, D., Tran, H.N., Grant Allen, D. (2013) A review of the properties of biosludge and its relevance to enhanced dewatering processes *Biomass and Bioenergy* Vol. 58 pp. 365-378

Murate, M., Noor, R., Nagamitsu, H., Tanaka, S and Yamada, M. (2012) Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*. *Genes to Cells* Vol. 17 pp. 234-247

Noor, R (2015) Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. *SpringerPlus* Vol. 4 pp. 599

Orzi, V., Cadena, E., D'Imporzano, G., Artola, A., Davoli, E., Crivelli, M., Adani, F. (2010) Potential odour emission measurement in organic fraction of municipal solid waste during anaerobic digestion: Relationship with process and biological stability parameters *Bioresource Technology* Vol. 101 pp. 7330-7337

Orzi, V., Scaglia, B., Lonati, S., Riva, C., Boccasile, G., Alborali, G.L., Adani, F. (2015) The role of biological processes in reducing both odour impact and pathogen content during mesophilic anaerobic digestion *Science of the Total Environment* Vol. 526 pp. 116-126

Pietronave, S., Fracchia, L., Martinotti, M. G. (2002) Researchers analyse how microorganisms suppress pathogen regrowth *Biocycle* Vol. 43, No. 10, pp. 57-60

Placha, I., Venglousky, J., Sasokova, N., Svoboda, I.F., (2001) The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solids of pig slurry. *J. Appl. Microbiol.* Vol. 91, pp. 1036–1043.

Qi, Y., Dentel, S. K., Herson, D. S. (2008) Effect of total solids on fecal coliform regrowth in anaerobically digested biosolids *Water Research* Vol. 42 pp. 3817-3825

Qi, Y., Dentel, S. K., & Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. *41*, pp. 571- 580.

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Rouch, D. A., Mandal, T., Pai, S., Glauche, F., Fleming, V. A., Thurbon, N., *et al*. (2011). Microbial safety of air dried and re-wetted biosolids. *Journal of Water and Health*,Vol. 9.2 pp. 403-414.

Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M., Adani, F., (2014) Sanitation ability of anaerobic digestion performed at different temperature on sewage sludge. *Sci. Total Environ*. No. 466-467, pp. 888–897.

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M., (2007). Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica serovar Typhimurium* in cow manure. FEMS Microbiol. Ecol. Vol. 60, pp. 419–428

Sidhu, J. P., & Toze, S. G. (2009). Human Pathogens and their Indicators in Biosolids: A Literature Review. *Environment International,* Vol. *35*, pp. 187- 201.

Sigma Aldrich (2016) Microbiology Introduction http://www.sigmaaldrich.com/technical-documents/articles/microbiology/microbiology-introduction.html [Accessed on 05/09/2016]

Sivertsvik, M., Jeksrud, W. K. and Rosnes, T. J. (2002) A review of modified atmosphere packaging of fish and fishery products- significance of microbial growth, activities and safety *International Journal of Food Science and Technology* Vol 37. pp. 107- 127

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manag.* Vol. 25, pp. 417–425

Sprigings, A. E., & Le, M. S. (2011). Growth Potential- A New Method For Estimation of the Risk of *E. coli* Regrowth in Digested Sludge Cake. *16th European Biosolids and Organic Resources Conference.*

Stevens, M., Ashbolt, N., & Cunliffe, D. (2003). Review of Coliforms as Microbial Indicators of Drinking Water Quality. *Recommendations to Change the Use of Coliforms as Microbial Indicators of Drinking Water Quality*. Canberra: Australian Government National Health and Medical Research Council, Biotext Pty Ltd.

Sun, F., Hu, W., Pei, H., Li, X., Xu, X., Ma, C. (2015) Evaluation on the dewatering process of cyanobacteria-containing $AlCl_3$ and PACl drinking water sludge. *Separation and Purification Technology* Vol. 150 pp. 52- 62

Todar, K (2012) Online Textbook of Bacteriology: Nutrition and Growth of Bacteria http://textbookofbacteriology.net/nutgro.html [Accessed on 05/09/2016]

Town, J.R., Links, M. G., Fonstad, T. A., Dumonceaux, T. J. (2013) Molecular characterisation of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. *BioResource Technology* Vol. 151 pp. 249- 257

Wang, L., Mankin, K. R., Marchin, G. L. (2004) Survival of Fecal Bacteria in Dairy Cow Manure *American Society of Agricultural Engineers* Vol 47 No. 4 pp. 1239-1246

Wang, H., Brown, S. L., Magesan, G. N., Slade, A. H., Quintern, M.,Clinton, P.W., Payn, T. W. (2008) Technological options for the management of biosolids *Environ Sci Pollut Res* Vol. 15 pp. 308-317

Water UK. (2010). Recycling of Biosolids to Agricultural Land. *(3)*. London: Water UK

Water UK (2013) Assurance Scheme for Biosolids Recycling http://www.water.org.uk/news-water-uk/latest-news/assurance-scheme-biosolids-recycling [Accessed on 31/08/2016]

Wen, Q., Chen, Z., Zhao, Y., Zhang, H., Feng, Y. (2010) Biodegredation of polyacrylamide by bacteria isolated from activated sludge and oil- contaminated soil *J. Hazard Mater* Vol. 175 pp. 955- 959

UKWIR. (1999). *E. coli in UK Mesophilic anaerobically digested sludge Report SL-06.* UK Water Industry Research Ltd.

US EPA (United States Environmental Protection Agency, (2012) A Plain English Guide to the EPA Part 503 Biosolids Rule [WWW Document]. URL http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm (accessed 05/01/2015).

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005a). Survival, growth and Regrowth of Enteric Indicator and Pathogenic Bacteria in Biosolids, Compost, Soil, and Land Applied Biosolids *Journal of Residuals Science and Technology* Vol. 2, No. 1 pp. 49- 63

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005b). Potential Regrowth and Recolonisation of *Salmonellae* and Indicators in Biosolids and Biosolids-Amended Soil *Applied and Environmental Microbiology* Vol. 71, No. 7, pp. 3701- 3708

Ziemba, C. and Peccia, J. (2011) Net energy production associated with pathogen inactivation during mesophilic and thermophilic anaerobic digestion of sewage sludge *Water Research* Vol. 45 pp. 4758- 4768

Chapter 3

Influence of Physical and Chemical Environmental Factors on *E. coli* Survival in Biosolids at a Laboratory Scale

Statement of author contributions:

S. Fane: Principal investigator, completion of experimental design and laboratory work, data analysis and chapter write up

Dr. A. Nocker: Provided supervisory support during experimental design and method development

A. Wilson: Industrial supervisor for Severn Trent Water Plc.

J. Tallec: 4 month placement student supporting laboratory trials on polyelectrolyte addition

C. Kanshio: 4 month MSc. thesis student supporting statistical analysis of polyelectrolyte addition data

Dr. M. Rivas Casado: Statistical consultant

Professor E. Cartmell: Developed overall concept of work with Severn Trent Water Plc.

Professor S. Tyrrel: Principal supervisor supporting research concept development, guidance on experimental design, data analysis and evaluation of final chapter write up

3 Influence of Physical and Chemical Environmental Factors on *E. coli* Survival in Biosolids at a Laboratory Scale

S. Fane[1], A. Nocker[2], A. Wilson[3], J. Tallec[4], C. Kanshio[1], M. Rivas Casado[1], E. Cartmell[5], S. Tyrrel[1]

1 Water Science Institute, School of Energy, Environment and Agrifood, Cranfield University, UK

2 IWW Rheinisch-Westfälisches Institut für Wasserforschung gemeinnützige GmbH, Germany

3 Severn Trent Water, UK

4 Polytech Nantes, Boulevard de l'université, Saint-Nazaire cedex, France

5 Scottish Water, Dunfermline, Scotland

3.1 Abstract

The effects of nutrient availability and polyelectrolyte addition on the growth of *Escherichia coli* (*E. coli*) bacteria in stored biosolids were examined. *E. coli* bacteria die-off rates were studied in manipulated storage treatments assessing the combined effect of modified atmosphere and temperature increase. The impact of dewatered sludge dry solids (DS) contents on *E. coli* behaviour was also investigated. All experiments were completed at a laboratory scale on mesophilic, anaerobic digested sludge, mechanically dewatered using a centrifuge or filter press. Polyelectrolyte addition during sludge dewatering showed no significant effect ($p > 0.05$) on peak *E. coli* level compared with control conditions. For both control and polyelectrolyte addition treatments *E. coli* showed an increase of approximately 2 orders of magnitude, consistent with on-site observations of indicator growth in post-dewatered biosolids products. Key findings from manipulated storage environments demonstrated that combining elevated temperatures with gas evacuated storage, led to a reduction of *E. coli* concentrations by up to 4.5 orders of magnitude after 14 days. Increased die-off rates were more pronounced at storage temperatures above 25°C. Manipulation of sludge moisture content between 17-35% DS had no significant effect (p

>0.05) on *E. coli* concentrations after a 21 day incubation period. The paper advances understanding of innate sludge qualities and identifies to what extent environmental parameters such as temperature and moisture content control the growth and death of *E. coli* indicator bacteria in stored biosolids.

3.2 Introduction

The use of biosolids on agricultural land has many benefits and provides a sustainable source of organic matter for soil conditioning (Deeks *et al* 2013). However, application is controlled by tight restrictions: microbial concentration limits stipulated and monitored by UK, EU and US environment agencies are based on acceptable levels of treatment for the reduction of indicator bacteria. *Escherichia coli* (*E. coli*) is an indicator organism whose detection provides a surrogate for the quantification of pathogens within a sample (Stevens *et al* 2003, Sidhu and Toze 2009). *E. coli* (indicator bacteria) compliance monitoring therefore provides verification that control of pathogen levels has been achieved by sludge treatment processes and storage. The microbial quality of biosolids is assured in the UK by agreements such as the Safe Sludge Matrix (SSM) (ADAS 2001) and in the USA through the US EPA Part 503 Biosolids Rule (US EPA 2012). Table 3.1 summarises agreed international compliance levels for biosolids treatment prior to agriculture reuse.

Table 3.1 International microbial compliance levels required/ proposed for treated biosolids prior to agricultural application

Origin	Organisation	Publication	Treatment Category	Treatment Requirement	References
UK	Environment Agency	Safe Sludge Matrix (SSM)		Log reduction between digester inlet and final biosolids product after 14 days storage	(Environment Agency 2003a)
			Conventional	2 Log_{10} *E. coli* reduction (99% pathogens destroyed)	(ADAS 2001)
				Maximum Allowable Concentration (MAC 5) threshold value 10^5 *E. coli* CFU per grams dry solid.	
			Enhanced	6 Log_{10} *E. coli* reduction (99.9999% pathogens destroyed)	
				Product free from *Salmonella*	
EU	European Environmental Agency (Biosolids requirements are the responsibility of individual member states)	Working Document on Sludge, 3rd Draft. ENV.E.3/LM	Conventional	>2 Log_{10} *E. coli* reduction	(European Union 2000)
			Advanced	>6 Log_{10} *E. coli* reduction (<500 CFU per gram wet sludge)	
				Treated sludge should not contain *Salmonella spp.* in 50g (wet weight)	
				Initial validation of treatment process through 6 Log_{10} reduction of test organism such as *Salmonella Senftenberg W775*	
USA	Environmental Protection Agency	Control of Pathogens and Vector Attraction in Sewage Sludge/40 CFR Part 503	Class A	Density of faecal coliform <1000 Most Probable Number (MPN) per gram total solids (dry weight basis)	(US EPA 2012)
				Density of *Salmonella sp.* <3 MPN per 4 grams of total solids (dry weight basis)	
			Class B	Geometric mean of 7 samples <2 x 10^6 MPN faecal coliforms per gram of total solids or <2 x 10^6 MPN faecal coliforms per gram of total solids at the time of use or disposal.	

Typically, sludge treatment includes anaerobic digestion (AD) followed by liquid sludge storage and mechanical dewatering before a minimum storage period acting as a tertiary treatment for pathogen reduction (Environment Agency 2003a). It is now well established that a resurgence of *E. coli* bacteria can occur in the biosolids product immediately after dewatering (Monteleone *et al* 2004, Higgins *et al* 2006, Higgins *et al* 2007a, Higgins *et al* 2007b, Dentel *et al* 2008, Chen *et al* 2011). Elevated concentrations of indicators can remain above compliance thresholds for several days or weeks in storage and therefore present a significant risk of non-compliance. Although resurgent *E. coli* levels are frequently observed in biosolids storage this is not the case at all treatment facilities. This unpredictable indicator bacterial behaviour in treatment and storage causes much concern for utility operators. The key factors responsible for indicator growth and survival in stored dewatered biosolids and their relative importance have not yet been clearly identified (Dentel *et al* 2008). *E. coli* survival during storage, post-dewatering, suggests characteristics of the storage environment may be the underlying reason (Higgins *et al* 2007a).

The sludge treatment process that precedes dewatering may provide some clues as to the relevant environmental parameters controlling *E. coli* dynamics. Knowledge of indicator bacteria concentrations in mesophilic anaerobic digestion (MAD) could be used to identify inhibitory environmental factors contributing to indicator bacteria reduction. In addition, physical and chemical changes to the sludge matrix during mechanical dewatering may indicate factors contributing to bacterial growth and the preservation of elevated concentrations during storage. Identifying the environmental factors influential on bacterial concentrations in upstream sludge treatment processes may help understand what storage conditions are relevant to their control.

Microbial cellular functions are controlled by external environmental factors. For example, temperature has been identified as a critical parameter controlling cell death rates and determining the biochemical conditions of AD (Wang *et al* 2004, Scaglia *et al* 2014). Le *et al* (2002) identified temperature as a key factor for pathogen deactivation in operational-scale secondary digesters. Tanks operating

at a maximum temperature of 28°C and 21°C showed a reduction of 1.7 and 1.0 Log respectively. Temperature regulates processes including substrate limitation and microbial competition which will have an influence on pathogen reduction and can therefore indirectly cause pathogen inactivation (Smith *et al* 2005, Rosenblum *et al* 2014). Conditions of MAD have been found to reduce *E. coli* concentrations by 1.35 to 3.36 Log (UKWIR 1999). An additional factor controlling bacterial reduction during digestion is oxygen availability. The combination of an oxygen limiting environment and other pernicious reactor conditions will have a controlling effect on pathogen concentrations. In particular, oxygen availability has a substantial effect on cell respiration and consequential energy production, regulating most cell activities (Alberts *et al* 2010). Although *E. coli* bacteria are facultative anaerobes, and therefore resilient to oxygen deprivation (Hutchison *et al* 2000, Henkel *et al* 2014), a combination of anaerobic conditions and additional inimical factors present in AD, such as temperature, may cause growth inhibition, and death. Cell nutrient availability during AD is likely to be an additional inimical factor, particularly for pathogenic bacteria that may be outcompeted for substrates in anaerobic digestion environments that favour the growth of methanogens (Orzi *et al* 2015, Town *et al* 2014). Substrate levels in AD are influenced by solids retention time which was found to be significantly related to faecal indicator quantities (Chen *et al* 2012, Rosenblum *et al* 2014). Under optimal conditions, organic matter is extensively degraded in AD, increasing biological stability as limited substrate availability reduces microbial activity (Orzi *et al* 2010). Therefore it is likely that the sludge cake is relatively nutrient limiting, specifically in readily biodegradable organic matter that has been removed during the digestion process. These results emphasise the influence nutrient availability can have on bacterial densities and rates of cell inactivation.

The rapid resurgence of *E. coli* post dewatering suggests processes of MAD may result in cell dormancy and loss of culturability (Higgins *et al* 2006, Higgins *et al* 2007b, Chen *et al* 2011). Mechanical dewatering causes substantial change of environmental conditions in the sludge product (Higgins *et al* 2007a, Chen *et al* 2011). The changes in environmental parameters acting upon the sludge matrix will influence bacterial growth and are likely to contribute to the resurgence

observed in biosolids immediately after mechanical dewatering (Higgins *et al* 2007b, Dentel *et al* 2008). Identifying these significant environmental factors may support the modification of biosolids storage practices for reduced pathogen indicator concentrations to meet compliance requirements.

The principal action of mechanical dewatering is to decrease the sludge water content. In the UK digested liquid sludge (~4% dry solids (DS)) is typically dewatered with centrifuges or belt presses and reaches approximately 25% DS (Sprigings and Le 2011, Severn Trent Water Plc. Operational Data 2008-2016). This limits capital, transportation and operational expenditure (Mowla *et al* 2013). Controlling the water content of sludge not only has operational benefits but may also be an influential factor for the control of indicator organisms (Berry and Miller 2005, Zaleski *et al* 2005a, Rouch *et al* 2011). Berry and Miller (2005) investigated the effect of moisture content on pathogenic *E. coli* 0157:H7 in cattle feedlot soil moisture. Results showed loss of *E. coli* viability at lower water contents (0.11 g H_2O g^{-1} Dry Feed Surface Material (DFSM)). In feedlot soils with higher water content (0.67 g H_2O g^{-1} DFSM) populations of pathogenic *E. coli* persisted and increased (Berry and Miller 2005). This study highlights the improved survival rate of pathogenic bacteria under higher moisture contents. Although other research studies show varied results, bacterial survival across a range of dry solids conditions has been observed (Jiang *et al* 2002, Zaleski *et al* 2005b, Lang and Smith 2007, Rouch *et al* 2011). As survival could be sufficient for stored biosolids to fail microbial compliance, understanding the impact of moisture content within the operational dry solids ranges experienced on treatment sites is necessary.

During mechanical dewatering the addition of polyelectrolyte flocculate determines the final solids concentration achieved for the biosolids product. Monteleone *et al* (2004) suggests that polyelectrolyte effects are an important consideration when explaining % *E. coli* increase in biosolids products. Research conducted by Qi *et al* (2007) concluded that polyelectrolyte addition has a significant influence on final coliform densities in dewatered biosolids after comparing two different concentrations of polyelectrolyte in dewatering operations, suggesting a substrate effect. Chang *et al* (2001) attributed coliform

increase to the physical effects of flocculation or the degradability of the polyelectrolyte itself. In mechanical dewatering, polymers (polyelectrolytes) condition the sludge to form a floc, aggregating bacteria and sludge organic matter. Research has shown that large amounts of bioavailable protein and polysaccharide exist in centrifuge dewatered cakes (Higgins *et al* 2006). These elements will be incorporated within floc matrices and could therefore provide a readily available nutrient source promoting bacterial growth. Considering the effects of polyelectrolyte addition and nutrient availability on biological processes in dewatered sludge will support operational decisions to maintain product quality.

A common variable between AD and dewatering is the modification of atmosphere conditions and the influence this may have on bacterial growth and survival. As mentioned above anaerobic conditions in digestion, when combined with other unfavourable factors, results in bacterial decrease. In mechanical dewatering opposing conditions are likely and an increase in oxygen exposure is a variable that may contribute to cell growth. Centrifuge dewatering produces smaller flocs with an overall larger surface area (Chen *et al* 2007) that is likely to increase oxygen exposure to bacteria cells. In addition, municipal wastewater treatment sites often use continuous flow decanter centrifuges which contain an internal scroll conveyor that can modify the atmosphere through amplified oxygen exposure (Chen *et al* 2011), providing a selective advantage for the facultative *E. coli* bacteria in comparison to methanogenic bacteria which are strict anaerobes (Town *et al* 2014).

The effects of modified atmosphere on bacteria survival has been studied in the food and agricultural industries where modified atmospheric packaging (MAP) and ensiling practices demonstrate the effect of controlled atmospheres. Oxygen exclusion restricts bacterial growth in silage production. The fermentation of residual biodegradable carbon reduces pH levels through lactic acid production (Herrmann *et al* 2011). Reduced pH, in the encapsulated silage, inhibits the growth of enterobacteria providing anaerobic conditions are maintained (Herrmann *et al* 2011). The combination of the modified atmosphere and the

additional pH reduction successfully inhibits microbial spoilage in storage. For food preservation methods, controlled atmospheres are directly linked to microbial inhibition. The replacement of air in MAP with different fixed gases such as nitrogen, carbon dioxide and oxygen, or the practice of active atmosphere modification with vacuum treatment, reduces oxidative chemical reactions and aerobic growth (Floros and Matsos 2005). Researchers attribute the absence of O_2 in vacuumed packaging and the partial dilution of O_2 and/or the presence of CO_2 in modified gas mixture treatments to reduced microorganism growth and increased microbial stability (Garrido *et al* 2016). Decreased concentrations of oxygen exposure appear to be a significant factor preserving food products and diminishing the growth of bacteria. It may therefore be possible that similar methods can be applied to enhance *E. coli* indicator die-off rates in biosolids storage environments.

The hypotheses tested in this chapter are:

1. Growth and survival of *E. coli* bacteria are enhanced by higher sludge nutrient concentrations
2. Polyelectrolyte addition will increase levels of *E. coli* growth in the sludge matrix
3. *E. coli* die-off in stored biosolids will be accelerated under elevated storage temperatures in combination with modified atmosphere conditions
4. Within the operational range of biosolids moisture contents, a higher level of moisture will cause *E. coli* numbers to increase.

This paper examines the effects of nutrient availability, and polyelectrolyte addition on the growth of *E. coli* bacteria in stored biosolids. Indicator bacteria die-off rates are studied in manipulated storage treatments assessing the combined effect of modified atmosphere and temperature increase. In addition to this, the impact of dewatered sludge moisture contents on *E. coli* behaviour is investigated.

3.3 Methodology

3.3.1 Sludge sampling and storage

Samples of sludge cake, which had been both mesophilic digested and centrifuge dewatered were collected from a wastewater treatment works serving a population equivalent (PE) of 440,000. Representative sub-samples were taken to form a bulk sample of 8 kg which were collected at a depth of 0.5 m using a spade or 'corer' device from central locations on the sludge stockpile after the 0.5 m surface 'crust' had been discarded. From this bulk (8 kg) sample sub-samples were taken for the subsequent experimental treatments. The sludge cake samples collected from the treatment site were less than 24 hours old from the point of centrifuge output and enabled laboratory tested storage environments to begin from a 'day 1' assumption. Samples were placed in screw lid sampling pots (Nalgene, Rochester, USA.) and transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2-8°C to suppress biological activity. Samples were processed within 24 hours of sampling**.**

For raw, undigested sludge samples a valve directly before the digesters was the point of sample collection. For digested liquid sludge samples a sampling valve between post-digestion retention tanks and polyelectrolyte injection was used. Samples were taken in 25 L screw-lid containers (FisherBrand, Loughborough, UK). Transport and storage was identical to the methods described above.

Samples collected from the treatment site were then used in the four sets of experiments described below.

3.3.2 Experimental treatments

3.3.2.1 Nutrient addition

Hypothesis 1: Growth and survival of E. coli bacteria are enhanced by higher sludge nutrient concentrations

A sludge-derived nutrient broth based on methods specified in Williams *et al* (2014) was prepared by centrifuging raw, undigested sludge at 168 g for 5 minutes (Sorvall Legend RT Plus centrifuge (DJB LabCare, Newport Pagnell,

England)). The supernatant was decanted, collected, and autoclaved at 121°C for 15 minutes followed by plating 1 mL of the nutrient broth on Membrane Lactose Glucuronide Agar (MLGA). A null count of colony forming units (CFU) on the agar after incubation at 30°C (± 1ºC) for 4 (± 1) hours and 37°C (± 0.5ºC) for 18 (± 2) hours (Environment Agency, 2003c) confirmed sterility.

Sub-samples of 2 x 400 g were taken from the bulk sludge cake (8 kg) and weighed into aluminum trays before being placed in a drying cabinet for 72 hours (maximum temperature 30°C, humidity controlled). During this time sludge was dried to 32% DS. 100 g of the dried samples were placed into each of 6 x 1000 mL capacity beakers (FisherBrand, Loughborough, UK) forming 3 replicates of 2 conditions (3 x nutrient addition and 3 x control). For the nutrient addition treatment, 16.6 mL of the sludge-derived nutrient broth was added to the prepared 100 g sludge cake samples. Similarly 16.6 mL of de-ionised water was added to the control conditions. The incorporation of 16.6 mL liquid with the 32% sludge cake samples reduced the DS% to 29 and 30% DS in both the nutrient addition and control conditions (respectively). 29 and 30% DS falls within the range measured on sludge treatment sites (Operational data 2008-2016, Severn Trent Water Plc.) and therefore represents conditions typically identified in stored sludge cake. Each replicate was mixed with a sterile spatula to ensure homogeneity and covered with parafilm M™ (FisherScientific, Loughborough, UK). Samples were incubated at 22°C for 30 days. *E. coli* concentrations were monitored at regular intervals over the test period.

3.3.2.2 Polyelectrolyte addition

Hypothesis 2: Polyelectrolyte addition will increase levels of E. coli growth in the sludge matrix

High cationic polyelectrolyte powders FLOPAM FO 4700 SH (SNF, Normanton, England) and ZETAG 8180 (BASF, Cheadle, England) were used in controlled dewatering experiments. A filter press with hydraulic closing (model 500 x 15 bar) (Latham International, CP14184, Newcastle-Under-Lyme, UK) and a Sorvall Legend RT Plus centrifuge (DJB LabCare, Newport Pagnell, England) were used to dewater separate samples of digested liquid sludge collected from an

operational wastewater treatment works (see Section 3.3.1). The manufacturer-recommended polyelectrolyte dosing concentration for sludge dewatering on the sampled wastewater treatment site is 0.3 g/ L (Severn Trent Water Plc.). This value was used in the experimental design when investigating polyelectrolyte addition, concentration and type on *E. coli* growth effects:

- In an experiment to determine the effect of concentration, 0.3 g/L and 0.5 g/L FLOPAM FO 4700 SH polyelectrolyte concentrations were compared.
- In experiments to investigate polyelectrolyte type, 0.3 g/L FLOPAM FO 4700 SH and 0.3 g/L ZETAG 8180 concentrations were used.

For both the concentration and type experiments the Latham International filter press dewatered large 20 L samples of digested sewage sludge. When investigating polyelectrolyte addition effects on *E. coli* growth the laboratory centrifuge was used to dewater 250 mL of sample containing 0.3 g/L FLOPAM FO 4700 SH polyelectrolyte or no polyelectrolyte addition (control). Samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant removed. Table 3.2 summarises the polyelectrolyte experiments conducted.

Table 3.2 Polyelectrolyte addition experiments; dewatering operations and polyelectrolyte dosing

Experimental trial	**A. Polyelectrolyte versus control (no polyelectrolyte)**	**B. Polyelectrolyte concentration**	**C. Polyelectrolyte type**
Dewatering equipment	Laboratory centrifuge	Filter press	Filter press
Polyelectrolyte type	FLOPAM	FLOPAM	FLOPAM and ZETAG
Dosing concentration	0.3 g/L / 0 g/L	0.3 g/L / 0.5 g/L	0.3 g/L / 0.3 g/L

The polyelectrolyte concentration solution was made 24 hours in advance of the dewatering operation with the cationic powder gradually added to a continuously mixed vessel of de-ionised water to ensure homogeneity. Before dewatering, the polyelectrolyte solution would be added to the digested liquid sludge and mixed, through inversion, in a screw-lid container. Samples would then be dewatered in either the laboratory centrifuge or the Latham International filter press in accordance with the manufacturer's instructions. Following dewatering, the fresh sludge cake samples were stored at 19°C (room temperature, ± 3°C) and *E. coli* concentrations measured at Time 0, Day 1, 2, 7 and 14. Each condition was repeated in triplicate.

3.3.2.3 Temperature and gas evacuated storage

Hypothesis 3: E. coli die-off in stored biosolids will be accelerated under elevated storage temperatures in combination with modified atmosphere conditions

Sub-samples of 250 g were taken from the bulk sludge cake (8 kg) and placed in sealed 'zip seal' plastic bags (18 cm x 18 cm). Eight 250 g sample conditions were prepared for each storage temperature treatment with four bags left open during the experiment and four bags gas evacuated using a vacuum pump (KNF Neuberger N035.1.2AN.18, Witney, UK). To ensure a secure seal, gas evacuated samples were bagged and exposed to the vacuum pump twice. Each 250 g sample bag was sacrificially used for each time point exclusively. Samples were incubated at temperatures of 19°C (room temperature, ± 3°C,), 25°C (± 1°C), 30°C (± 1°C) and 37°C (± 0.5°C). *E. coli* concentrations, pH and total oxides of nitrogen (TON) levels were monitored at time intervals of 0, 24 hours, 7 days, 14 days and 21 days.

3.3.2.4 Moisture content

Hypothesis 4: Within the operational range of biosolids moisture contents, a higher level of moisture will cause E. coli numbers to increase

Similar to Section 3.3.2.1 sub-samples of 6 x 400 g were taken from the representative bulk sludge cake sample and weighed into aluminum trays before being placed in a drying cabinet for 72 hours (maximum temperature 30°C,

humidity controlled). During this time sludge was dried to 35% DS. To alter the DS% within the range typically experienced in stored sludge cake de-ionised water was added to 60 g of the dried sludge sample contained within a glass beaker (FisherBrand, Loughborough, UK). Table 3.3, below shows the amount of de-ionised water added to each 60 g of dried sludge sample and the arising DS% measured in the modified cake.

Table 3.3 Modified sewage sludge cake DS% after sludge drying to 35% and de-ionised water addition

Sludge cake mass (g)	**De-ionised water volume addition (mL)**	**Final DS% of sludge cake sample**
60	60	17
60	40	20
60	24	25
60	None (control sample)	35

Samples were prepared in triplicate forming 3 replications of each DS% condition. Following sludge cake DS% modification, samples were covered with Parafilm M™ (FisherScientific, Loughborough, UK) and incubated at 22°C for 21 days. *E. coli* concentrations were measured at time 0 and days 1, 7, 14 and 21.

3.3.3 Analytical methods:

3.3.3.1 Microbial enumeration

Membrane filtration (Environment Agency, 2003c) was used for bacterial enumeration using Membrane Lactose Glucuronide Agar (MLGA) (OXOID, Basingstoke, UK) plates to distinguish *E. coli* from coliforms. From each sample condition, 3 g of material were removed and mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube. To ensure effective homogenization, samples were subjected to vortexing for 1 minute (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA.) prior to settling for a further 20 seconds. The supernatant was then removed for serial dilution. Samples were serial diluted with MRD to ensure CFUs were <80/plate. Samples

were passed through 0.45 µm filters (Millipore S-PAK® 47 mm, Watford, UK) using a 3-way vacuum manifold (CombiSart®, Sartorius UK Ltd., Surry, UK). Filters were placed on MLGA plates and incubated at 30°C (± 1ºC) for 4 (± 1) hours and 37°C (± 0.5ºC) for 18 (± 2) hours as described by the Environment Agency (Environment Agency, 2003c). Arising green colonies were counted as presumptive *E. coli*, yellow colonies were counted as coliforms and pink colonies were recorded as non-coliforms. All *E. coli* enumeration results were normalized against percentage dry solids (DS%). A 3 g sludge sample was used for DS determination and analysis was performed as per Environment Agency (2003b) guidance.

3.3.3.2 pH measurement

Measurements of pH were conducted following recommended soil pH analysis techniques (GEB, 2007), a 3 g sub-sample was removed and mixed with approximately 15 mL de-ionised water in a universal tube. The solution was homogenised by vortexing (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA) for 1 minute followed by resting for 30 minutes to allow stabilisation. A pH probe (FisherBrand Hydrus 300, Loughborough, UK) was used to record pH fluctuations.

3.3.3.3 Total oxides of nitrogen (TON) analysis

Total oxides of nitrogen (TON) determination was conducted using a Series 2000 auto-sampler (Burkard, Uxbridge, UK) following methods supplied by Burkard Scientific and Method 53 of the Ministry of Fisheries and Food (MAFF), Reference Book 427 (1986). Nitrate extraction utilised 10 g of tested sludge cake material placed in a screw lid container (Nalgene, Rochester, USA) and mixed with 50 mL of 2 M potassium chloride solution. Samples were subsequently placed on a reciprocating shaker (Stuart SSL2, Bibby Scientific, Stone, UK) set at 220 rpm for 2 hours (±10 minutes). The automated hydrazine reduction method was used to measure TON (Standard Methods for the Examination of Water and Wastewater, 1995).

3.3.3.4 Statistical analysis

Using STATISTICA (Version 12, Tulsa, USA.) a normality assessment was conducted on the dataset using the histogram and normal probability plot program function. Variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá, 2007). A repeated measures analysis of variance (ANOVA) was performed to identify statistical significant difference ($p < 0.05$) between and within the conditions tested. *E. coli* concentration (CFU Log_{10} g^{-1} DS) was set as the dependent variable and the treatments tested as categorical factors. Similarly, for pH and TON analysis these variables were set as dependant and the treatments as categorical factors. The validity of the ANOVA assumptions, such as the normality of residuals (Gujarati 2011), were tested through residual analysis. A post-hoc analysis (Fisher LSD) was carried out to assess the distinct differences between experimental conditions (Marques de Sá, 2007).

3.4 Results

3.4.1 Nutrient addition

Although no significant difference ($p > 0.05$) in *E. coli* counts between the nutrient addition treatment and control were found, a significant change from time 0 to day 1 was identified in the sludge-derived nutrient addition treatment (Figure 3.1). *E. coli* concentrations rapidly increased to 7.1 Log in the first 24 hours of storage whilst the control condition remained relatively stable at 6.6 Log. In the following 5 days *E. coli* concentrations in the nutrient addition treatment remained consistently higher than control conditions. No increased die-off rate was observed in the nutrient addition treatment and for both conditions *E. coli* reduced steadily to 4 Log between day 10 and day 30 of the test period.

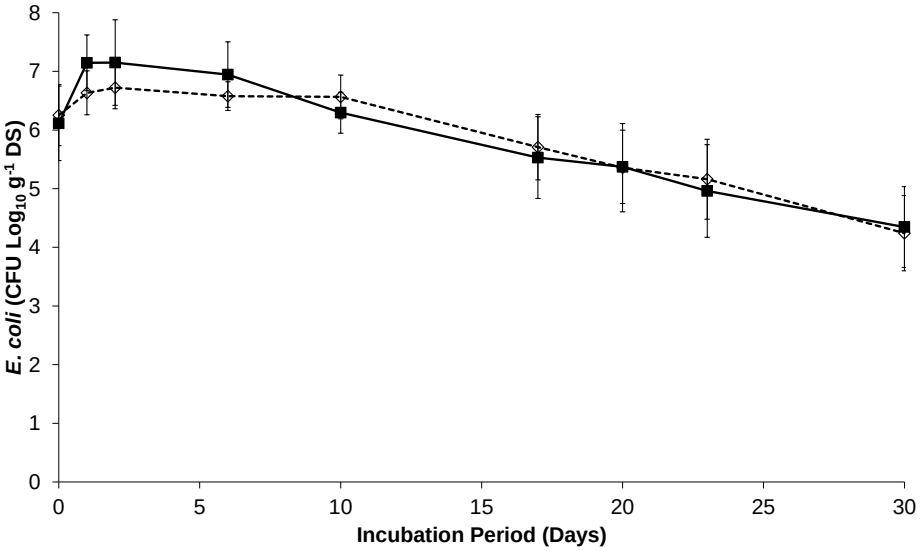

Figure 3.1 Impact of sludge-derived nutrient broth addition on *E. coli* concentration monitored over 30 day incubated (22°C) storage period. Key: --◇-- Control (de-ionised water addition), —■— Nutrient addition (nutrient broth added to sludge cake). Error bars represent the standard deviation of 2 repeats with 6 replications in total.

3.4.2 Polyelectrolyte impact on *E. coli* dynamics

3.4.2.1 Polyelectrolyte addition versus control

There was a significant difference between time 0 and day 1 for both the control (no polyelectrolyte) and 0.3 g/L FLOPAM addition treatments, with numbers of *E. coli* increasing by approximately 1 Log after 24 hours of storage at 19°C (Figure 3.2). No significant difference between the two treatments occurred on day 1 of storage. Following the peak *E. coli* level both treatments showed a gradual reduction in bacterial concentrations with no significant difference identified between the two treatments. *E. coli* concentrations reduced to approximately 2.5 Log after 14 days of storage.

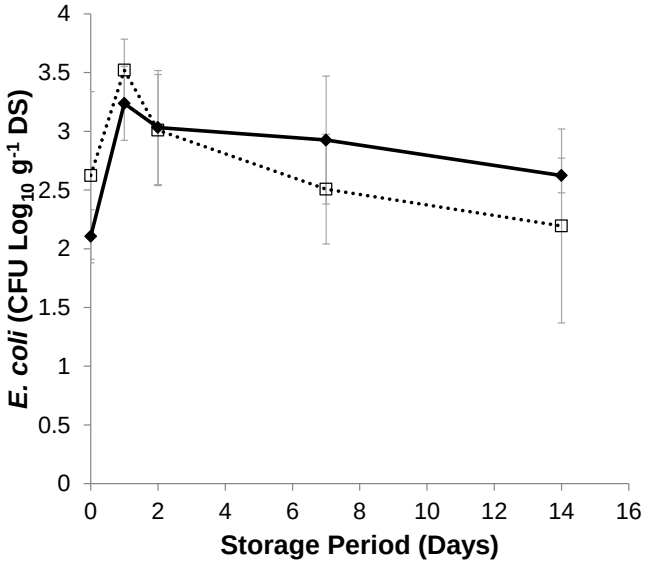

Figure 3.2 Effect of laboratory scale centrifuge dewatering on *E. coli* concentrations with and without polyelectrolyte addition. Key: —◆— Control (no polyelectrolyte addition), ··⊡·· 0.3 g/L FLOPAM addition. Samples stored at 19°C for 14 days. Error bars represent the standard deviation of 3 replicates (n=3)

3.4.2.2 Polyelectrolyte concentration

A rapid and significant increase to 4.6 and 4.1 Log in *E. coli* concentrations was observed in the first 48 hours after mechanical dewatering for the 0.3 g/L and 0.5 g/L treatments respectively (Figure 3.3). This increase in *E. coli* number occurred for both concentrations tested and for both types of polyelectrolyte, as shown in Figures 3.3 and 3.4.

There was a significant difference between *E. coli* concentrations in the 0.3 g/L and 0.5 g/L concentrations tested ($p < 0.05$). The 0.5 g/L treatment maintained a lower *E. coli* concentration by approximately 0.6 Log throughout the test period. In both conditions levels of *E. coli* bacteria display a gradual reduction to 4 Log (0.3 g/L) and 3.6 Log (0.5 g/L) over the 14 days of storage.

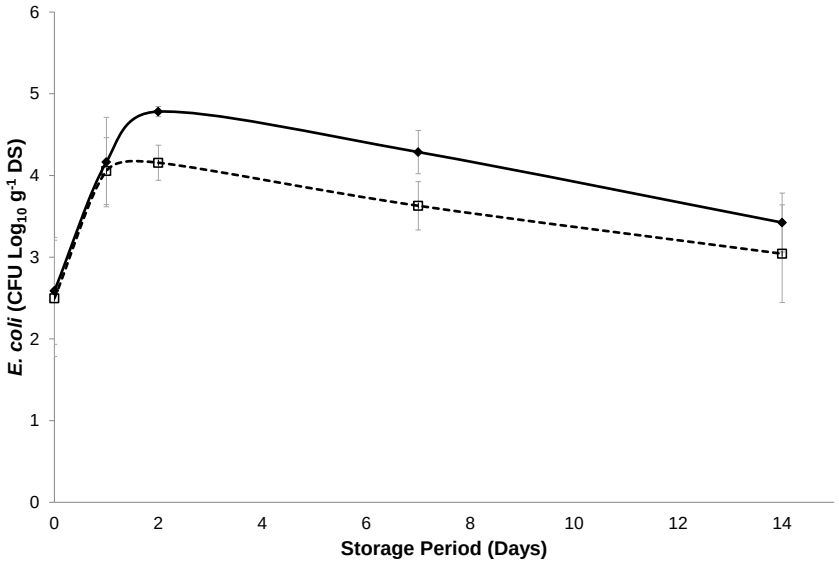

Figure 3.3 FLOPAM polyelectrolyte concentration effect on *E. coli* dynamics in controlled biosolids storage for 14 days after filter press dewatering. Key: —◆— 0.3 g/L FLOPAM, - -□- - 0.5 g/L FLOPAM. Error bars represent the standard deviation of replicates (n=3).

3.4.2.3 Polyelectrolyte type

There was a significant increase in *E. coli* concentration in the first 48 hours after dewatering with a peak of 4.8 and 4.4 Log for the FLOPAM and ZETAG products respectively (Figure 3.4). In addition to producing similar *E. coli* growth curves to those in Figure 3.3, the results displayed no significant effect ($p > 0.05$) of polyelectrolyte type on *E. coli* concentrations in filter press dewatered sludge. Following the peak *E. coli* concentration after 48 hours post-dewatering, concentrations of bacteria showed a gradual reduction to approximately 3.4 Log by day 14 of storage.

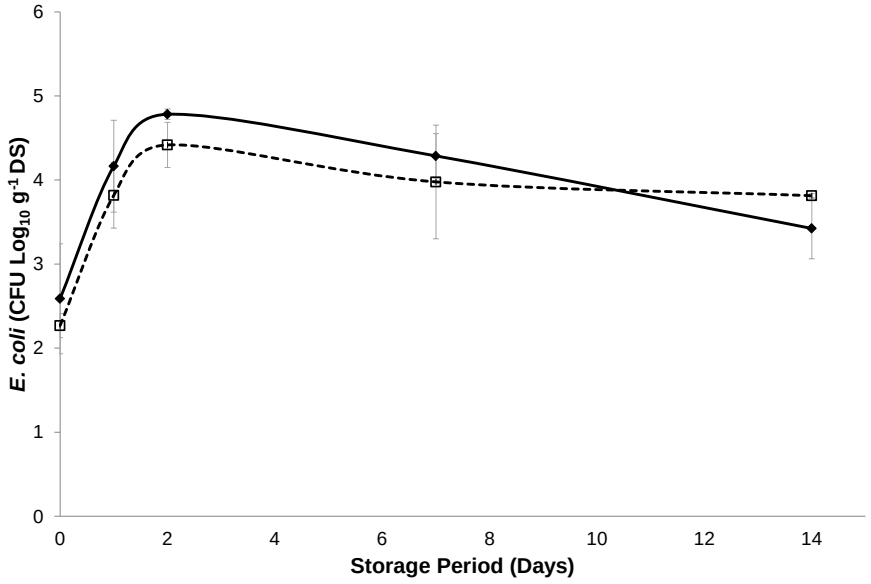

Figure 3.4 Comparison of FLOPAM and ZETAG polyelectrolyte types on *E. coli* dynamics in controlled biosolids storage for 14 days after filter press dewatering. Key: —◆— 0.3g/L FLOPAM, -□- 0.3 g/L ZETAG. Error bars represent the standard deviation of replicates (n=3).

3.4.3 Temperature and modified atmosphere

The effect of different storage temperatures on *E. coli* survival in sludge cake samples kept in open and gas evacuated storage conditions was investigated. A gradual reduction in *E. coli* concentrations was displayed across all conditions tested, however the extent of reduction was significantly different between open and gas evacuated conditions, and dependent upon storage temperature (Figure 3.5). The smallest change in *E. coli* concentrations between open and gas evacuated storage occurred at 19°C with an average reduction less than 0.25 Log by day 21 of storage. In open bags, temperatures above 19°C resulted in a greater decline of indicator bacteria. At day 21, *E. coli* concentrations showed a significant difference of approximately 1.8 Log units between 19°C and elevated storage at 25, 30 and 37°C. Only a small difference in *E. coli* concentration was displayed between the 25, 30 and 37°C conditions in open bags (Figure 3.5A). The temperature effect was more pronounced under gas evacuated storage with a statistically significant difference ($p < 0.05$) being demonstrated between the *E. coli* values in open and gas evacuated conditions at days 7, 14 and 21 (Figure 3.5B). At 25°C *E. coli* values were recorded at 5.3 and 4.0 Log units on days 14

and 21, respectively. At 30°C and 37°C, *E. coli* numbers were reduced to 3.9 Log and 2.4 Log by day 14 and no colonies were detected after 21 days. Statistical analysis confirmed the effect of higher temperature increasing *E. coli* die-off rate with conditions at 37°C under gas evacuated storage being significantly different from all other conditions tested at day 14.

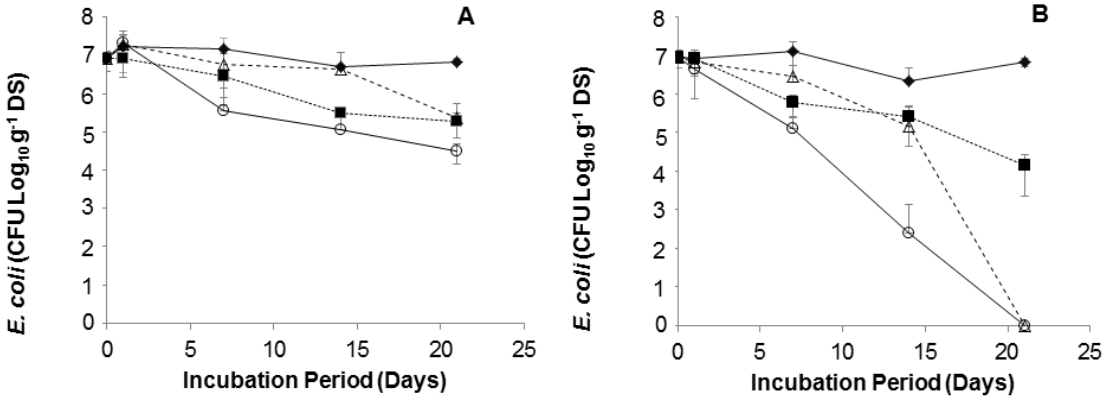

Figure 3.5 Effect of temperature and modified atmosphere storage on *E. coli* concentrations during 21 day incubation period. Results show colony forming units (CFU) per gram dry solids change in concentration and represent averages taken from three experimental repeats. Figure 3.5A shows open treatment condition, Figure 3.5B: shows gas evacuated storage. Key: —◆— Room temperature (19°C); --■--25°C; - -△-30°C; —○—37°C. Error bars represent the standard deviation of experimental repeats (n=3).

Under gas evacuated storage the sludge pH remained stable at approximately 8.6 over the 21 days of storage (Figure 3.6). In comparison, observations of the open condition showed a gradual reduction in pH level from 8.4 to 7.0 over the 21 day test period. At day 14 pH levels between open and gas evacuated conditions differed by 1.2 pH units and after 21 days this difference increased to 1.8 pH units. These differences were identified as statistically significant ($p < 0.05$) suggesting a substantial change in the chemical environment between the open and gas evacuated treatments.

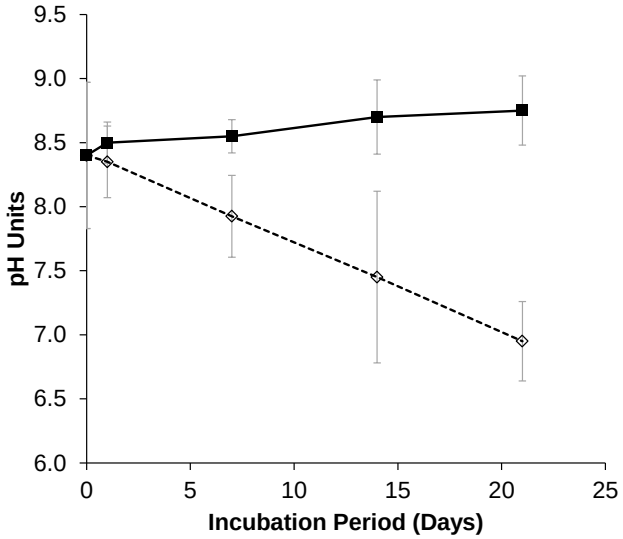

Figure 3.6 Changes in average pH during sample storage under open and modified atmosphere conditions. Key: --⟐--open treatment condition, —■—gas evacuated storage. Error bars represent the standard deviation of three experimental repeats (n=3).

To determine which chemical parameters could have been impacted, supplementary data was gathered from the analysis of TON performed at time intervals of 0, 24 hours, 7 days and 14 days (Figure 3.7). Levels of TON remained stable during the first 7 days of storage across all conditions, remaining within the range of 0.84 to 1.84 (mg/kg DS) after 24 hours and 2.84 to 7.86 (mg/kg DS) at day 7. Analysis at day 14 revealed a substantial change in levels of TON under the open treatment condition. At temperatures of 25°C and 30°C TON increased significantly to 170 and 171 mg/kg DS respectively. Samples held at 37°C under open storage however, showed no increase and remained at 2.51 mg/kg DS. In contrast, gas evacuated treatments at day 14 showed no change in concentrations and remained within the range of 2.34 to 5.52 (mg/kg DS).

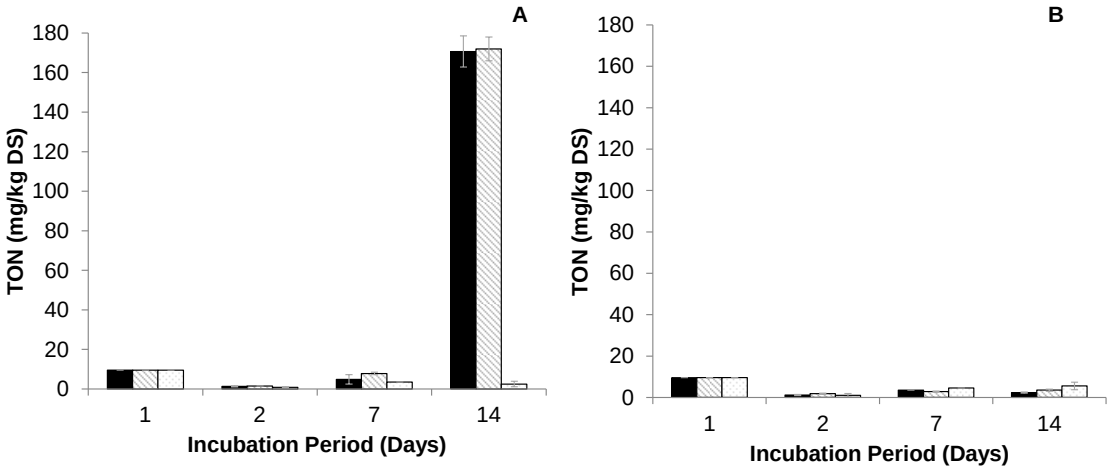

Figure 3.7 Effect of temperature and modified atmosphere storage on levels of total oxides of nitrogen (TON) (mg/kg DS) in biosolids stored for 14 days in aerobic condition (open bags) (A) or gas evacuated condition (sealed bags) (B). Key: ■ 25°C; ▨30°C; □ 37°C. (No data from 19°C storage condition was collected). Error bars represent the standard deviation of replicates (n=3).

3.4.4 Moisture content

Moisture contents within the DS% range of 17-35% were tested over a 21 day storage period. A gradual decrease in *E. coli* concentrations was displayed across all conditions (Figure 3.8), however the extent of reduction was not significantly different ($p > 0.05$) between the moisture contents tested. A slow reduction of approximately 1 Log was observed over the 21 day storage period in all treatment conditions.

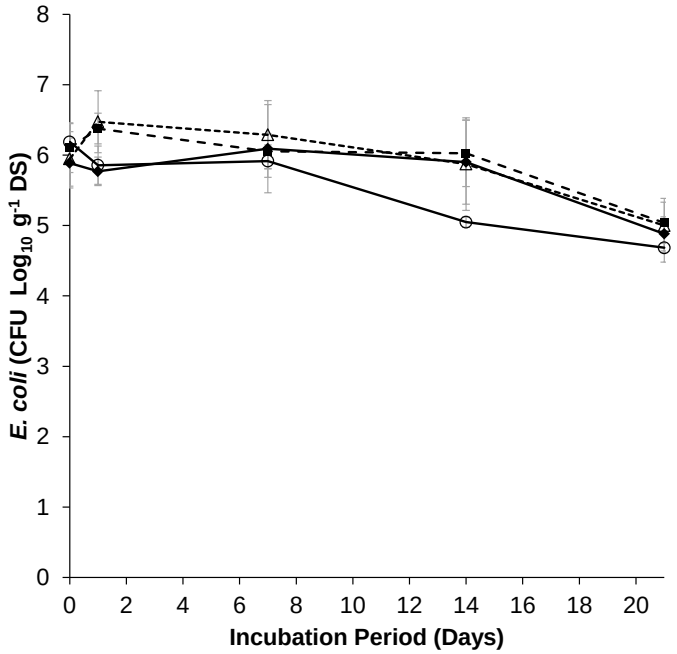

Figure 3.8 Effect of modified biosolids DS% on *E. coli* dynamics during incubated storage at 22°C for 21 days. DS% modified within range identified on sludge treatment sites between 2008- 2016 (Severn Trent Water Plc.). Key: —◆— 35% DS, - ■ - 25% DS, --△-- 20% DS, —○— 17% DS. Error bars represent the standard deviation of replicates (n=3).

3.5 Discussion

3.5.1 *E. coli* growth response to sludge-derived nutrient addition

The effect of adding nutrient derived from sludge on the growth of *E. coli* bacterial cells was investigated. Nutrients are essential for cell energy generation and biosynthesis (Todar 2012), an increase in nutrient availability post-dewatering may be a contributory factor to bacterial growth in the biosolids storage environment. In AD substrate competition is, in part, responsible for pathogen reductions (Orzi *et al* 2015) as *E. coli* experience competitive stress for nutrients. It is therefore plausible that increased nutrients within the biosolids matrix after dewatering operations could lead to elevated indicator concentrations. Identifying the level of bacterial response to nutrient addition in biosolids would support understanding of the mechanisms contributing to the peak *E. coli* concentrations observed after dewatering treatments. The results showed a significant increase (0.5 Log) in *E. coli* concentration between time 0 and 24 hours of storage in samples containing added nutrients. Elevated bacterial concentrations continued

in the following 5 days of storage when compared to stable control conditions which remained at 6.6 Log. Although no significant treatment effect was identified, the higher average *E. coli* level in nutrient addition treatments is likely to be a true response to the increased nutrient content. This is consistent with Williams *et al* (2015) who generated *E. coli* regrowth in thermally treated (62°C for 45 minutes) digested sludge cake at a laboratory bench scale. The mean generation time of *E. coli* bacteria was lower in experiments with nutrient broth addition compared to control treatments, emphasising the ability of *E. coli* bacteria to respond quickly to chemical changes in the immediate environment. Reasons for the absence of a significant difference between treatments tested in this study might be attributed to limitations in the experimental design. Reduced variability in the results may have been achieved with a greater number of experimental repeats. The sludge cake matrix is expected to be a relatively limited environment with regards to readily bioavailable nutrients, as organic matter is extensively degraded in AD increasing substrate limitation and microbial stability (Orzi *et al* 2010). Although Higgins *et al* (2006) suggest centrifuge dewatered cakes contain bioavailable protein and polysaccharides the research results generated here indicate the sludge matrix to be a nutrient limited environment. The short term growth response identified and the continued elevation in concentration suggests that *E. coli* bacteria survival can be enhanced under higher sludge nutrient concentrations, confirming the first hypothesis tested.

3.5.2 *E. coli* response to polyelectrolyte addition

Results showing *E. coli* growth curves from centrifuge and filter press dewatered sludge are consistent with on-site bacterial increases identified by Higgins *et al* (2007a), Qi *et al* (2008) and Chen *et al* (2011). Within 48 hours of fresh biosolids storage, observed *E. coli* concentrations rose by a minimum of 1 Log in all treatments tested. This result dispels arguments of substrate effects from polyelectrolyte degradation or flocculation influences (Chang *et al* 2001). In contrast, Qi *et al* (2007) found faecal coliform numbers to increase by 2 orders of magnitude in samples exposed to greater polymer concentrations. Our laboratory tests confirmed a higher level of bacterial die-off in samples with increased

polyelectrolyte concentration (0.3 g/L compared to 0.5 g/L). This observation points to a possible toxicity effect from the slow degradation of polyacrylamide copolymer to acrylamide which is toxic to cells (Junqua *et al* 2015). Therefore the second research hypothesis can be rejected as results have shown polyelectrolyte addition does not encourage *E. coli* growth in the sludge matrix.

Experimental limitations exist in the trials investigating effects between polyelectrolyte addition and control treatments with no polyelectrolyte. Up-scaled dewatering with the hydraulic filter press was not possible in control samples and therefore a laboratory centrifuge was used. This prevents a direct comparison between centrifuge and filter press dewatering for the polyelectrolyte versus no polyelectrolyte treatment.

3.5.3 Modified biosolids storage environment

3.5.3.1 Elevated storage temperature impacts on die-off

The effect of elevated storage temperatures and modified atmosphere on *E. coli* die-off was demonstrated. A significant reduction in *E. coli* bacteria was observed under gas evacuated treatments at temperatures above 25°C. The effect was increasingly pronounced under elevated storage temperatures of 30 and 37°C which reduced *E. coli* levels to below detectable limits after 21 days (Figure 3.5). Findings are consistent with results from studies on microbial dynamics in livestock manure and soils, which have greater associated datasets and form an analogous environment to biosolids. In a study by Semenov *et al*. (2007) testing the effects of temperatures ranging from 7 to 33°C on *Salmonella* and *E. coli* 0157:H7 survival in cow manure microcosms, survival of both pathogens declined significantly with increasing mean temperatures. The authors hypothesised that the reduced survival at higher temperatures may be a consequence of greater stress and energy expenditure for a particular organism (Semenov *et al* 2007). In addition, microorganisms antagonistic to enteropathogens in manure are more competitive at temperatures between 16 and 33°C, possibly due to increased temperature initially causing faster growth (Semenov *et al* 2007). Reduced antagonistic activity and competition for indigenous microorganisms is likely at lower temperatures (Cools *et al* 2001). In samples of manure Semenov *et al*

(2007) demonstrated that at temperatures of 7°C *E. coli* 0157:H7 reached the detection limit of 1 Log CFU g^{-1} DS after 159 days, highlighting prolonged survival times at colder temperatures. The argument for increasing biosolids storage temperature is supported by evidence from Plachá *et al* (2001) who showed that lower temperatures during winter months cause prolonged cell survival in pig slurry. This literature supports experimental results as storage at ambient room temperature (19ºC) caused less cell suppression, with concentrations always remaining above the critical 10^5 (MAC 5) threshold (Environment Agency 2003a).

Our results showed increased bacterial reduction under 37°C storage temperatures. Although 37°C is an optimal growth temperature for *E. coli* bacteria (Alberts *et al* 2010) heat tolerance might be reduced under hostile environmental conditions, such as oxygen depletion, causing physiological stress (Lang and Smith, 2008). This can influence microbial cell function, providing further evidence of enhanced indicator reduction under combined high temperature and modified atmosphere storage.

3.5.3.2 Influence of gas evacuated storage on rate of indicator die-off

Temperature seems to be a critical environmental parameter governing microbial growth and survival, however, modified atmosphere appears to exert a complementary effect. The significant *E. coli* reductions shown under gas evacuated conditions demonstrated increased inactivation efficiency (Figure 3.5B). The physical effects of gas evacuated storage will have reduced pore spaces in the sludge cake sample and caused mild compression effects in the material. Containment in a sealed bag would have additionally prevented oxygen diffusion. Whether complete anoxia was achieved is uncertain and methods would have benefitted from a measure of the oxygen concentration in the sealed bags. As such, it is difficult to attribute the enhanced die-off observed in samples stored under gas evacuated treatments to oxygen depletion. However, investigations into vacuum and modified atmosphere conditions in food preservation by Garrido *et al* (2016) support ideas of oxygen depletion suppressing biological activity. Results found significant inhibition of viable bacteria in vacuum and modified atmosphere treatments compared to control

conditions. This provides an argument for biosolids storage under gas evacuated treatments in order to accelerate indicator bacteria death and resonates with results. Despite the facultative nature of *E. coli*, aerobic respiration is greatly preferred as it yields more energy in the form of adenosine triphosphate (ATP) (30 ATP produced in aerobic respiration versus 2 ATP molecules in anaerobic respiration (Alberts *et al* 2010)). Therefore *E. coli* under oxygen depleted conditions, as in AD and the modified biosolids storage environments tested, are likely to persist but unlikely to grow. As the *E. coli* bacteria move into a phase where cell death rate is greater than cell growth, the conditions created through gas evacuation and incubation accelerate the reduction in viable cells. Unlike in silage conditions where pH decrease inhibits the growth of detrimental microorganisms (Herrmann *et al* 2011) a constant pH was maintained throughout the gas evacuated storage treatment.

3.5.3.3 Chemical changes to storage environments

Another potential biochemical pathway that can result in acidification is nitrification based on the release of hydrogen ions when ammonium is oxidised to nitrite and nitrate (Rowell 1994). A notable reduction in pH was observed under open storage (aerobic) conditions, which was not apparent in the gas evacuated treatment. A strong indication of nitrification occurring in aerobic sample bags was the increase of TON after 14 days. Nitrifying bacteria are strict aerobes (Gerardi 2002, Rowell 1994) and therefore the gas evacuated samples that were likely to be oxygen depleted may present an inhibitory environment for growth. As full air exchange was created in the aerobic (open) treatment and concentrations of NH_4-N in samples were recorded at approximately 1606 mg/kg DS, nitrification is more likely to have occurred. Autotrophic microorganisms active in the nitrification process grow very slowly (Bougard *et al* 2006). Temperature has a significant effect on the growth of nitrifying bacteria and rates of nitrification (Gerardi 2002). The optimal temperature range for nitrification is between 28°C and 32°C (Gerardi 2002), therefore the higher temperature condition of 37°C may have inhibited the nitrifying bacteria, resulting in the low TON levels displayed in Figure 3.7 at day 14. Results demonstrated the biochemical change induced through the modification of the biosolids storage

environment in the laboratory conditions tested. The data suggest that the third research hypothesis tested can be accepted as the difference in *E. coli* die-off was a direct result of the modification of environmental factors occurring in the stored biosolids matrix.

3.5.4 Effect of DS% on bacterial dynamics

Bacterial survival is possible across a range of dry solids conditions (Jiang *et al* 2002, Zaleski *et al* 2005b, Lang and Smith 2007, Rouch *et al* 2011). Understanding the impact of operational biosolids DS% on *E. coli* dynamics is important for benchmarking cake quality targets and operational decisions relating to polyelectrolyte dosing. Sprigings and Le (2011) defined the operational DS% range representative of centrifuge dewatered biosolids as 25-30% on a UK treatment site operating mesophilic digestion. To study extremes, and based on operational records (Severn Trent Water Plc. Operational data 2008- 2016), investigations across 17-35% DS were undertaken. Manipulating the biosolids DS% presented practical challenges and therefore the experimental design incorporating drying and de-ionised water addition to increase sludge moisture contents comes under criticism. Nevertheless, results from *E. coli* enumerations during 21 days of storage showed no significant difference between treatments and therefore the fourth hypothesis tested can be rejected. A gradual reduction in *E. coli* concentration across all conditions tested was exhibited over the storage period. This result emphasises that the sludge moisture content achieved in current dewatering operations does not influence *E. coli* die-off rates. Moreover, no increase in *E. coli* numbers was observed. It is therefore feasible to assume that elevated bacterial density is not related to sludge moisture content and that effects from other environmental factors such as temperature and nutrient availability are more dominant.

3.6 Conclusions

1. Sludge-derived nutrient addition to the biosolids matrix resulted in a significant increase of *E. coli* concentration within 24 hours of storage. This result emphasises the ability of *E. coli* organisms to respond quickly to chemical changes in the nutrient-limited sludge environment.

2. Post-dewatering *E. coli* growth identified on sludge treatment sites was verified at a laboratory scale. In addition, experiments investigating the impact of polyelectrolyte on *E. coli* growth confirmed no effect of addition. Results indicated possible toxicity effects on *E. coli* cells from increasing polyelectrolyte concentrations giving confidence that polyelectrolyte dosing can be optimised against DS% estimates and cake quality targets without increasing bacterial concentrations.
3. In these experiments there has been a synergistic effect of higher temperature (>25°C) and gas evacuated storage, which accelerated *E. coli* die-off. This suggests that oxygen depletion increases cell stress under the modified storage conditions. These results indicate benefits in controlling biosolids storage temperatures and levels of oxygen exposure after mechanical dewatering for sites challenged by compliance requirements.
4. No effect of biosolids moisture content (tested within the operational DS% range experience on treatment sites in the UK) was identified on *E. coli* growth dynamics. Therefore suggesting that moisture content is not the dominant factor directly influencing *E. coli* behaviour within current biosolids storage environments.

3.7 Acknowledgements

The authors would like to thank the Natural Environment Research Council (NERC) and Severn Trent Water Plc. for their financial support.

3.8 References

ADAS, (2001) THE SAFE SLUDGE MATRIX: Guidelines for the Application of Sewage Sludge to Agricultural Land. Gleadthorpe Research Centre.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Berry, E. D. and Miller, D. N. (2005) Cattle Feedlot Soil Moisture and Manure Content: II Impact on *Escherichia coli* 0157 *Journal of Environmental Quality* Vol. 34 pp.656- 663

Bougard, D., Bernet, N., Chèneby, D., Delgenès, J.P. (2006) Nitrification of a high-strength wastewater in an inverse turbulent bed reactor: Effect of temperature on nitrite accumulation. *Process Biochem*. Vol. 41, pp. 106–113.

Chang, L.L., Raudenbush, D.L., Dentel, S. K., (2001) Aerobic and anaerobic biodegradability of a flocculant polymer *Water Science and Technology* Vol. 44 No. 2-3, pp. 461- 468

Chen, Y., Higgins, M. J, Beightol, S. M, Araujo, G. G., Murthy, S. N., Barben, E. J., Toffey, W. E. (2007) Odour Generation and Pathogen Indicator Regrowth after Dewatering: Are They Related? In: Proceedings of the Mid-Atlantic Biosolids Assocation Conference

Chen, Y. C., Higgins, M. J., Beightol, S. M., Murthy, S. N., & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research,* Vol. *45*, pp. 2616- 2626.

Chen, Y., Fu, B., Wang, Y., Jiang, Q., Liu, H. (2012) Reactor performance ad bacterial pathogen removal in response to sludge retention time in a mesophilic anaerobic digester treating sewage sludge *Bioresource Technology* Vol. 106 pp. 20-26

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., (2001) Survival of *E. coli* and *Enterococcus spp*. derived from pig slurry in soils of different texture. *Appl. Soil Ecol*. Vol. 17, pp. 53–62.

Deeks, L. K., Chaney, K., Murray, C., Sakrabani, R., Gedara, S., Le, M. S., Tyrrel, S., Pawlett, M., Read, R., and Smith, G. H. (2013) A new sludge-derived organo-mineral fertilizer gives similar crop yields as conventional fertilizers. *Agronomy for Sustainable Development*, Vol. 33, pp. 539–549.

Dentel, S. K., Qi, Y., & Herson, D. S. (2008). Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration, and assessment. *Water Science and Technology,* Vol. 57 No. 2.

Environment Agency, (2003a) The Microbiology of Sewage Sludge-Part 1- An overview of the treatment and use in agriculature of sewage sludge in relation to its impact on the environment and public health. Bristol.

Environment Agency, (2003b) The Microbiology of Sewage Sludge-Part 2- Practices and procedures for sampling and sample preparation. Bristol

Environment Agency, (2003c) The Microbiology of Sewage Sludge- Part 3- Methods for the isolation and enumeration of *Escherichia coli*, including verocytoxigenic *Escherichia coli*. Bristol.

European Union, 2000. Working Document on Sludge, 3rd Draft. ENV.E.3/LM. Brussels.

Floros, J. D and Matsos, K. I. (2005) Introduction to modified atmosphere packaging. In Han, J. H. (Ed.), *Innovations in Food Packaging* (pp. 159- 184). Elsvier Ltd.

Gerardi, M.H., 2002. Nitrification and Denitrification in the Activated Sludge Process. John Wiley and Sons Inc., New York.

Garrido, M. D., Hernández, M. D., Espinosa, M. C., López, M. B. (2016) Enhanced quality characteristics of refrigerated Seabream (Sparus aurata) fillets packed under different systems (modified atmosphere Vs. vacuum) *Journal of Aquatic Food Product Technology* Vol. 25, No. 2, pp. 156-168

Geotechnical Engineering Bureau (GEB), 2007. Geotechnical Engineering Bureau: Test method for determination of pH value of water or soil.

Gujarati, D. (2011) 'Econometrics by Example' PALGRAVE MACMILLAN, Hampshire, UK

Herrmann, C., Heiermann, M., Idler, C., 2011. Effects of ensiling, silage additives and storage period on methane formation of biogas crops. *Bioresour. Technol.* Vol. 102, pp. 5153–5161.

Higgins, M. J., Chen, Y. C., Murthy, S. N., & Hendrickson, D. (2007a). Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering. *Procedings on moving forward wastewater biosolids sustainability: Technical, managerial and public synergy*, pp. 204-19.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Henderickson, D., Farrel, J., & Schafer, P. (2007b). Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Research*, Vol. 41 pp. 665-673.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Hendrickson, D., Schafer, P., & Farrell, J. (2006). WERF Phase 2: The Impact of Digestion and Dewatering on Reactivation and Regrowth of Viable but Non-Culturable Indicator Bacteria. WEFTEC.

Hutchison, M.L., Nicholson, F.A., Smith, K.A., Keevil, C.W., Chambers, B.J., Moore, A., (2000) Study on Farm Manure Applications To Agricultural Land and an Assessment of the Risks of Pathogen Transfer Into the Food Chain FS2526.

Henkel, S.G., Ter Beek, A., Steinsiek, S., Stagge, S., Bettenbrock, K., Teixeira de Mattos, M.J., Sauter, T., Sawodny, O., Ederer, M., (2014) Basic Regulatory Principles of *Escherichia coli's* Electron Transport Chain for Varying Oxygen Conditions. *PLOS one*. Vol. 9 Iss. 9, e107640

Jiang, X., Morgan, J. and Doyle, M. P. (2002) Fate of *Escherichia coli* 0157:H7 in manure amended soil *Applied Environmental Microbiology* Vol. 68, pp.2605-2609

Junqua, G., Spinelli, S., Gonzalez, C. (2015) Occurrence and fate of acrylamide in water-recycling systems and sludge in aggregate industries *Environ Sci Pollut Res* Vol. 22 pp. 6452- 6460

Lang, N. L. and Smith, S. R. (2007) Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions *Journal of Applied Microbiology* Vol. 103, pp. 2122- 2131

Lang, N.L. and Smith, S.R. (2008) Time and temperature inactivation kinetics of enteric bacteria relevant to sewage sludge treatment processes for agricultural use. *Water Res*. Vol. 42, pp. 2229–2241.

Le, M.S., Mayhew, M.E., Back, P.A., (2002) Effectiveness of Secondary Digesters as a Pathogen Controller in Winter. *J. CIWEM* Vol. 16, pp. 292–295.

Marques de Sa, J.P. (2007) Applied Statistics using SPSS, STATISTICA, MATLAB and R. Springer- Verlag, Berlin.

Ministry of Fisheries and Food (MAFF), (1986) The Analysis of Agricultural Materials, RB427 ed. Her Majesty's Stationary Office, London.

Monteleone, M. C., Furness, D., Jefferson, B., & Cartmell, E. (2004). Fate of *E. coli* Across Mechanical Dewatering Processes. *Environmental Technology*, Vol. 25 No. 7 pp. 825-831.

Mowla, D., Tran, H.N., Grant Allen, D. (2013) A review of the properties of biosludge and its relevance to enhanced dewatering processes *Biomass and Bioenergy* Vol. 58 pp. 365-378

Orzi, V., Cadena, E., D'Imporzano, G., Artola, A., Davoli, E., Crivelli, M., Adani, F. (2010) Potential odour emission measurement in organic fraction of municipal solid waste during anaerobic digestion: Relationship with process and biological stability parameters *Bioresource Technology* Vol. 101 pp. 7330-7337

Orzi, V., Scaglia, B., Lonati, S., Riva, C., Boccasile, G., Alborali, G.L., Adani, F. (2015) The role of biological processes in reducing both odour impact and pathogen content during mesophilic anaerobic digestion *Science of the Total Environment* Vol. 526 pp. 116-126

Placha, I., Venglousky, J., Sasokova, N., Svoboda, I.F. (2001) The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solids of pig slurry. *J. Appl. Microbiol*. Vol. 91, pp. 1036–1043.

Qi, Y., Dentel, S. K., & Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. 41, pp. 571- 580.

Qi, Y., Dentel, S. K., Herson, D. S. (2008) Effect of total solids on fecal coliform regrowth in anaerobically digested biosolids *Water Research* Vol. 42 pp. 3817-3825

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Rouch, D. A., Mandal, T., Pai, S., Glauche, F., Fleming, V. A., Thurbon, N., *et al*. (2011). Microbial safety of air dried and re-wetted biosolids. *Journal of Water and Health*, Vol. 9.2 pp. 403-414.

Rowell, D.L., 1994. Soil Science: Methods and Applicatons. Longman Scientific and Technical, Harlow, UK.

Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M., Adani, F., 2014. Sanitation ability of anaerobic digestion performed at different temperature on sewage sludge. *Sci. Total Environ.* 466-467, pp. 888–897.

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M. (2007) Influence of temperature fluctuations on *Escherichia coli O157:H7* and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol*. Vol. 60, pp. 419–428.

Sidhu, J. P., & Toze, S. G. (2009). Human Pathogens and their Indicators in Biosolids: A Literature Review. *Environment International,* Vol. 35, pp. 187- 201.

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manag*. Vol. 25, pp. 417–425.

Sprigings, A. E., & Le, M. S. (2011). Growth Potential- A New Method For Estimation of the Risk of *E. coli* Regrowth in Digested Sludge Cake. *16th European Biosolids and Organic Resources Conference.*

Standard Methods for the Examination of Water and Wastewater, 1995. Automated Hydrazine Reduction Method (19th Edn). Washington DC, USA.

Stevens, M., Ashbolt, N., & Cunliffe, D. (2003). Review of Coliforms as Microbial Indicators of Drinking Water Quality. *Recommendations to Change the Use of Coliforms as Microbial Indicators of Drinking Water Quality*. Canberra: Australian Government National Health and Medical Research Council, Biotext Pty Ltd.

Todar, K (2012) Online Textbook of Bacteriology: Nutrition and Growth of Bacteria http://textbookofbacteriology.net/nutgro.html [Accessed on 05/09/2016]

Town, J.R., Links, M. G., Fonstad, T. A., Dumonceaux, T. J. (2014) Molecular characterisation of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. *BioResource Technology* Vol. 151 pp. 249- 257

UKWIR. (1999). *E. coli in UK Mesophilic anaerobically digested sludge Report SL-06.* UK Water Industry Research Ltd.

US EPA (United States Environmental Protection Agency, 2012. A Plain English Guide to the EPA Part 503 Biosolids Rule [WWW Document]. URL http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm (accessed 05/01/2015).

Wang, L., Mankin, K. R., Marchin, G. L. (2004) Survival of Fecal Bacteria in Dairy Cow Manure *American Society of Agricultural Engineers* Vol 47 No.4 pp. 1239-1246

Williams, T., Harris, J and Tyrrel, S. (2014) Competitive exclusion as a means to reduce *Escherichia coli* regrowth in digested sludge. Unpublished PhD. thesis. School of Energy, Environment and Agrifood, Cranfield University, UK

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005a). Survival, growth and Regrowth of Enteric Indicator and Pathogenic Bacteria in Biosolids, Compost, Soil, and Land Applied Biosolids *Journal of Residuals Science and Technology* Vol. 2, No. 1 pp. 49- 63

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005b). Potential Regrowth and Recolonisation of *Salmonellae* and Indicators in Biosolids and Biosolids-Amended Soil *Applied and Environmental Microbiology* Vol. 71, No. 7, pp. 3701- 3708

Chapter 4

Long Term Characterisation of the Biosolids Storage Environment: Implications for *E. coli* Dynamics

Statement of author contributions:

S. Fane: Principal investigator, completion of experimental design and laboratory work, data analysis and chapter write up

A. Wilson: Industrial supervisor for Severn Trent Water Plc.

Dr. M. Rivas Casado: Statistical consultant

Professor J. Harris: Supported evaluation of trial design

Professor E. Cartmell: Developed overall concept of work with Severn Trent Water Plc.

Professor S. Tyrrel: Principal supervisor supporting research concept development, guidance on experimental design, data analysis and evaluation of final chapter write up

4 Long Term Characterisation of the Biosolids Storage Environment: Implications for *E. coli* Dynamics

S. Fane[1], A. Wilson[2], M. Rivas Casado[1], J. Harris[1], E. Cartmell[3], S. Tyrrel[1]

1 Water Science Institute, School of Water, Energy and Environment, Cranfield University, UK

2 Severn Trent Water, UK

3 Scottish Water, Dunfermline, Scotland

4.1 Abstract

To meet the requirements for conventional treatment in the Safe Sludge Matrix, a 2 log reduction in *Escherichia coli (E. coli)* concentration is needed between the digester inlet and the final biosolids product after 14 days of storage. Observations have identified survival of *E. coli* bacteria in stored biosolids after mechanical dewatering and in the following 14 days of storage. Indicator bacteria survival presents a significant risk of non-compliance. Parameters of the biosolids storage environment may encourage prolonged *E. coli* survival and presently characteristics of the storage environment are not well profiled. This study aimed to identify the characteristics of biosolids storage through a long term, up-scaled, environmental monitoring programme which tested the hypothesis that physical and chemical environmental factors within biosolids storage are the controlling parameters of bacterial concentrations. Mesophilic anaerobically digested, centrifuge dewatered biosolids were stored in 8 tonne stockpiles over a 12 month period, divided into 4 study quarters to investigate seasonal effects. Monthly *E. coli* levels were monitored in addition to sludge dry solids and pH. Stockpile temperature and redox potential levels were recorded at hourly intervals and ambient weather data collected. *E. coli* bacteria showed significant reductions across each study quarter with a greater level of die-off observed in the summer storage quarter between June and September. A significant effect of stockpile temperature on *E. coli* dynamics was identified with a significant reduction of 4.5 Log in summer months coinciding with peak stockpile temperatures of 31°C. Stockpile temperature did not change with depth in the stored material but was

found to be significantly influenced by the monthly average ambient temperature. In lower winter temperatures *E. coli* survival was prolonged and concentrations remained at approximately 6.5 Log. Redox potential levels showed a significant reduction of -218 mV in the first 48 hours of storage after stockpiling, suggesting mechanical dewatering operations may increase oxygen exposure to the sludge matrix that is subsequently depleted over time. Stockpile moisture content was shown to be significantly different with depth through the stockpile and changes across the 12 month study period were significantly related to temporal patterns in precipitation. Substantially higher moisture content was recorded in wetter months of the year. Findings from the study have enhanced understanding of biosolids storage environments at an operational scale. The assessment of multiple variables in analysis has identified the significant roles physical environmental factors have on *E. coli* dynamics.

4.2 Introduction

Indicator bacteria resurgence is frequently observed in biosolids storage and therefore poses a risk of non-compliance with sludge quality requirements (Sprigings and Le 2011). Although anaerobic digestion provides stabilisation and additional benefits of reduced biological activity in sludge (Chen *et al* 2011), high concentrations of indicator bacteria in anaerobically digested biosolids have been observed immediately after mechanical dewatering (Monteleone *et al* 2004, Higgins *et al* 2007a, Dentel *et al* 2008, Qi *et al* 2008, Chen *et al* 2011). Post-dewatering indicator changes have been known to range from -0.4 Log to +6.4 Log units (Qi *et al* 2007) emphasising the highly variable bacterial behaviour amongst treatment plants. This unpredictable indicator fluctuation in storage causes much concern for utility operators and limits the quality and value of sludge products. Higgins *et al* (2007a) argues that conditions in the biosolids after mechanical dewatering favour the growth of indicator organisms. A study assessing the *E. coli* densities in biosolids stored at 35°C produced after thermophilic and mesophilic digestion treatments and centrifuge dewatering found *E. coli* concentrations increased within the first three days to between 10^8 and 10^9 colony forming units (CFU) /g DS (Higgins *et al* 2007a).

Limited research has been completed on the characteristics of the biosolids storage environment in temperate climates and the effects of physical parameters on *E. coli* bacteria dynamics. Studies are often limited to laboratory scale experiments and short term study (Iranpour *et al* 2005, Lang and Smith 2008, Qi *et al* 2008, Sprigings and Le 2011). This is possibly due to the practicalities involved with operational scale studies and the reduced level of experimental control achieved when working at such scales. The key factors responsible for indicator growth and survival in stored dewatered biosoilds and their relative importance have not yet been clearly identified (Dentel *et al* 2008). The immediate elevation in indicator numbers post-dewatering and the prolonged survival during subsequent biosolids storage, suggests parameters of the storage environment may be controlling factors (Higgins *et al* 2007a).

The understanding of *E. coli* behaviour in biosolids could be enhanced by studies in analogous environments where findings may infer the trends expected in biosolids material. In particular, identifying the environmental characteristics affecting bacterial survival in manure and biosolids-amended soils in seasonal monitoring studies including field investigations and operational-scale tests may provide clues to characterise the post-dewatering biosolids storage environment. Plachá *et al* (2001) studied the survival of *Salmonella typhimurium* during field storage of solid fraction pig slurry. Research was carried out between June and August (summer) and from January to June (winter/ spring). Findings showed temperature to be the most important factor affecting the survival of microorganisms in the environment (Plachá *et al* 2001). In the summer and winter/spring tests the maximum survival period of *Salmonella typhimurium* was 26 days and 85 days respectively. The temperature of the solid slurry fraction ranged from 17 to 26°C during summer periods and -1 to 30°C in winter/ spring (Plachá *et al* 2001). The volume of the solid fraction slurry tested was limited to 50 L which is substantially smaller than operational scale volumes. It is therefore possible that the temperatures recorded are not representative of a larger storage facility. However, researchers found a significant correlation between *Salmonella* survival and temperature for summer periods. Likewise, significant correlations between temperature and *Salmonella* survival were identified for winter/ spring

indicating that the survival of indicator bacteria was considerably affected by the temperature of the solid fraction in field storage experiments. First year results obtained by Nicholson *et al* (2005) on the survival times of pathogens in livestock solid manure heaps provide evidence of elevated temperatures promoting pathogen die-off. Observations of *E. coli* 0157 showed bacterial concentrations reached undetectable levels in less than 10 days of storage in unturned dairy farmyard manure between June and July where temperatures greater than 55°C were maintained (Nicholson *et al* 2005).

Similar findings on temperature were identified by Iranpour *et al* (2005) and Sprigings and Le (2011) on sludge treatment sites. Iranpour *et al* (2005) recorded temperature in the post-digestion train during pilot tests on a battery of 6 thermophilic digesters. Monitoring showed a large drop in the biosolids temperature; digester outlet 52.6°C, centrifuge outlet 48.2°C, silo at truck loading facility 41°C, farm storage 40.3°C. The reoccurrence of faecal coliforms in post-digestion biosolids was attributed to this temperature reduction and researchers argue that maintaining a minimum temperature of 50°C (representative of the upstream thermophilic digestion process) may prevent growth of faecal coliforms (Iranpour *et al* 2005). A comparative on-site study by Sprigings and Le (2011) focused on determining a temperature value representative of the biosolids storage conditions. Cake pad temperature monitoring was undertaken from the centre of the cake pile and recorded alongside ambient temperature measurements at two treatment sites over a period of 48 hours. The cake temperature immediately after centrifuge dewatering was approximately 30°C (±2.5°C) with the cake retaining this higher temperature for approximately 12 hours, possibly an effect of residual heat from the digestion process. Over the following 48 hours the temperature profile gradually reduced and for both treatment sites fell below the ambient temperature recorded (Sprigings and Le 2011). Sprigings and Le (2011) suggest that the role of residual heat in the first 12 to 24 hours is an important factor in aiding the proliferation of *E. coli* with peak concentrations measured at 10^7 CFU g/DS in the study. Arguably these temperature datasets are not fully representative of the biosolids storage environment as treatment requires a minimum 14 day storage period after

mesophilic digestion (DEFRA 2006). As Sprigings and Le (2011) show the temperature value to gradually reduce within the first 48 hours of storage it may be that cooler temperatures in the proceeding days are capable of preserving the growth and survival of bacteria.

A more detailed long term, up-scaled, monitoring study was completed by Lang *et al* (2007) on field investigations studying the survival of *E. coli* in biosoilds-amended agricultural soil. Temperature monitoring highlighted seasonal trends in the data with warmer temperatures of approximately 19°C in spring/ summer seasons compared to winter months where the maximum temperature recorded was 13°C. Analysis confirmed 'soil temperature' to be the key environmental parameter responsible for the general seasonal patterns in background *E. coli* numbers observed between the different field trials.

Empirical statistical models were developed by Lang *et al* (2007) and summarised the relationship between soil temperature, moisture content, time and *E. coli* populations. Results from three field trials completed between June to August, October to February and April to July on plots of 1.5 m^2 showed that soil moisture declined with seasonal increases in air temperature and soil was generally drier during warm periods. *E. coli* concentrations in spring/ summer trials varied with soil drying and wetting cycles. An increase in soil moisture content observed following heavy rainfall (44.5 mm) prior to sampling coincided with an increase of 2-3 Log_{10} CFU/100 g^{-1} DS (Lang *et al* 2007). The seasonal monitoring study completed by Plachá *et al* (2001) additionally suggested that prolonged survival of *Salmonella* during winter months may be an effect of dry matter content as the average % dry solids (DS) was reduced between summer and winter test periods, although this decrease was marginal. In the on-site tests completed by Sprigings and Le (2011) researchers suggested an interesting effect of moisture retaining heat in the biosolids matrix. A comparison of sites A and B showed differences in the DS% of the dewatered sludge cake (Site A: 25% DS; Site B: 31% DS) which corresponded with site A maintaining a higher sludge cake temperature. A lower DS% will help the fresh cake retain heat from the AD process for a longer period, delaying cake cooling to lower ambient temperatures which have been shown to

prolong cell survival (Plachá *et al* 2001, Cools *et al* 2001, Semenov *et al* 2007). In this particular study site B showed the highest peak *E. coli* concentration with levels >10^7 CFU g/DS suggesting an increase in DS% may not be a contributing factor to increased *E. coli* growth in post-dewatered biosolids. However, this study was limited to 48 hours and therefore a detailed indication of the long term characteristics of moisture content in stored biosolids and the relative impact on *E. coli* survival is not clear.

Changes in moisture content can affect oxygen and nutrient pools available for organisms (Banach *et al* 2009, Peralta *et al* 2014). Increases in moisture content can encourage anaerobic conditions to develop resulting in a redox potential decrease. As conditions become more anoxic microorganisms switch to alternative electron acceptors such as nitrate, iron or sulphur, which alters the dominant metabolic activity in the tested material (Keddy 2000, Mitsch and Gosselink, 2007). In up-stream sludge treatment processes, such as digestion, the combination of an oxygen limiting environment and other pernicious reactor conditions has a controlling effect on pathogen concentrations. In particular, oxygen availability has a substantial effect on cell respiration and consequential energy production, regulating most cell activities (Alberts *et al* 2010). Mechanical dewatering causes substantial change of environmental conditions in the sludge product (Higgins *et al* 2007b, Chen *et al* 2011) and often continuous flow decanter centrifuges are employed, containing an internal scroll conveyor that can amplify oxygen exposure (Chen *et al* 2011). Oxygen can disrupt methanogens (Chen *et al* 2007, Chen *et al* 2011) and therefore provide a selective advantage for *E. coli* bacteria growth. Chen *et al* (2011) suggests that this selective advantage may quickly disappear as the storage conditions of biosolids return to an anaerobic (anoxic) state. Redox potential measurements can give an indication of the oxygen availability and a shift in microbial metabolic pathways (Peralta *et al* 2014). Datasets on the redox conditions of biosolids storage environments are very limited and may provide some indication of the spatial differences in oxygen availability and microbial dynamics exhibited across storage stockpiles.

The studies above highlight the effects of physical parameters on bacterial survival in biosolids and analogous environments. Limited trials have been completed to understand the characteristics of the biosolids storage environment, particularly at an operational scale and incorporating the assessment of multiple variables. Previous studies tend to have been based on short term measurements over a period of days and are often focussed upon indictor bacteria growth immediately after mechanical dewatering, rather than any long term die-off dynamics. Measurements at operational sludge treatment facilities are often confronted with safety risks in addition to sampling access challenges which discourage operational scale study of the biosolids storage environment. The trial described in this chapter aims to identify the characteristics of the biosolids storage environment through a long term, up-scaled, environmental monitoring programme. Furthermore it aims to test the hypotheses:

1. Temperature of stored biosolids is a significant factor controlling *E. coli* concentrations.
2. pH changes in biosolids stockpiles is a parameter significantly influencing *E. coli* dynamics.
3. Dry solids % prevailing within the biosolids material has a significant effect on *E. coli* survival.
4. Redox potential conditions of stored biosolids are a significant factor controlling *E. coli* concentrations.

4.3 Methodology

4.3.1 Sludge sample source

Representative bulk samples of sludge cake, which had been both mesophilic digested and centrifuge dewatered were collected from a wastewater treatment works serving a population equivalent (PE) of 440,000. The samples were transported to Cranfield University (Bedfordshire, England) in 25 tonne capacity haulage trucks. For practical purposes the sewage sludge cake was transported in individual 1 tonne capacity bulk bags (Travis Perkins, UK). The cake was taken immediately after centrifuge dewatering to ensure the product was less than 24

hours old when reaching site, this allowed tested storage environments to begin from a day 1 assumption.

4.3.2 Programme schedule

The full-scale monitoring programme ran for a period of 12 months segregated into quarters with 3 or 4 months of monitoring undertaken on a fresh load of sludge cake. Quarter 1 ran from October 2015 to December 2015, Quarter 2 January to April 2016, Quarter 3 April to June 2016 and Quarter 4 July to September 2016. The 4 study quarters were incorporated to enable the observation of seasonal differences in the characteristics of stored sludge cake. Although 3 months storage is substantially greater than the post-digestion 14 day storage period recommended on treatment sites operating mesophilic digestion (DEFRA 2006), it is not uncommon for sewage sludge cake to be stored for up to 6 months if the product fails compliance tests. Additionally, stockpiles can be stored on agricultural land for long periods of time before spreading.

4.3.3 Sludge containment cages

Galvanised steel cages (1.5 m^3) were designed and made to hold approximately 8 tonnes of mesophilic anaerobically digested and centrifuge dewatered sewage sludge cake (J.R. Trolleys, Bedfordshire, England). The cages were situated on an open concrete pad at Cranfield University. Cages were lined with an interlocked, knitted high-density polyethylene (HDPE) mesh to allow drainage and gas exchange whilst holding the sludge cake in the cages (Secure Covers, Shropshire, UK).

4.3.4 Preliminary trials to determine sludge sampling strategy

Preliminary trials were conducted before the full-scale monitoring study was started and used a spiral auger to obtain sludge samples for laboratory analysis. Observations identified increased disturbance to the tested stockpile as compression around the sides of an augured sampling hole prevented the sludge from collapsing in after the auger was removed. This finding raised concerns as the increasing number of holes generated by sampling over a study quarter in the full-scale trial would cause a cooling effect on the tested stockpiles.

To prevent augured holes having a cooling effect sampling tubes of acrylic plastic (Amari Plastics, Bedford, UK) were designed (Figure 4.1). These acrylic semi-circular tubes were 250 mm in depth and designed to be in lengths of 2 m which would be installed across the cage during filling. The tubes contained small 150 mm diameter holes to allow natural drainage though the sewage sludge cake and regular baffles were fitted to ensure sludge was retained in the tube when pulled out of the monitoring cage during sampling. The tubes were used sacrificially for each sampling time point. Sampling with the tubes was considered the most appropriate method able to maintain the natural storage environment with a low level of disturbance within the tested stockpiles when sampling.

Figure 4.1 Prototype of 150 mm acrylic sampling tube containing 2 x end baffles (250 mm depth) and 3 x 15 mm drainage holes. At an up-scaled size, for the monitoring project, 4 lengths of 0.5 m were joined together with resin to form a 2 m length that would be placed across the whole cage width when filling with sewage sludge cake.

4.3.5 Temperature and redox potential data recording

Temperature and redox potential data were collected at hourly intervals using a CR800 measurement and control system powered by a 12 V alkaline battery (Campbell Scientific, Loughborough, UK). Temperature data was collected from 9 probes in each cage (CS655-DS, Campbell Scientific, Loughborough, UK), arranged as shown in Figure 4.3. Redox probes were designed with three sensors fitted into a 1 m length probe (Figure 4.2) (Paleo Terra, Amsterdam, Netherlands). 3 x 1 m probes were installed into each full test cage. Separate reference electrodes (Paleo Terra, Amsterdam, Netherlands) were installed into each of the three cages and individually connected to the data logger, maintaining a separate redox data recording system for each test cage. The final redox potential value (E_h) was calculated by adding the potential from the reference

electrode (E_{ref} = -144 mV) to the measured potential (E_m) (Vorenhout van der Geest and Hunting 2011):

$$E_h = E_m + E_{ref} \quad \textbf{(4-1)}$$

Probes were connected to the data logger system through two AM16/32B 16/32 channel relay multiplexers (Campbell Scientific, Loughborough UK), which were housed in mounted enclosures to protect the equipment during the 12 month study (TM-ENC-MOUNT, Campbell Scientific, Loughborough, UK). All temperature and redox probes were placed into the cages during filling and data was downloaded at the end of each storage period.

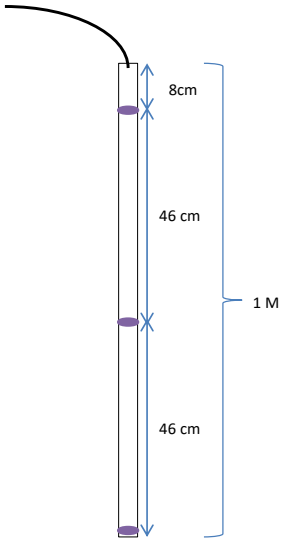

Figure 4.2 Redox probe sensor arrangement. 3 x 1 m probes (9 redox sensors recording data) in each of the three test cages (Paleo Terra, Amsterdam, Netherlands). Key: ⬬ redox sensor location.

4.3.6 Ambient weather data

Ambient weather fluctuations were monitored for the duration of each storage period using a wireless weather centre (Maplin Electronics, Rotherham, England). Specific focus was given to precipitation levels and ambient temperature which were measured and recorded at 30 minute intervals. Recorded data was downloaded at the end of each study period. The weather

centre was located on the roof of a building adjacent to the three test cages and set up according to the manufacturer's instructions.

4.3.7 Sludge treatment site stockpile temperature comparison

Operational sludge treatment site cake pad temperature recordings were taken on 28/04/2016 and 20/07/2016 to provide comparative data with the 3 x 8 tonne environmental monitoring storage trials. The operational sludge stores measured were estimated to be 200 tonnes in capacity with dimensions of approximately 50 m x 30 m x 1.5 m. A hand held meter reader and temperature probe (TPA1-N 1 AWE Ltd., Stafford, UK) was used for data recording. A transect from the storage bay back wall to the front of the stockpile was used and temperature readings taken every 2 m. The transect was created with a loading shovel vehicle removing a 2 m wide strip of the stored material next to the side wall of the storage bay facility. The first reading was taken from approximately 5 m in off the back wall.

Temperature recording on 28/04/2016 was taken when the stored biosolids were approximately 4 weeks old (from centrifuge output). On 18/07/2016 the stored cake was approximately 6-8 weeks old (from centrifuge output).

The ambient temperature on the treatment site at the time of temperature monitoring was identified on Weather Underground (2016) from the IDERBY108 weather station which records meteorological measurements at 5 minute intervals. Data is automatically uploaded to the online weather database.

4.3.8 Full-scale experimental set up

Samples of mesophilic digested and centrifuge dewatered sludge cake were transported from the treatment site (Section 4.3.1) in 1 tonne bulk bags and cages were filled following a sequence of sludge, sampling tubes and monitoring probes to build up the grid layers designed in the programme (Figure 4.3). The cages had a capacity of 8 tonnes and typically 2 tonne loads separated each of the sampling tube and probe location clusters. Figure 4.3 shows a schematic of the sample tube and monitoring probe arrangement. The monitoring was completed in a triplicate study with three independent cages forming 3 repeats. Replicates of the top, middle and lower depths across a vertical plane were taken from

horizontal layers 1, 2 and 3 (Figure 4.3) which gave 3 repeats and 3 replicates for each result value output.

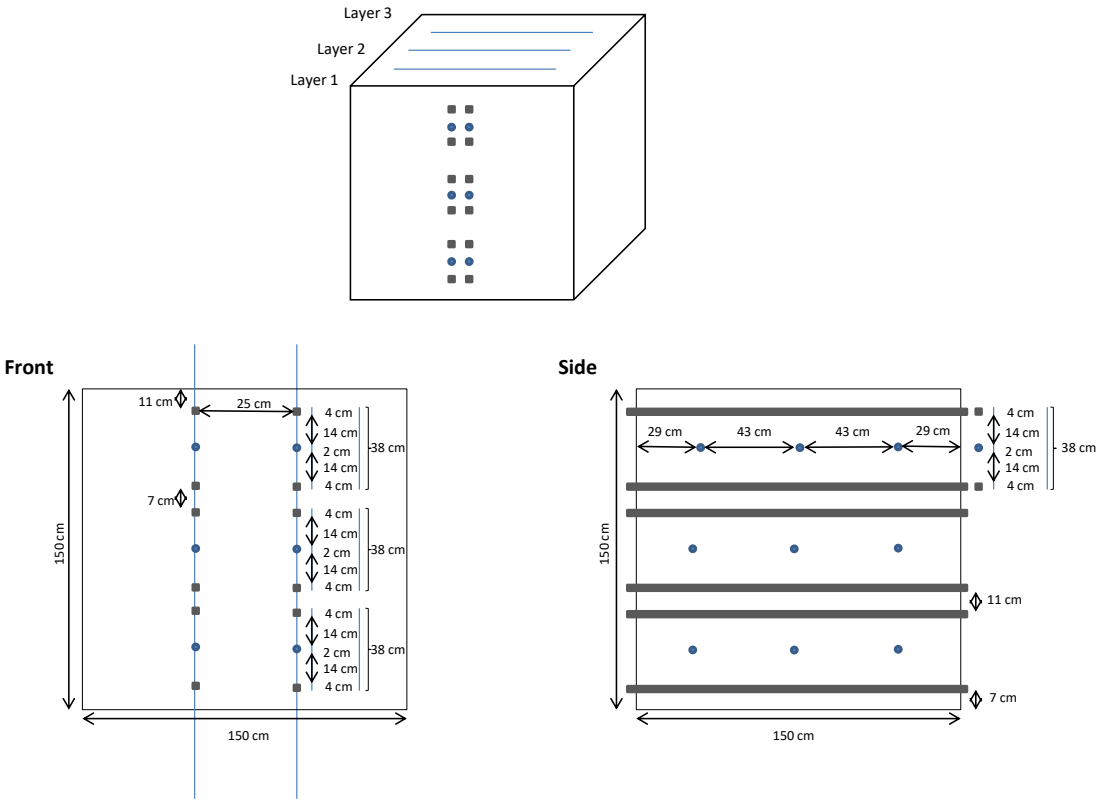

Figure 4.3 Schematic of sample tube and monitoring probe arrangement within 3 x 1.5 m^3 cages, dimensions are approximate values. Key: ■ sample tube, ● monitoring probe.

4.3.9 Microbial analysis

Over each quarter during the 12 month programme measurements of *E. coli* concentrations and dry solids were taken at monthly intervals from the approximate locations displayed in Figure 4.3. The first sample collection was completed on day 1 of storage and samples were taken from the top, middle and lower sections of each cage on a vertical transect and from the front, middle and back of each test cage horizontally (layers 1, 2 and 3). This gave a grid of 9 samples removed from the cages using the sampling tubes. Following day 1 the sub-sequential sampling campaigns were taken every 4 weeks representing 1 month of storage between each time point tested. Day 1 represented month 1, day 28 represented month 2 and day 56 represented month 3.

Approximate samples of 20 g were taken from the removed 2 m acrylic sampling tubes and placed into autoclaved, screw cap, glass universal tubes (FisherBrand, Loughborough, UK). The samples were transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2-8°C to suppress biological activity. Samples were processed on the day of sampling following the standard protocol for membrane filtration and dry solids determination.

Membrane filtration (Environment Agency 2003) was used for bacterial enumeration using Membrane Lactose Glucuronide Agar (MLGA) (OXOID, Basingstoke, UK) plates to distinguish *E. coli* from coliforms. From each sample, 3 g of material were removed and mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube. To ensure effective homogenization, samples were subjected to vortexing for 1 minute (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA.) prior to settling for a further 20 seconds. The supernatant was then removed for serial dilution. Samples were serial diluted with MRD to ensure CFUs were <80/plate. Samples were passed through 0.45 µm filters (Millipore S-PAK® 47 mm, Watford, UK) using a 3-way vacuum manifold (CombiSart®, Sartorius UK Ltd., Surry, UK). Filters were placed on MLGA plates and incubated at 30°C (± 1ºC) for 4 (± 1) hours and 37°C (± 0.5ºC) for 18 (± 2) hours as described by the Environment Agency (Environment Agency, 2003). Arising green colonies were counted as presumptive *E. coli*, yellow colonies were counted as coliforms and pink colonies were recorded as non-coliforms. All *E. coli* enumeration results were normalized against percentage dry solids (DS%). A 3 g sludge sample was used for DS determination and analysis was performed as per Environment Agency (2003) guidance.

4.3.10 pH monitoring

Measurements of pH were conducted on sludge cake samples removed from the acrylic sampling tubes and followed recommended soil pH analysis techniques (GEB, 2007), a 3 g sub-sample was removed from the 20 g bulk sample and mixed with approximately 15 mL de-ionised water in a universal tube. The solution was homogenised by vortexing (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA) for 1 minute followed by resting for 30 minutes to allow

stabilisation. A pH probe (FisherBrand Hydrus 300, Loughborough, UK) was used to record pH fluctuations.

4.3.11 Statistical analysis

The assumption of normality for each of the variables was visually assessed via histograms and normal probability plots using STATISTICA (Version 12, Tulsa, USA). Variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá, 2007). A repeated measures analysis of co-variance (ANCOVA) was performed to identify statistical significant differences ($p < 0.05$) between the conditions tested. In individual tests '*E. coli',* 'stockpile temperature', 'stockpile pH', 'stockpile DS%' and 'stockpile redox' were set as dependent variables. Experimental factors including 'quarter' and 'month' were classified as categorical and therefore the length of storage time was set as an independent variable. Factors set as co-variates for ANCOVA analysis included ambient temperature data (including monthly average, range and maximum), the total monthly rainfall and the measured stockpile parameters including temperature, pH, DS% and redox when not set as the dependent variable. The validity of the ANCOVA assumptions, such as normality of residuals, was tested through residual analysis (Gujarati 2011). A post-hoc analysis (Fisher LSD) was completed to assess the distinct differences between groups based on *E. coli* concentrations (Marques de Sá, 2007).

4.4 Results

E. coli concentrations (Figure 4.4) in study quarters 1 and 4 showed a gradual and significant reduction between the start and the end of each storage period. No significant difference was identified between the starting *E. coli* concentrations (average was 6.8 Log) in each of the 4 storage quarters. In quarters 1 and 4 a significant reduction of 0.8 Log and 4.5 Log was shown (respectively) between months 1 and 2 of storage. For quarters 1, 2 and 3 no significant change in *E. coli* concentration was identified between month 2 and the end of the storage quarter. Quarter 4 showed an enhanced level of die-off with *E. coli* concentrations significantly reducing from 6.5 to 2 Log over the 3 month storage period. Statistical analysis confirmed no effect of depth on the *E. coli* concentrations

measured from the top, middle and low sampling points in the tested stockpiles. Co-variate (ANCOVA) analysis showed *E. coli* values to be significantly affected by stockpile temperature (p= 0.017) (Figure 4.5).

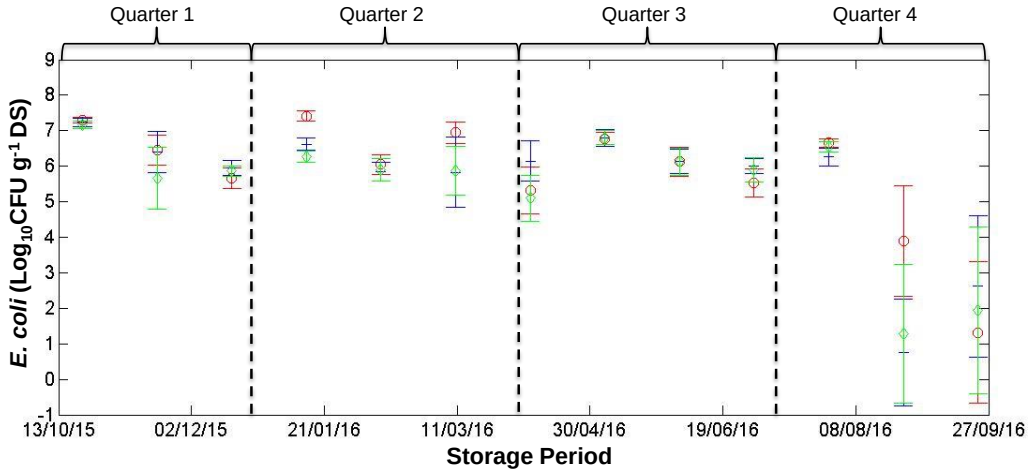

Figure 4.4 *E. coli* concentration data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: ○ samples taken from low cage depth, + samples taken from middle cage depth, ◊ samples taken from top of stored biosolids (~0.1 m below surface). Error bars represent the standard deviation of 3 repeats (n=3).

No significant change in stockpile temperature was identified with stockpile depth for each individual quarter. A significant reduction of 3.6°C (average) was displayed between quarters 1 and 2 followed by a significant stockpile temperature increase between quarters 2 and 3 of 6.6°C (average) (Figure 4.5). In addition, a significant increase of 7.5°C was measured between quarters 3 and 4. Across the months of storage in each quarter a significant decrease of 5.8°C was shown in quarter 1, no significant change in temperature was shown from the start to end of quarter 2. In quarter 3 a significant 6.2°C increase in stockpile temperature was displayed between months 1 and 4 and in quarter 4 no significant difference was shown between the months of storage. ANCOVA analysis showed stockpile temperature to be significantly affected by the monthly average ambient temperature (p= 0.028). For ambient temperature (Figure 4.5), variation was high between daily measurements. A notable increase in measurement variability occurred on 12/04/2016 and continued for the remaining

6 months of testing as a result of the weather station being moved to a higher elevation due to access restrictions. For the monthly average temperature data, a significant reduction was displayed between quarters 1 and 2 whilst a significant increase was shown between quarters 2 and 4. The average quarterly ambient temperatures observed were 10°C, 5.5°C, 12.3°C and 17.9°C for quarters 1, 2, 3 and 4 respectively. The average stockpile temperature for each quarter always remained above the average ambient temperature by a minimum of 0.4°C in quarter 1 and a maximum of 3.1°C in quarter 4.

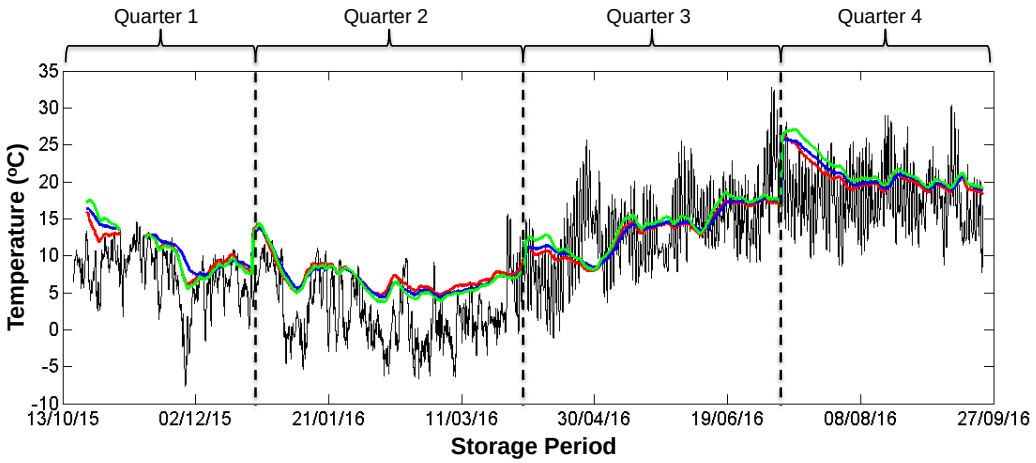

Figure 4.5 Temperature data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured lines show the average temperature of 3 repeats with 3 replicates per repeat. Cage temperature data was recorded at hourly intervals. Key: — data recorded from low cage depth, — data recorded from middle cage depth, — data recorded from top of stored biosolids (~0.25 m below surface). — Ambient temperature recorded on-site every 30 minutes.

Operational sludge treatment site cake pad temperature recordings taken in April 2016 and July 2016 are shown in Figure 4.6. Recordings on 28/04/2016 are comparable with data in Figure 4.5 and show the biosolids temperature in the storage bays on the operational treatment site to be approximately 2.8°C higher than the stockpile temperature in the monitoring study. The ambient temperature on 28/04/2016 was 6°C and below the average ambient temperature (12.3°C) recorded for that study period (quarter 3).

On the 18/07/2016 an average stockpile temperature of 17°C was recorded on the operational treatment site for stored cake approximately 6-8 weeks old (from centrifuge output). Although this time point was between quarters 3 and 4, and therefore stockpile temperature recording was not active in the storage trial, the final biosolids temperature at the end of quarter 3 was 17°C (30/06/2016) and therefore shows similarity in the on-site results. The average ambient temperature on the treatment site was 21.5°C and directly comparable with the temperature at the long term storage trial location which was 21.8°C.

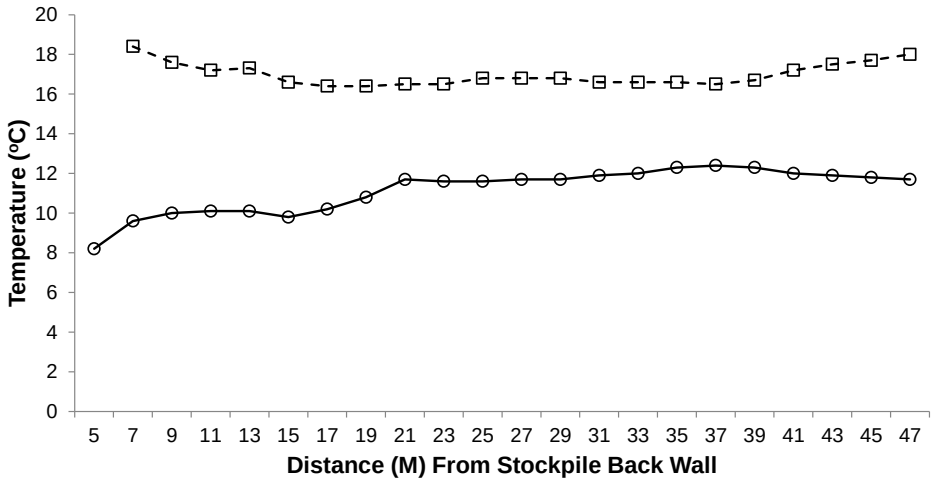

Figure 4.6 Temperature data recorded from sludge treatment site stockpiles on 28/04/2016 (—○—) and 18/07/2016 (– ⊟ –). Transect taken from back wall of biosolids storage bay. X-axis indicates distance from back wall along transect. Ambient temperature at site on 28/04/2016 was 6ºC and for 18/07/2016 ambient temperature was recorded as 21.5ºC (data from local weather station (IDERBY108 Weather Underground 2016)).

The pH showed no significant difference with depth in the stored stockpiles (Figure 4.7). No significant change between quarters 1, 2 and 3 was shown in the pH levels recorded. However, a significant increase in the biosolids pH was identified between quarters 3 and 4 with the average pH rising from 7.8 in quarter 3 to 8.1 in quarter 4. Over the months of storage in each quarter a significant change in pH value was observed for quarter 1 between months 2 and 3 with the pH rising by 0.4 units. No significant change in pH was identified between the storage months in quarter 2 however, quarter 3 showed a steady and significant rise in pH from 7.6 to 8.1 units. No significant change in pH was shown across

the storage period in quarter 4. ANCOVA analysis confirmed a relationship with pH and stockpile temperature significant to p= 0.049 and analysis of results showed higher stockpile temperature to coincide with a rise in pH level. In addition, a significant effect of DS% on pH was also identified (p= 0.01).

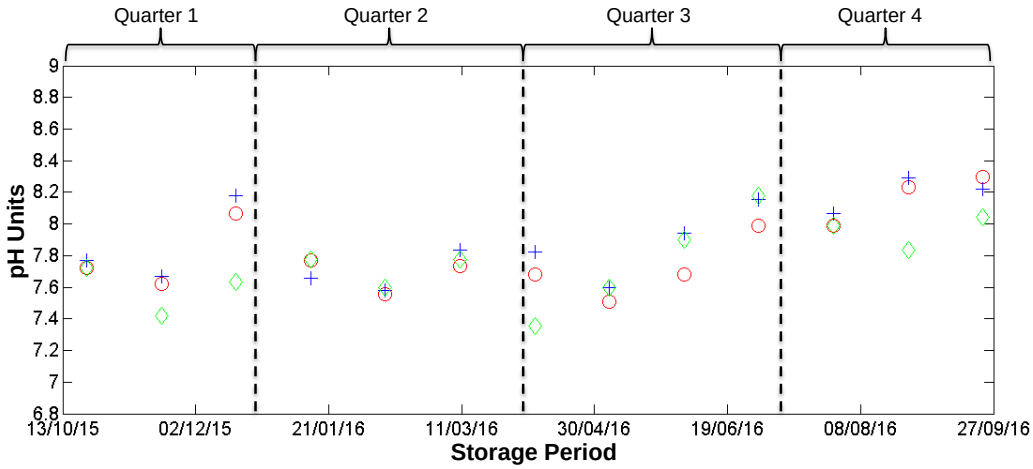

Figure 4.7 pH data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: ○ samples taken from low cage depth, + samples taken from middle cage depth, ◇ samples taken from top of stored biosolids (~0.1 m below surface).

A significant change in DS% was observed between each of the consecutive quarters studied (Figure 4.8). Within study quarters, a significant change between month 1 and month 3 was shown in quarter 1 with the average DS% decreasing by 1.3%. Quarter 2 showed a significant increase in DS% between months 1 and 3 of storage, whilst results from quarter 3 displayed a 2% decrease. In quarter 4 a significant increase in DS% was observed between month 1 and month 2 by 1.9% however, no significant difference was shown between month 2 and month 3 of storage. A significant difference was identified between the top, middle and lower sections of the stored biosolids material. The total monthly rainfall over each quarter significantly affected the DS% of the stored biosolids to a p value of 0.005. The total rainfall measured for each consecutive quarter was 235 mm, 224 mm, 343 mm and 176 mm respectively and analysis confirmed a significant difference between each of the 4 quarters measured. The peak rainfall events in quarter 3 coincide with the low DS% of 18.6% in month 3. The average annual

precipitation for the storage location area is 597.6 mm (1981-2010 records, Met Office 2010) therefore, the precipitation recorded between October 2015 and September 2016 can be considered above average.

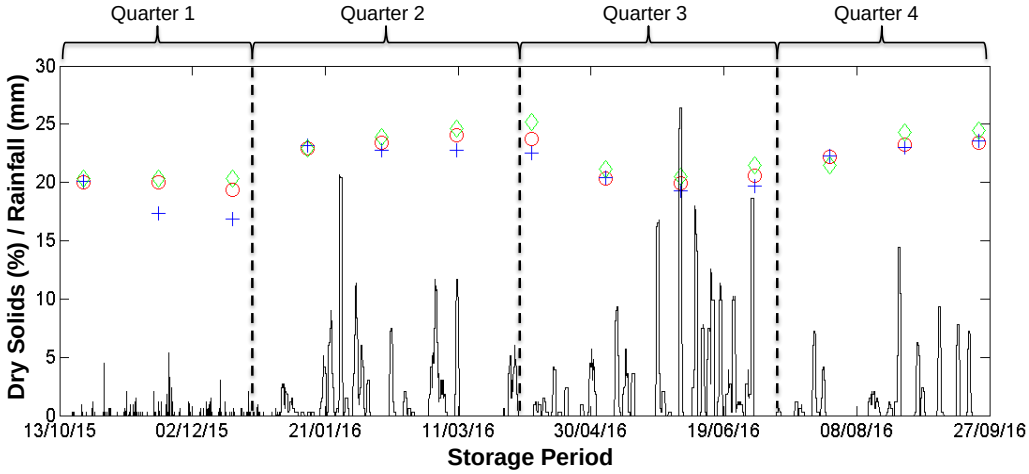

Figure 4.8 Dry solids (%) data and rainfall measurements recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 3 repeats with 3 replicates per repeat. Key: ○ samples taken from low cage depth, + samples taken from middle cage depth, ◊ samples taken from top of stored biosolids (~0.1 m below surface). — Rainfall measurements recorded on-site over 24 hours displayed.

Redox potential levels (Figure 4.9) demonstrated a high degree of temporal variability. Only redox data from probes in the top level of the stored biosolids are displayed for quarter 1 as equipment failure restricted full data collection. No significant difference was identified between redox potentials in the top, middle and lower sections of the stored stockpiles in quarter 1. Only in quarter 3 was a significant difference found between the middle and lower sections of the stored material with a reduced redox potential recorded in the middle of the stockpile; -362.8 mV (middle depth average) in comparison to -332.7 mV (low depth average). No significant difference was shown between quarters 1, 2 and 4 for redox measurements. However, a significant difference between quarters 1 and 3 was identified with the average redox level changing from -496.4 mV in quarter 1 to -551.5 mV in quarter 3. No significant difference was identified between the months of storage within each quarter for the redox data although in quarters 1 and 4 a distinct reduction in the first 48 hours of storage was displayed. The

average redox potential in the first 48 hours for quarter 1 reduced from -266 to -502.4 (quarter 1 average was -496.4 mV). Similarly in quarter 4 the redox potential after 1 hour of storage was -362.9 however, following 48 hours records showed a reduction to -563.5 mV, the average redox potential for quarter 4 was -630.9 mV. ANCOVA analysis confirmed no significant relationship between redox potential and the other variables measured over the study quarters.

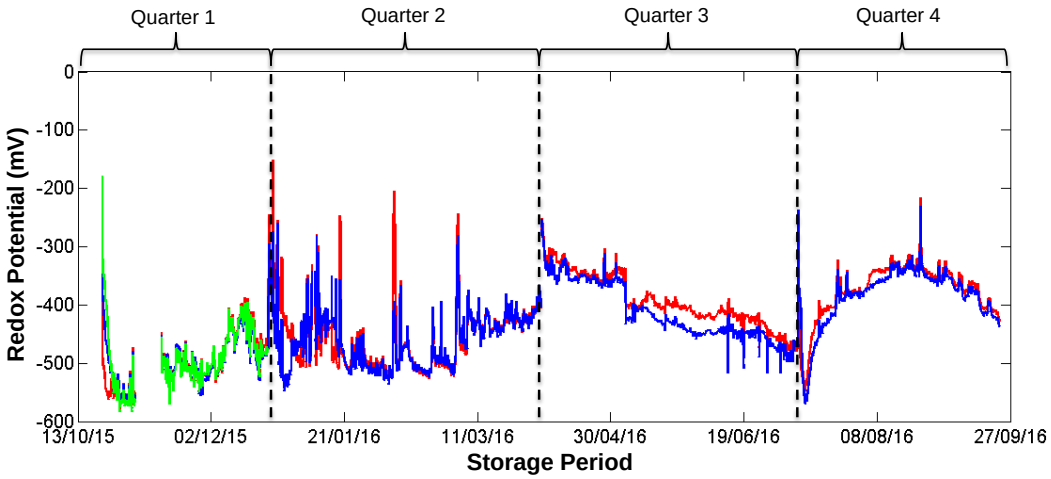

Figure 4.9 Redox potential data recorded during the long term environmental monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured lines show the average redox potential of 3 repeats with 3 replicates per repeat. Cage redox potential data was recorded at hourly intervals. Key: — data recorded from low cage depth, — data recorded from middle cage depth, — data recorded from top of stored biosolids (~0.25 m below surface). Please note redox probes measuring 'top' data were corrupted after first quarter and therefore 'top' data for quarters 2, 3 and 4 are not shown.

4.5 Discussion

4.5.1 *E. coli* dynamics during storage

E. coli concentrations in the fresh cake did not vary significantly between the 4 study quarters suggesting stability of on-site treatment processes throughout the 12 month monitoring programme. Across all 4 study quarters, *E. coli* showed a gradual reduction in concentration (Figure 4.4). Interestingly, the greatest change in *E. coli* concentrations was shown in the first 4 weeks of storage and statistical analysis confirmed significant reductions between months 1 and 2 in study quarters 1, 2 and 4. Lang *et al* (2007) found that 'time' had a highly significant

effect on *E. coli* numbers in agricultural soil. In addition Higgins *et al* (2007a) identified that continued storage of mesophilic cake decreased *E. coli* and faecal coliform concentrations substantially with results showing bacteria to reach a non-detectable level after 20 days of storage at 35°C. In contrast to results from Higgins *et al* (2007a) and Lang *et al* (2007), this long term study showed *E. coli* levels after 4 weeks of storage to plateau with no significant change in concentration after the first month. The only quarter to show a continued reduction in *E. coli* density was quarter 4. In this case, the *E. coli* concentration reduced by 4.5 Log between July and September 2016. Significant die-off in the first 4 weeks of storage was also observed by Holley *et al* (2006) who studied the length of *Salmonella* survival in manure-amended soils under different seasonal temperature sequences. The reduction in *Salmonella* concentrations in samples stored under the summer-winter regime was greater during the first month of storage (90% concentration reduction). In addition, prolonged *Salmonella* survival continued for >180 days after the first storage month and in our study levels of *E. coli* bacteria persisted at concentrations >5.2 Log over the following months of storage in quarters 1, 2 and 3. Rogers *et al* (2011) suggests that an explanation for a slower decay of organisms following a rapid reduction in culturable bacteria is that organisms enter a viable but non culturable (VBNC) state, resulting in a rapid decay until stressed cells alter their physiology. The rapid decline is then followed by a slower decay of more resilient organisms that remain culturable or as cells transition in and out of a VBNC state (Rogers *et al* 2011). Findings by Li *et al* (2014) suggest that a VBNC *E. coli* bacterium may be resuscitated in conditions including rich medium and a temperature upshift. Methods to enumerate the *E. coli* bacteria include plating on a nutrient rich agar and incubation for 18 hours at 37°C (Section 4.3.9) which could create suitable conditions for resuscitation. Therefore, the prolonged survival shown over the study quarters may be the transition of VBNC cells into a culturable state as a result of enumeration methods. Pinto *et al* (2011) observed more resuscitation of VBNC *E. coli* at 37°C than at 25°C indicating the importance of a temperature upshift as a stimulating factor to reverse the VBNC state. An alternative argument for the maintenance of culturability over the long storage period is the lower

temperatures *E. coli* bacterial cells were exposed to during the study. Pinto *et al* (2011) suggests that at lower temperatures the metabolism of cells is reduced allowing them to remain culturable for longer.

Research on *E. coli* dynamics in sewage sludge suggests that the *E. coli* indicator bacteria may enter a VBNC state during digestion that is reversed after centrifuge dewatering under favourable biosolids storage conditions (Higgins *et al* 2006, Higgins *et al* 2007b). Evidence for this was shown by a comparison of standard culturing methods and quantitative polymerase chain reaction (qPCR) which showed a three order of magnitude increase in *E. coli* per g DS from qPCR measurements after thermophilic digestion. This demonstrates that cells in a VBNC state were present in the post-digestion samples. Researchers attribute the observed increase in culturable bacteria often seen after centrifuge dewatering (to levels of 10^8 *E. coli* per g DS) to the reactivation and regrowth of VBNC bacteria (Higgins *et al* 2006, Higgins *et al* 2007b). Although no significant level of growth was observed in the present monitoring study, no comparisons were made with on-site *E. coli* concentrations before transportation to the storage site. It would have been beneficial to increase the frequency of *E. coli* measurements in the first 1-3 days of storage to understand whether increases in *E. coli* density, as commonly experienced on treatment sites, was occurring (Monteleone *et al* 2004, Higgins *et al* 2007a, Qi *et al* 2008, Chen *et al* 2011). Completing *E. coli* enumerations once a month limited any evaluation of short term fluctuations in bacterial density during storage.

4.5.2 Effects from temperature on storage environments

The enhanced level of die-off (4.5 Log reduction) observed in quarter 4 coincided with the highest stockpile temperatures recorded during the storage trials (peak stockpile temperature was 31°C) and outputs from ANCOVA testing confirmed that stockpile temperature was having a significant effect on *E. coli* concentrations ($p= 0.0017$). Storage temperatures increased in quarter 4 by 7.5°C from the average stockpile temperature recorded in quarter 3 (12.3°C). Under warmer conditions microbial metabolic activity and increased competition for nutrients is expected (Cools *et al* 2001, Jiang *et al* 2002, Holley *et al* 2006). In

environments such as sewage sludge cake, where substrates can be limiting, nutrient exhaustion can increase cell death rate (Holley *et al* 2006). The microbial activity per unit of biomass showed a positive correlation with concentrations of dissolved organic carbon (DOC) in a study measuring biotic and abiotic characteristics in manure-amended soils (Franz *et al* 2008). It may therefore be possible that nutrients, such as DOC, released as a result of dewatering operations (Chen *et al* 2011, Sun *et al* 2015) are degraded and become a limiting factor to microbial growth over time. Findings suggest the rate of nutrient degradation is increased under higher temperatures, such as those recorded in the summer months of storage.

It had been originally expected that *E. coli* bacteria survival may vary with depth in the biosolids as stockpile temperatures in the centre of the material may be greater. However, *E. coli* levels showed no significant change between the top, middle and lower sections of the stored biosolids. This coincided with no significant difference in stockpile temperature between the sampling depths. Due to the dimensions of the stockpiles tested in the storage trial (1.5 m^3) the mass of sludge cake monitored (8 tonnes) was significantly smaller than a typical treatment site storage bay (200-250 tonnes). Although a similar depth (1.5 m), the larger treatment site stockpile area may show differences across a horizontal transect as the stockpile width is greatly increased. The biosolids temperatures could therefore be influenced by the larger surface area of the dark organic material, lowering albedo and enhancing surface temperatures (Carson *et al* 2014). In the present study this effect is unlikely to have occurred, but a measurement of daily sunlight hours would have been an interesting co-variate to assess against the stockpile temperatures recorded. Sunlight has been shown to raise soil temperatures considerably above ambient levels with surface heating able to penetrate to a depth of 300 mm (Lake *et al* 2016). In order to verify and compare the stockpile temperatures recorded during the long term storage study with operational biosolids stockpiles, on-site cake pad temperature measurements were taken (Figure 4.6). Only a 2.8°C difference was measured between operational sludge treatment site stockpiles and the biosolids stored in the monitoring study (on 28/04/2016) suggesting that the tested storage

environment was not substantially different from operational sites. This conflicts with findings from Sprigings and Le (2011) who calculated 30°C as the representative temperature of stored biosolids. However, this value was identified after only 48 hours of biosolids temperature monitoring during which time the material may retain residual heat from up-stream digestion processes. Therefore, findings from the present study suggest that the representative temperature of stored biosolids is approximately 11°C if generalising over a 12 month period, with an average temperature of 8°C in winter and 15°C in summer.

Stockpile temperature changed substantially between each storage quarter and was shown to be significantly affected by the monthly average ambient temperature ($p= 0.028$). Interestingly the biosolids stockpile temperature always remained above the ambient temperature (Figure 4.5) suggesting a possible insulating effect of the biosolids material, preserving higher temperatures even when conditions in winter months were below freezing. Heat generation is affected by biological processes (Kanaan *et al* 2016) and materials containing organic matter such as biosolids will have increased microbial activity, boosting temperatures.

Stockpile temperature was shown to significantly affect the biosolids pH level recorded monthly over the storage period. pH increased significantly between quarters 3 and 4 (Figure 4.7) which coincided with a significant change in stockpile temperature. However, the pH change between quarters 3 and 4 was 0.3 pH units and therefore not considered sufficient to alter microbial dynamics in the biosolids material.

4.5.3 Influence of moisture content in stored biosolids

Stockpile DS% did not significantly affect *E. coli* concentrations across the 12 month study period. This result is in contrast to other studies (Plachá *et al*, 2001, Holley *et al* 2006, Lang *et al* 2007, Roberts *et al* 2016) and is likely to be due to the relatively stable DS% of the stored biosolids material in the present study which always remained within a range of 17-24% (Figure 4.8). Monthly rainfall was shown to significantly affect the DS% of the biosolids with peak rainfall

events coinciding with a reduced DS%. Findings suggest that storage practices may benefit from covered treatments if cake quality, such as stockpile integrity, is an important feature to preserve.

DS% and rainfall were expected to significantly influence the redox levels recorded over the 12 month study period, however no such effect was observed. This conflicts with previous studies which suggest that an increase in moisture content will lower redox potential and oxygen concentrations (Rubol *et al* 2012, Peralta *et al* 2014). Rubol *et al* (2012) argues that water content is directly linked to oxygen concentration and redox potential, which regulate microbial metabolism and chemical transformations in the environment. Findings from a long term experiment on a 1.5 m depth soil column showed increased soil moisture lowered the redox potential and oxygen concentrations of the soil (Rubol *et al* 2012). The redox potential levels measured in the current biosolids storage study were typically always below -300 mV and above -600 mV (Figure 4.9), which is within the redox range dominated by heterotrophic anaerobic bacteria, sulphate reducing bacteria and methanogenic bacteria (Sigma Aldrich 2016). The measurements of redox confirm an anoxic state of the biosolids stockpile and as no significant change in depth was shown (with exception of quarter 3) it can generally be accepted that the anoxic state of the biosolids material is homogeneous throughout. Similar to our findings is a study by Hall *et al* (2013) who identified a limited effect of moisture on redox potential when humid tropical forest soils where exposed to extended periods of elevated moisture. No influence on temporal patterns in redox levels were shown and researchers suggest that the clay rich soils under study had a high affinity for moisture and a well-developed aggregate structure which enabled anaerobic conditions to persist. Likewise, the sludge matrix appears able to withstand the variable precipitation events without substantial change to the DS% or redox levels. It therefore seems likely that the consistency of conditions in the sludge matrix could support microbial population stability and provide a buffer to fluctuations in environmental conditions, such as pH, which showed little variation throughout the study.

One significant change observed in the redox levels was the steady decline over the first 48 hours of stockpiling where levels reduced by approximately -218 mV. This phenomenon is hinted at by Chen *et al* (2011) who suggested that the shearing effects from mechanical dewatering possibly introduce oxygen to the sludge matrix. Over time this increase is reduced as oxygen is depleted during storage for microbial respiration (Chen *et al* 2011). An additional explanation for the reduction in redox potential is stockpile settling over the first 48 hours of storage where oxygen diffusion through the stockpile will be diminished as sludge pores become smaller and fissures across the stockpiled matrix close during the settling process.

Redox measurements were limited by practical restrictions in the experimental set up, notably maintaining electrical isolation with the power supply potential which possibly influenced redox signals in connections for probes in the top layer of the stored biosolids. This effect was most likely due to moisture on the connections reducing the isolation resistance possibly as a result of condensation (Paleo Terra communication 2016). Every time the datalogger enclosure is opened moisture will enter with the fresh air. Although silica beads were placed inside the datalogger cabinet a small heating unit or increased ventilation may have reduced the variability recorded in the sensors which ultimately prevented the use of redox potential data gathered from the top section of the cages. The stability of the middle and lower probes may have been because these were wired to a different multiplexer or an alternative channel where the effect of moisture on the wiring was reduced.

This study has successfully profiled the biosolids storage environment and confirmed the first hypothesis, that temperature is a significant factor controlling *E. coli* concentrations. Within the ranges measured in this trial; pH, DS%, and redox potential conditions did not have a significant effect on *E. coli* dynamics and therefore the other hypotheses in this study cannot be accepted.

4.6 Conclusions

1. *E. coli* concentrations in stored biosolids are significantly affected by stockpile temperature. Higher stockpile temperatures during summer

months led to greater cell die-off in comparison to cooler winter stockpile temperatures.

2. Ambient temperature (monthly average) is a parameter that has significant influence on stockpile temperature and ultimately indicator bacterial survival.
3. Biosolids stockpiles showed an insulating effect able to maintain a minimum core temperature of 4°C when ambient temperatures fell below freezing (-4°C).
4. The greatest reduction in *E. coli* concentrations was exhibited in the first 4 weeks of biosolids storage. Therefore efforts to reduce bacteria levels should target this period of increased die-off to enhance mechanisms, such as nutrient exhaustion, which cause cell death in order to achieve compliance limits.
5. The significant reduction in redox potential over the first 48 hours of storage after stockpiling indicates that increased oxygen availability does occur in the sludge matrix as a result of mechanical dewatering processes. However this increase is rapidly depleted over time as redox potential measurements became more negative.
6. Results on sludge DS%, pH and rainfall showed no significant effect on *E. coli* concentrations in the storage conditions tested. However, rainfall was shown to significantly impact upon sludge DS%. Therefore, if DS% and stockpile integrity are important qualities to preserve, storage areas may benefit from covered treatments.

4.7 Acknowledgements

The authors would like to thank the Natural Environment Research Council (NERC) and Severn Trent Water Plc. for their financial support. The technical advice given by Paleo Terra during the experimental set up and the support throughout the 12 month monitoring programme is also acknowledged.

4.8 References

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Banach, A. M., Banach, K., Visser, E. J. W., Stepniewska, Z., Smits, A. J. M., Roelofs, J. G. M., Lamers, L. P. M. (2009) Effects of summer flooding on floodplain biogeochemistry in Poland; implications for increased flooding frequency. *Biogeochemistry* Vol. 92, pp. 247- 262

Carson, A.W., Rutherford, M.P., Burton, P.J. (2014) Desulpjurised tailings serve as a useful soil suppliment for mine reclamation *Canadian Journal of Soil Science* Vol. 94 pp.529- 541

Chen, Y., Higgins, M. J, Beightol, S. M, Araujo, G. G., Murthy, S. N., Barben, E. J., Toffey, W. E. (2007) Odour Generation and Pathogen Indicator Regrowth after Dewatering: Are They Related? In: Proceedings of the Mid-Atlantic Biosolids Assocation Conference

Chen, Y. C., Higgins, M. J., Beightol, S. M., Murthy, S. N., & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research,* Vol. *45*, pp. 2616- 2626.

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., (2001) Survival of *E. coli* and *Enterococcus spp*. derived from pig slurry in soils of different texture. *Appl. Soil Ecol.* Vol. 17, pp. 53–62.

DEFRA (2006) Code of Practice for Agricultural Use of Sewage Sludge sludge http://adlib.everysite.co.uk/adlib/defra/content.aspx?id=000IL3890W.16NTC17O1X82UF [accessed on 31/08/2016]

Dentel, S. K., Qi, Y., & Herson, D. S. (2008). Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration, and assessment. *Water Science and Technology,* Vol. 57 No. 2.

Environment Agency, (2003) The Microbiology of Sewage Sludge- Part 3- Methods for the isolation and enumeration of *Escherichia coli*, including *verocytoxigenic Escherichia coli*. Bristol.

Franz, E., Semenov, A.V., Termorshulzen, A.J., de Vos, O.J., Bokhorst, J.G., van Bruggen, H.C. (2008) Manure-amended soil characteristics affecting the survival of *E. coli 0157:H7* in 36 Dutch soils *Environmental Microbiology* Vol. 10 No. 2 pp. 313- 327

Geotechnical Engineering Bureau (GEB), 2007. Geotechnical Engineering Bureau: Test method for determination of pH value of water or soil.

Gujarati, D. (2011) 'Econometrics by Example' PALGRAVE MACMILLAN, Hampshire, UK

Hall, S. J., McDowell, W. H., Silver, W. L. (2013) When wet gets wetter: Decoupling of moisture, redox biogeochemistry, and greenhouse gas fluxes in a humid tropical forest soil *Ecosystems* Vol 16 pp. 576- 589

Higgins, M. J., Chen, Y. C., Murthy, S. N., & Hendrickson, D. (2007a). Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering. *Procedings on moving forward wastewater biosolids sustainability: Technical, managerial and public synergy*, pp. 204-19.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Henderickson, D., Farrel, J., & Schafer, P. (2007b). Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Research*, Vol. 41 pp. 665-673.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Hendrickson, D., Schafer, P., & Farrell, J. (2006). WERF Phase 2: The Impact of Digestion and Dewatering on Reactivation and Regrowth of Viable but Non-Culturable Indicator Bacteria. WEFTEC.

Holley, R.A., Arrus, K.M., Ominski, K.H., Tenuta, M., Blamk, G. (2006) *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure *Journal of Environmental Quality* Vol 35 pp. 1170- 1180

Iranpour, R., Palacios, R., Cox, H.H.J., Abkain, V. (2005) Solving fecal coliform growth/ reactivation in biosolids during full-scale post-digestion processes *Water Science and Technology* Vol 52, No. 1-2, pp. 283- 288

Jiang, X., Morgan, J. and Doyle, M. P. (2002) Fate of *Escherichia coli* 0157:H7 in manure amended soil *Applied Environmental Microbiology* Vol. 68, pp.2605-2609

Kanaan, H., Minz, D., Medina, S., Krasnovsky, A., Raviv, M. (2016) The independent effects of solar disinfection and compost maturity level on soil microbial activity *Phytoparasitica* Vol. 44 pp. 55- 64

Keddy, P. A. (2000) Wetland Ecology: Principles and Conservation. Cambridge University Press, Cambridge

Lake, J.A., Johnson, I., Cameron, D. D. (2016) Carbon capture and storage (CCS) pipeline operating temperature effects in UK soils: The first empirical data *International Journal of Greenhouse Gas Control* Vol. 53 pp. 11-17

Lang, N.L. and Smith, S.R., (2008) Time and temperature inactivation kinetics of enteric bacteria relevant to sewage sludge treatment processes for agricultural use. *Water Res*. Vol. 42, pp. 2229–2241.

Lang, N. L., Bellett-Travers, M. D. and Smith, S. R. (2007) Field Investigations on the survival of *Escherichia coli* and presence of other enteric micro-organisms in biosolids-amended agricultural soil *Journal of Applied Microbiology* Vol. 103, pp. 1868- 1882

Li, L., Mendis, N., Trigui, H., Oliver, J. D., Faucher, S. P. (2014) The importance of the viable but non-culturable state in human bacterial pathogens *Frontiers in MICROBIOLOGY* Vol. 5 Article. 258 pp. 1-20

Marques de Sá, J.P., 2007. Applied Statistics using SPSS, STATISTICA, MATLAB and R. Springer- Verlag, Berlin.

Met Office (2010) Bedford Climate http://www.metoffice.gov.uk/public/weather/climate/gcr8td8jb [accessed on 11/10/2016]

Mitsch, W. J. and Gosselink, J. G. (2007) Wetlands (4th Ed.) John Wiley and Sons. Hoboken

Monteleone, M. C., Furness, D., Jefferson, B., & Cartmell, E. (2004). Fate of *E. coli* Across Mechanical Dewatering Processes. *Environmental Technology*, Vol. 25 No. 7 pp. 825-831.

Nicholson, F. A., Groves, S. J., Chambers, B. J. (2005) Pathogen survival during livestock manure storage and following land application *Bioresource Technology* Vol. 96, pp. 135- 143

Peralta, A. L., Ludmer, S., Matthews, J. W., Kent, A. D. (2014) Bacterial community response to changes in soil redox potential along a moisture gradient in restored wetlands *Ecological Engineering* Vol. 73 pp. 246- 253

Pinto, D., Almeida, V., Almeida Santos, M., Chambel, L. (2011) Resusitation of *Escherichia coli* VBNC cells depends on a variety of environmental or chemical stimuli *Journal of Applied Microbiology* Vol. 110 pp. 1601- 1611

Plachá, I., Venglousky, J., Sasokova, N., Svoboda, I.F., 2001. The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solids of pig slurry. *J. Appl. Microbiol*. Vol. 91, pp. 1036–1043.

Qi, Y., Dentel, S. K., Herson, D. S. (2008) Effect of total solids on fecal coliform regrowth in anaerobically digested biosolids *Water Research* Vol. 42 pp. 3817- 3825

Qi, Y., Dentel, S. K., Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. 41, pp. 571- 580.

Roberts, B.N., Bailey, R.H., McLaughlin, M.R., Brooks, J.P. (2016) Decay rates of zoonotic pathogens and viral surrogates in soils amended with biosolids and manures and comparison of qPCR and culture derived rates *Science of the Total Environment* Vol. 573 pp. 671- 679

Rogers, S.W., Donnelly, M., Peed, L., Kelty, C.A., Mondal, S., Zhong, Z., Shanks, O.C. (2011) Decay of bacterial pathogens, fecal indicators and real-time quantitative PCR genetic markers in manure-amended soils *Applied and Environmental Microbiology* Vol. 77 No. 14 pp. 4839- 4848

Rubol, S., Silver, W.L., Bellin, A. (2012) Hydrologic control on redox and nitrogen dynamics in a peatland soil *Science of the Total Environment* Vol. 37 pp. 37- 46

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M., (2007) Influence of temperature fluctuations on *Escherichia coli O157:H7* and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol.* Vol. 60, pp. 419–428.

Sigma Aldrich (2016) Microbology Introduction http://www.sigmaaldrich.com/technical-documents/articles/microbiology/microbiology-introduction.html [Accessed on 05/09/2016]

Sprigings, A. E., & Le, M. S. (2011). Growth Potential- A New Method For Estimation of the Risk of *E. coli* Regrowth in Digested Sludge Cake. *16th European Biosolids and Organic Resources Conference.*

Sun, F., Hu, W., Pei, H., Li, X., Xu, X., Ma, C. (2015) Evaluation on the dewatering process of cyanobacteria-containing $AICI_3$ and PACI drinking water sludge. *Separation and Purification Technology* Vol. 150 pp. 52- 62

Vorenhout M., van der Geest H.G., and Hunting E. R. (2011) ‘An Improved Datalogger and Novel Probes for Continuous Redox Measurements in Wetlands’ *International Journal of Environmental Analytical Chemistry* Vol. 91:7-8, pp. 801-810

Weather Underground (2016) Spondon, Derby (IDERBY108) https://www.wunderground.com/personal-weather-station/dashboard?ID=IDERBY108 [accessed on 28/04/2016]

Chapter 5

E. coli Death Rates in a Summer and Winter Pilot Trial Testing Modified Biosolids Storage Environments

Statement of author contributions:

S. Fane: Principal investigator, completion of experimental design and laboratory work, data analysis and chapter write up

Dr. A. Nocker: Provided supervisory support during experimental design

A. Wilson: Industrial supervisor for Severn Trent Water Plc.

Dr. M. Rivas Casado: Statistical consultant

Professor E. Cartmell: Developed overall concept of work with Severn Trent Water Plc.

Professor S. Tyrrel: Principal supervisor supporting research concept development, guidance on experimental design, data analysis and evaluation of final chapter write up

5 *E. coli* Death Rates in a Summer and Winter Pilot Trial Testing Modified Biosolids Storage Environments

S. Fane[1], A. Nocker[2], A. Wilson[3], M. Rivas Casado[1], E. Cartmell[4], S. Tyrrel[1]

1 Water Science Institute, School of Water, Energy and Environment, Cranfield University, UK

2 IWW Rheinisch-Westfälisches Institut für Wasserforschung gemeinnützige GmbH, Germany

3 Severn Trent Water, UK

4 Scottish Water, Dunfermline, Scotland

5.1 Abstract

Current controls on biosolids storage environments focus upon surface and ground water contamination (Environment Agency 2012) and provide limited guidelines for the best practices to support bacterial die-off. Stringent criteria (ADAS 2001) limiting the risk of pathogen transfer govern the biosolids to land application route. In contrast to up-stream sludge treatment processes, which are highly controlled and monitored, biosolids storage post-dewatering appears to be overlooked. This study investigated modifications to the biosolids storage environment for the control of physical environmental parameters such as temperature, oxygen availability and biosolids moisture content in a 2-phase pilot study. The trials aimed to increase the *Escherichia coli* (*E. coli)* death rate in response to physical parameter changes. Weekly measurements were made of stockpile conditions, ambient weather and *E. coli* concentrations over summer and winter pilot trials. *E. coli* die-off rates were enhanced during the summer trial. A significant reduction in *E. coli* concentration was observed (3.7 Log) under covered treatments able to maintain stockpile temperatures >25°C. Pilot trial treatments, including sealed-bag conditions, which aimed to reduce oxygen diffusion into stockpiles showed no significant effect on measured redox levels in comparison to control conditions. Redox level and moisture content in the biosolids remained relatively stable for the duration of storage. *E. coli* suppression in cooler winter months was limited and high concentrations (>6 Log) persisted for the trial duration. Temperature is a critical environmental parameter controlling

E. coli death rates. Covering sludge stockpiles in summer months could contribute to the consistent achievement of compliance targets but is unlikely to be a successful standalone strategy in colder months.

5.2 Introduction

Sludge may be disposed on landfill or incinerated, however the most practicable environmental option recommended by EU and UK governments is agricultural land application (Water UK 2010). To ensure that a safe product is applied to agricultural land sludge treatment processes must be validated for treatment efficacy (European Union 2000). The Code of Practice for the agricultural use of sewage sludge (DEFRA 2006) stipulates that sludge, subject to primary mesophilic anaerobic digestion, must be stored for a minimum period of 14 days. The primary purpose of this final treatment is to significantly reduce the potential health hazard posed by the risk of pathogens in sewage sludge and provide a final barrier preventing the transmission of wastewater contaminants to agricultural land (DEFRA 2006). In Europe and the USA biosolids storage is employed with microbial compliance monitoring to verify effective treatment and removal of indicator bacteria such as faecal coliforms, *Salmonella* and *Escherichia coli* (*E. coli*). In the UK, the Safe Sludge Matrix (SSM) agreement (ADAS 2001) issues guidelines for the application of biosolids to agricultural land. The Matrix, developed by ADAS, Water UK (representing 14 UK Water and Sewage Operators) and the British Retail Consortium (BRC) consists of a table of crop types with clear direction on the minimum acceptable level of treatment of any sewage sludge (biosolids) based product which may be applied to that crop (ADAS 2001). The agreement stipulates two levels of treatment. Conventional treatments will have achieved a 2 Log reduction in levels of *E. coli* bacteria between the digester inlet and the final biosolids product. An enhanced product will have achieved a 6 Log reduction in levels of *E. coli* indicator bacteria and be free of *Salmonella* (ADAS 2001). The agreement allows the beneficial properties of sewage sludge to continue to be used as a valuable source of nutrients and organic matter whilst ensuring the highest possible standards of food safety.

Although regulations exist for the final outputs of biosolids storage, limited regulation or control of the dewatered sewage sludge storage environment exists. Controls on the storage environment focus upon preventing surface and ground water contamination (Environment Agency 2012) with no advisory comments on the best practice to support bacterial die-off. A typical sludge cake bay holds between 200-250 tonnes of sewage sludge and is constructed with a concrete base where run off is channelled into surface drains. Concrete side walls segregate individual bays and no top cover is present. When evaluated in the context of up-stream sludge treatment processes such as anaerobic digestion where environmental parameters are highly monitored and controlled, the tertiary storage treatment of the biosolids material appears to be overlooked.

Indicator bacteria resurgence is frequently observed in biosolids storage which may indicate the presence of pathogens and therefore a risk of non-compliance (Monteleone *et al* 2004, Higgins *et al* 2007a, Dentel *et al* 2008, Qi *et al* 2008, Chen *et al* 2011, Sprigings and Le 2011). Examples of indicator concentration change range from -0.4 Log to +6.4 Log units after centrifuge dewatering (Qi *et al* 2007). The unpredictable indicator bacterial behaviour in storage causes much concern for utility operators and limits the quality and value of sludge products. Sprigings and Le (2011) demonstrated that *E. coli* regrowth in both enhanced and conventionally treated biosolids follows the typical microbial growth curve after centrifuge dewatering. The immediate elevation in indicator numbers post-dewatering and the prolonged survival during subsequent biosolids storage, suggests characteristics of the storage environment may be controlling factors of indicator growth (Higgins *et al* 2007a). Presently the key factors responsible for indicator growth and survival in stored biosolids and their relative importance have not been clearly identified (Dentel *et al* 2008).

Demonstrated in Section 3.2, an evaluation of the sludge treatment process can provide indication of the relevant environmental parameters controlling *E. coli* dynamics. Mesophilic anaerobic digestion (MAD) research on indicator bacteria concentrations has identified dominant environmental factors that may contribute to *E. coli* die-off. Temperature and oxygen availability are two key elements tightly

controlled in AD conditions. Temperature was identified by Scaglia *et al* (2014) as a critical parameter controlling cell death rate and determining the biochemical conditions of AD. Trials completed by Le *et al* (2002), Smith *et al* (2005) and Rosenblum *et al* (2014) demonstrated temperature as a factor regulating pathogen deactivation through influences on microbial competition and substrate limitation. Coupled with temperature, the anaerobic environment in digestion will have a substantial effect on cell respiration and consequential energy production, regulating cell activity (Alberts *et al* 2010). Although *E. coli* bacteria are facultative anaerobes, and therefore resilient to oxygen deprivation (Hutchison *et al* 2000, Henkel *et al* 2014), the combination of an oxygen limiting environment and other pernicious reactor conditions will have a controlling effect on *E. coli* concentrations.

Following AD, the changes in environmental parameters acting upon the sludge matrix during and after mechanical dewatering may contribute to the observed increase in bacterial concentrations (Higgins *et al* 2007b, Dentel *et al* 2008). Identifying the influential environmental factors will support the implementation of modified storage practices able to reduce indicator bacteria concentrations back to post-digestion levels, and meet compliance requirements.

The principal action of mechanical dewatering is to decrease the sludge water content, limiting capital, transportation and operational expenditure (Mowla *et al* 2013). Controlling sludge water content has operational benefits but may also be a factor controlling bacterial survival (Berry and Miller 2005, Zaleski *et al* 2005a, Rouch *et al* 2011). In the study conducted by Sprigings and Le (2011) researchers noted a possible effect of moisture retaining heat in the biosolids matrix. A comparison of sites A and B showed differences in the DS% of the dewatered sludge cake (Site A: 25% DS; Site B: 31% DS) which corresponded with site A maintaining a higher sludge cake temperature. A lower DS% will help the fresh cake retain heat from the AD process for a longer period, delaying cake cooling to ambient temperatures (Sprigings and Le 2011). Cooler temperatures have been shown to support prolonged cell survival (Plachá *et al* 2001, Cools *et al* 2001, Semenov *et al* 2007) suggesting temperature is a controlling factor of

bacterial behaviour. Bacterial survival is possible across a range of dry solids conditions (Jiang *et al* 2002, Zaleski *et al* 2005b, Lang and Smith 2007, Rouch *et al* 2011).

A common variable between AD and dewatering processes is the modification of atmosphere conditions and the influence this may have on bacterial growth and survival. In mechanical dewatering opposing conditions to that of AD are likely as municipal wastewater treatment sites often use continuous flow decanter centrifuges which contain an internal scroll conveyor that can amplified oxygen exposure (Chen *et al* 2011). In addition, centrifuge dewatering produces smaller flocs with an overall larger surface area (Chen *et al* 2007) which is expected to increase the availability of oxygen to bacteria cells and provide a selective advantage for the facultative *E. coli* bacteria.

Evidence suggests the control of temperature and oxygen availability in AD contributes to bacterial die-off. In mechanical dewatering, the increased oxygen availability may be a factor influencing *E. coli* growth in biosolids storage. It is therefore plausible to assume that the control of temperature and oxygen availability in the biosolids storage environment may have a beneficial effect on *E. coli* behaviour, enabling treatment operators to consistently achieve stipulated compliance levels.

The effects of temperature modifications in biosolids storage have been demonstrated by Iranpour *et al* (2005) who monitored temperature in the post-digestion train during pilot tests on a battery of 6 thermophilic digesters. Tests showed a large drop in the biosolids temperature between the dewatering centrifuges (48.2°C) and storage silos (41°C). The reoccurrence of faecal coliforms in post-digestion biosolids was attributed to this temperature reduction and researchers suggested that maintaining a minimum temperature of 50°C (representative of the up-stream thermophilic digestion process) may prevent growth of faecal coliforms. Trials with insulated silos and electrical heat-tracing confirmed that faecal coliform levels could be controlled by preventing cooling of stored biosolids (Iranpour *et al* 2005). Faecal coliform regrowth tests after silo incubation showed undetectable bacterial levels, highlighting the importance of

maintaining a higher storage temperature for the prevention of coliform growth (Iranpour *et al* 2005).

The effects of a modified atmosphere negatively impacting bacterial growth can be studied in food and agricultural industries with modified atmospheric packaging (MAP) and ensiling practices. In silage production the fermentation of biodegradable carbon reduces pH levels in the encapsulated silage, inhibiting bacterial growth, providing anaerobic conditions are maintained (Herrmann *et al* 2011). Similarly in food preservation, controlled atmospheres such as MAP or the practice of active atmosphere modification with vacuum treatment reduces oxidative chemical reactions and aerobic growth (Floros and Matsos 2005). Researchers attribute the absence of O_2 in vacuumed packaging and the partial dilution of O_2 in modified atmosphere treatments to reduced microorganism growth and increased microbial stability (Garrido *et al* 2016). Decreased oxygen exposure appears to be a significant factor diminishing the growth of bacteria and therefore similar treatments may enhance *E. coli* indicator die-off rates when applied to biosolids storage environments.

This research aims to investigate the effects of modified storage environments on *E. coli* bacteria die-off rates in response to physical parameter change at an operational scale. The hypotheses tested in this research are that:

1. *E. coli* die-off will be increased under manipulated storage environments where stockpile temperature is elevated.
2. *E. coli* die-off will increase in conditions where exposure to oxygen is reduced.

5.3 Methodology

5.3.1 Sludge sample source

Representative bulk samples of sludge cake, which had been both mesophilic digested and centrifuge dewatered were collected from a wastewater treatment works serving a population equivalent (PE) of 440,000. The samples were transported to the pilot trial site in 25 tonne capacity haulage trucks. A total of 240 tonnes and 300 tonnes were transported to the pilot trial site for the summer and

winter trials respectively. The cake was taken immediately after centrifuge dewatering to ensure the product was less than 24 hours old when reaching site, this allowed storage environments to begin from a day 1 assumption.

5.3.2 Pilot trial location

Pilot trials were conducted on a Severn Trent Water Plc. farm site located in the North West Midlands, England. The pilot trial test site was a flat field chosen because of its open surroundings, field accessibility for heavy plant equipment and free draining soil which would reduce the impact of water clogging on the tested storage treatments.

5.3.3 Summer trial set up

For the summer trial, sewage sludge cake was placed in 20 tonne stockpiles under four storage conditions intended to test the effect of different stockpile covers, bags and storage environments on the temperature, moisture content, redox conditions and *E. coli* concentrations of the biosolids. These included an open control (typical of storage practices on operational treatment sites), naturally ventilated barn storage, a covered treatment using a membrane designed to keep rain off for improved stockpile integrity (Airbeam Roller Stockpile Cover, Tim Evans Environment, Surrey, UK), and a sealed-bag technology (Ag-Bag, AB Systems, Devon, UK) used to reduce rainfall effects and air exposure to stockpiles (Figures 5.1 and 5.2). The stockpiles for the control, barn and covered treatments were formed with a front-bucket loading vehicle. Straw bales were used to segregate stockpiles in the barn treatment and for the covered condition the manufacturer's instructions were used to deploy the membrane and cover the prepared stockpiles. For the sealed-bag technology, sewage sludge cake was tipped (using a front-bucket loading vehicle) into a trailer containing a hydraulic ram which forced the material into cylindrical end-sealed plastic bags. The trial ran during June and July 2014. Due to practical restrictions, the sealed-bag treatment began 28 days after the other treatments and ran during July and August 2014. All treatments were performed in triplicate and ran for 28 days.

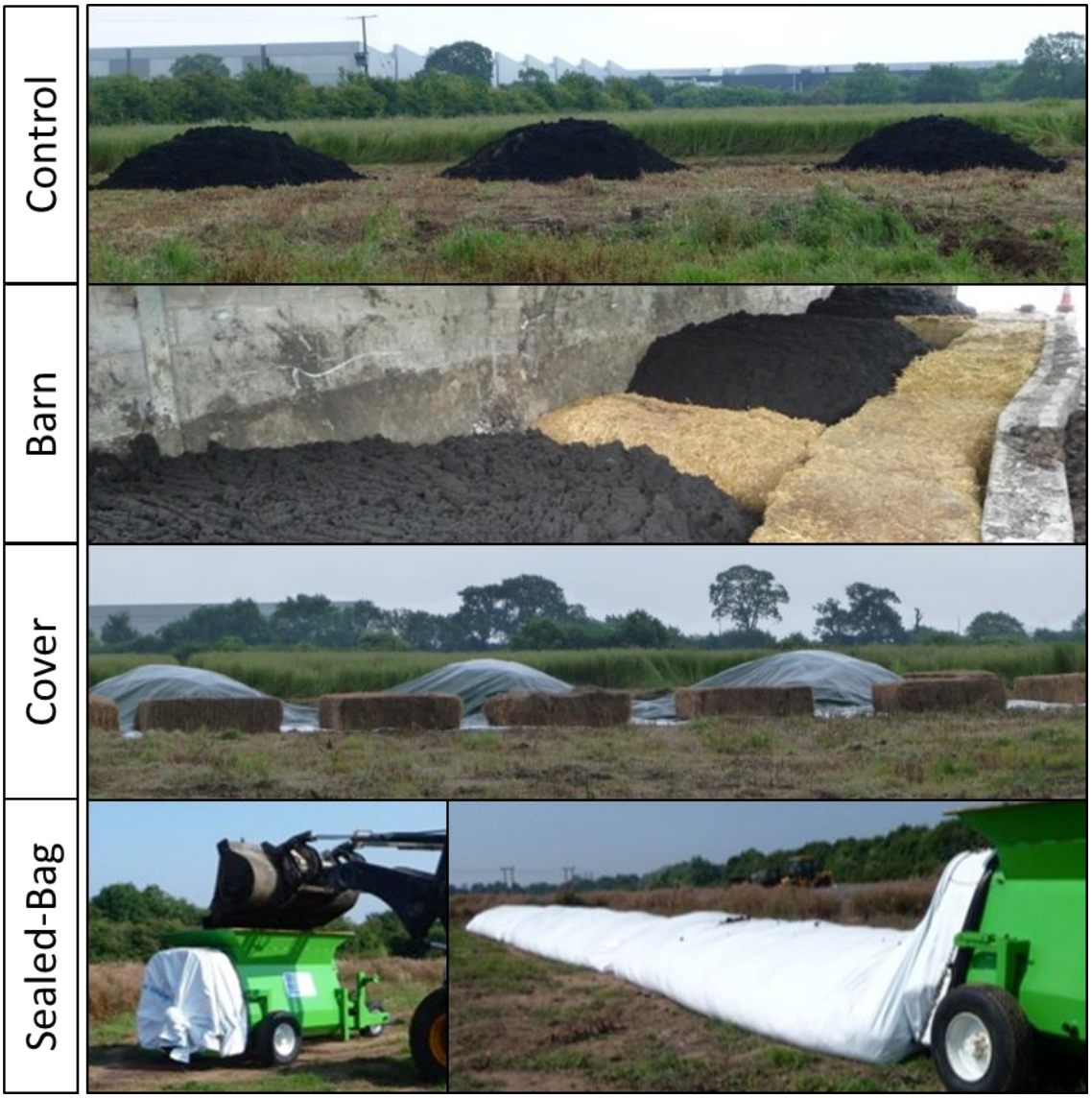

Figure 5.1 Photographs from summer pilot trial 2014. Treatment condition specified in text boxes on left side of photographs.

5.3.4 Winter trial set up

Maintaining consistency with the summer pilot trial, 20 tonne stockpiles of mesophilic digested and centrifuged sludge cake were place under five storage conditions. These included an open control, a covered condition using an impermeable membrane and a sealed-bag technology (replicating treatments tested in the summer pilot trial). Two additional variations of the membrane cover and the sealed-bag technology were included with the aim of achieving two insulated storage conditions. Sewage sludge cake was forced into long 1.5 m x

13 m and 1.5 m x 6 m end-sealed plastic cylinder bags using a hydraulic ram (same protocol as for the sealed-bag treatment tested) and placed around a second membrane covered treatment and sealed-bag condition. Both insulated treatments were protected with 2 additional membrane covers over the top of stockpiles with the aim of providing an insulating layer to the stored sludge. The trial conditions ran for 10 weeks during January and March 2016.

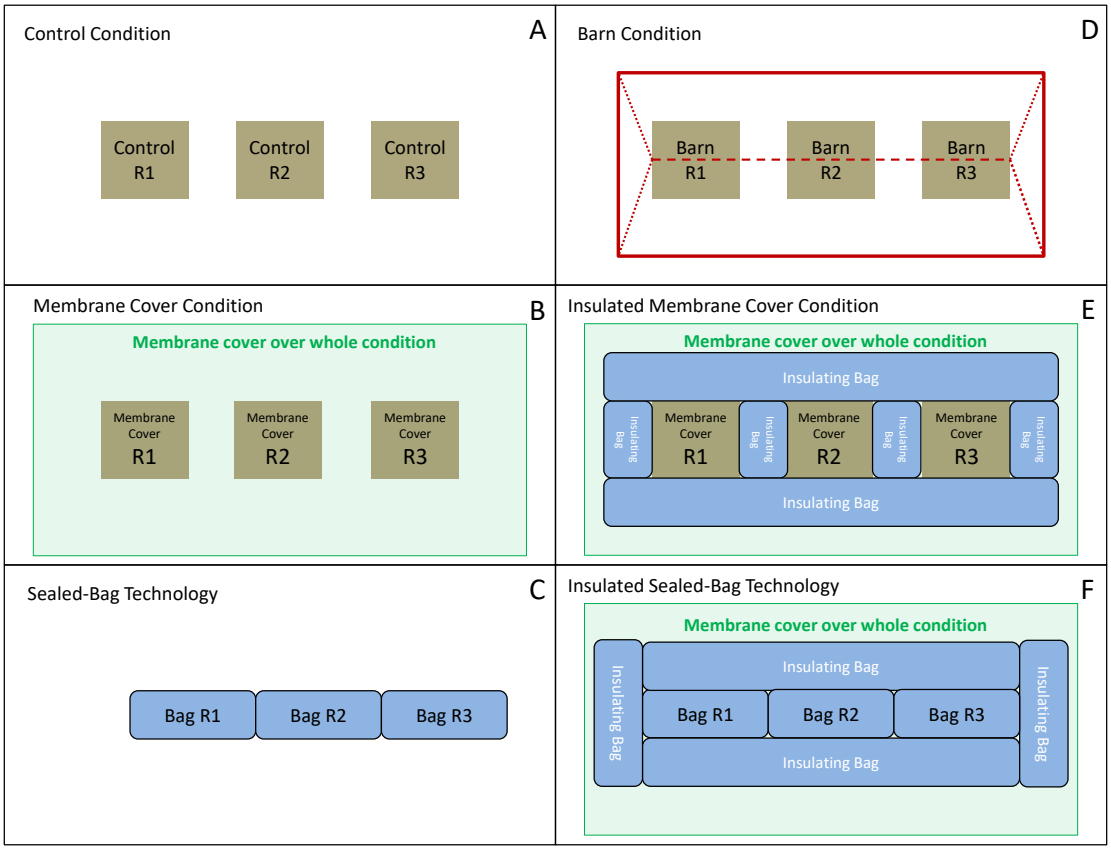

Figure 5.2 Detailed schematic of conditions in summer and winter pilot trials with repeats. Summer trial included conditions A, B, C and D. Winter trial included conditions A, B, C, E and F.

5.3.5 Data recording of environmental factors

Recording environmental parameters during the pilot trials was conducted using hand held meter readers. Probes were not permanently installed in the stockpiles. Monitoring probes recorded pH (PS90S, AWE Ltd., Stafford, UK) and temperature data (TPA1-N 1 AWE Ltd, Stafford, UK), moisture content (SM 150 soil moisture sensor, Delta-T, Cambridge, England) and redox potential measurements (Paleo Terra, Amsterdam, Netherlands). Before use, and on the

day of sampling, all probes were calibrated following the manufacturer's instructions in the laboratory. Between trialled conditions the pH probe was recalibrated to prevent any drift in the meter affecting recorded results. When taking readings, the probes were inserted individually into the 20 tonne stockpiles on a horizontal plane and to a depth of 0.5 m following a standard W-shape sampling regime (Carter and Gregorich 2008) before recording the output. To ensure consistency, particularly with redox and pH measurements a timer was used and readings recorded after 1 minute when the probe output had settled. The final redox potential value (E_h) was calculated by adding the potential from the reference electrode (E_{ref} = -144 mV) to the measured potential (E_m) (Vorenhout van der Geest and Hunting 2011):

$$E_h = E_m + E_{ref} \quad \textbf{(5-1)}$$

During the winter pilot an external laboratory service was contracted to complete the environmental parameter data recording (WatStech Ltd., Wolverhampton, UK). A schematic of the pilot trial sampling regime for the stockpiles in each condition is shown in Figure 5.3.

Weather fluctuations were monitored for the duration of each pilot trial at 30 minute intervals using a wireless weather centre (Maplin Electronics, Rotherham, England). Specific focus was given to precipitation levels and ambient temperature. The weather centre was located in a field adjacent to the pilot trial and set up according to the manufacturer's instructions.

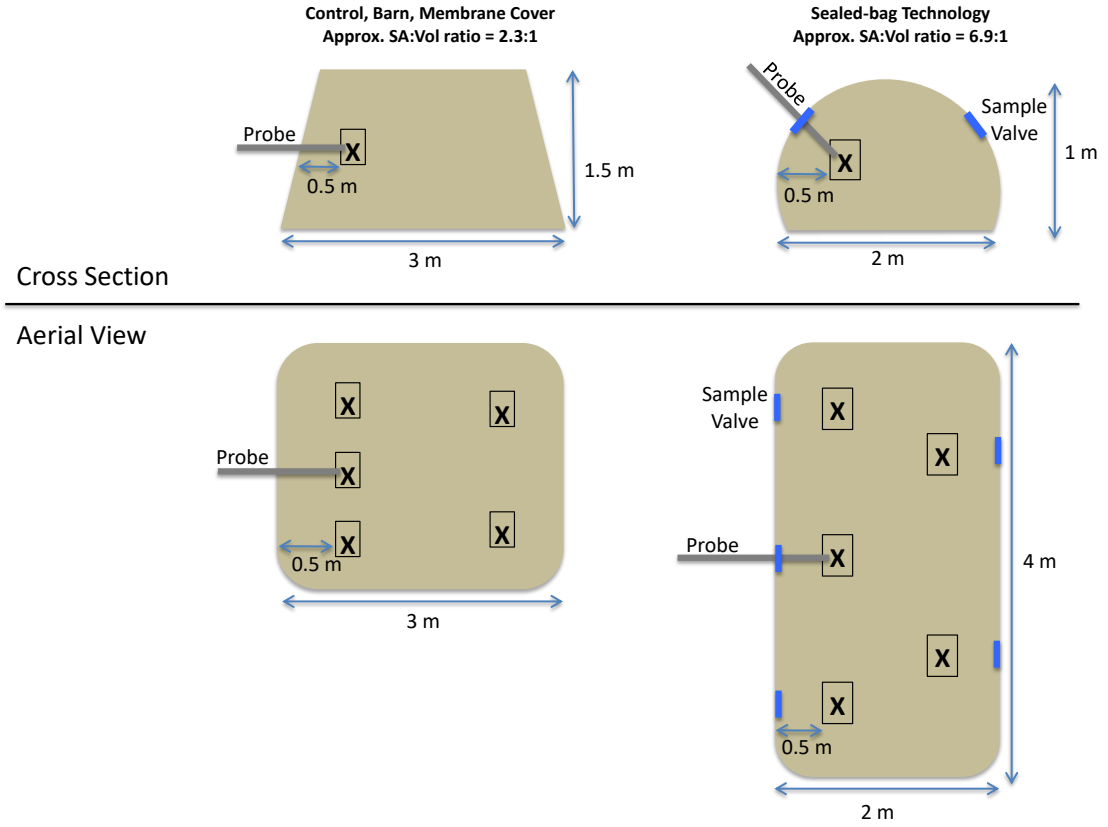

Figure 5.3 Cross section and aerial view of pilot trial stockpiles (including approximate dimensions) and the sampling points used (x). The bagged treatment was held in a sealed cylindrical bag altering the stockpile shape (sampling valves only present for summer pilot trial).

5.3.6 Microbial enumeration

Samples for *E. coli* enumeration and dry solids (DS) determination were collected from each 20 tonne stockpile in the summer and winter trials. 5 sub-samples of approximately 30 g were taken from a stockpile depth of 0.5 m on a horizontal plane using a clean spiral auger (Delta-T, Cambridge, England) following a standard W-shape sampling regime (Carter and Gregorich 2008). Samples were placed in screw lid sampling pots (Nalgene, Rochester, USA.) and transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2-8°C to suppress biological activity. Samples were processed within 24 hours of sampling**.** During the winter pilot an external laboratory service was contracted to complete the on-site sampling (WatStech Ltd., Wolverhampton, UK).

During the summer pilot trial all *E. coli* enumeration was conducted in Cranfield University laboratories. An external laboratory service was contracted to complete the microbial enumeration and DS analysis during the winter pilot trial (ALS Environmental, Coventry, England). Standard protocols were used to ensure homogenous methods were applied in both trials. The standard method for microbial enumeration and DS determination is described below.

Membrane filtration (Environment Agency 2003) was used for bacterial enumeration using Membrane Lactose Glucuronide Agar (MLGA) (OXOID, Basingstoke, UK) plates to distinguish *E. coli* from coliforms. From each sample condition, 3 g of material were removed and mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube. To ensure effective homogenization, samples were subjected to vortexing for 1 minute (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA.) prior to settling for a further 20 seconds. The supernatant was then removed for serial dilution. Samples were serial diluted with MRD to ensure colony forming units (CFU) were <80/plate. Samples were passed through 0.45 µm filters (Millipore S-PAK® 47 mm, Watford, UK) using a 3-way vacuum manifold (CombiSart®, Sartorius UK Ltd., Surry, UK). Filters were placed on MLGA plates and incubated at 30°C (± 1^{o}C) for 4 (± 1) hours and 37°C (± 0.5^{o}C) for 18 (± 2) hours as described by the Environment Agency (Environment Agency 2003). Arising green colonies were counted as presumptive *E. coli*, yellow colonies were counted as coliforms and pink colonies were recorded as non-coliforms. All *E. coli* enumeration results were normalized against DS%. A 3 g sludge sample was used for DS determination and analysis was performed as per Environment Agency (2003) guidance.

5.3.7 Statistical analysis

Normality assumptions for each of the variables measured was assessed via histograms and normal probability plots using STATISTICA (Version 12, Tulsa, USA). Variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá, 2007). A repeated measures analysis of co-variance (ANCOVA) was performed to identify statistical significant differences (p

<0.05) between the conditions tested. In separate tests '*E. coli*' 'stockpile temperature', 'stockpile pH', 'stockpile moisture content' and 'stockpile redox potential' were set as dependent variables. Experimental factors including 'season' ('winter' or 'summer') and 'week' were classified as categorical and therefore the length of storage time was set as an independent variable. Factors set as co-variates for ANCOVA analysis included ambient temperature data, rainfall and the measured stockpile parameters including temperature, pH, moisture content and redox potential when not set as the dependent variable. The validity of the ANCOVA assumptions was tested through residuals analysis (Gujarati 2011). A post-hoc analysis (Fisher LSD) was completed to assess the distinct differences between groups based on *E. coli* concentrations (Marques de Sá, 2007).

5.4 Results

The starting *E. coli* concentration was significantly 2.9 Log higher ($p < 0.05$) in the winter compared to the summer trial (Figure 5.4). The *E. coli* density gradually reduced in all storage conditions over the course of both trials. The greatest decrease in *E. coli* densities were observed in the summer trial. The highest level of *E. coli* die-off in the summer trial was in the covered treatment which reduced by 3.7 Log over the 28 day period. A significant reduction of approximately 2.1 Log from time 0 to day 28 was observed across the summer storage period for the control, barn and sealed-bag conditions as *E. coli* concentrations decreased. The levels of *E. coli* in the control, barn and bagged conditions during the summer trial were not significantly different from each other but were significantly different from the covered condition after day 14 of storage. In the winter trial, no significant treatment effect between the control, cover and bagged conditions (Figure 5.4A) was displayed up to day 42 of storage. The control and cover treatments maintained a higher level of die-off (0.5 Log) between day 42 and 69. At day 69 both the control and covered conditions contained *E. coli* concentrations of 6.1 Log. Bagged and insulated treatments (Figure 5.4B) maintained a level above 6.4 Log. Insulated treatments had no enhanced effect on death rate and *E. coli* concentrations reduced steadily to 6.5 Log over the 10 week period. Co-variate

analysis identified *E. coli* concentrations in the stockpiles for both the summer and winter trial to be significantly affected by stockpile temperature (p= 0.008).

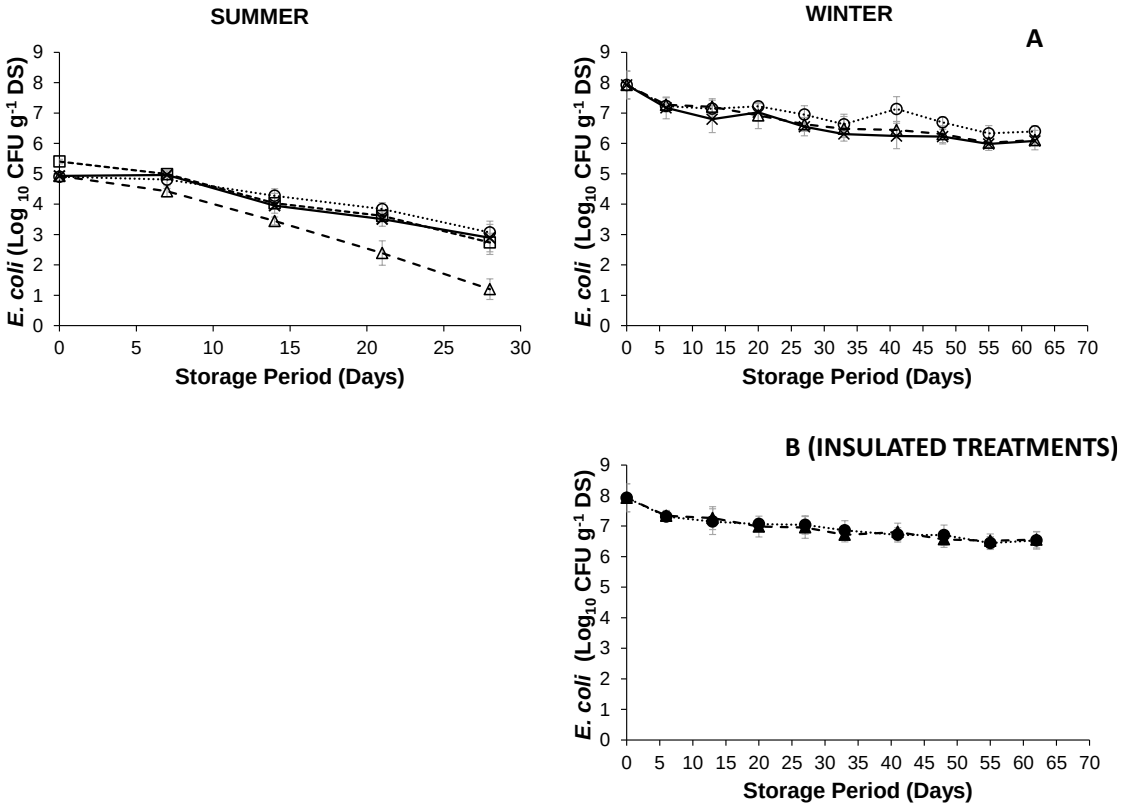

Figure 5.4 *E. coli* concentrations recorded during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: open control, barn storage, membrane covered treatment, sealed-bag technology. Additional insulated treatments included: insulated membrane covered treatment and insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3).

Stockpile temperatures were significantly different between the two pilot trials with a reduction of approximately 12.2°C between summer and winter seasons at the start of storage (Figure 5.5). This coincided with an average 13.7°C reduction in ambient temperature at time 0. The covered condition in the summer trial was significantly different from all other treatments by day 7 of storage and maintained a temperature above 25°C over the 28 day period (Figure 5.5A). The most significant stockpile temperature decrease was in the bagged treatment, reducing by 12.8°C (Figure 5.5B). The open control condition maintained a significantly higher temperature than the barn and bagged treatments during the

summer trial with a 20.3°C stockpile temperature recorded on day 28. In the summer months the stockpile temperatures consistently remained above the ambient field site temperature. Fluctuations in ambient temperature over the 28 day summer trial were limited and the overall range experienced was 6.3°C and 13.7°C (Figures 5.5A and 5.5B respectively). In the winter pilot, ambient temperature and stockpile temperatures were more similar with the greatest difference (10.6°C) at time 0. In the winter trial the ambient temperature range was 12.1°C. ANCOVA analysis across both seasons confirmed stockpile temperature was significantly affected by the 24 hour average ambient temperature ($p < 0.05$). During the winter trial, the covered treatment was able to maintain the highest stockpile temperature (Figure 5.5C) and was significantly different from the other treatments tested after the first month of storage. On the final day of storage the covered stockpile temperature was recorded as 9.3°C, at least 2.4°C higher than the other treatments tested. The insulated conditions (Figure 5.5D) did not maintain a higher stockpile temperature. The insulated sealed-bag treatment was not significantly different from the bagged condition and the insulated covered treatment was significantly cooler than the covered condition. Over the winter storage period there was a significant difference in stockpile temperatures between the time points tested and in all treatments stockpile temperatures showed a gradual reduction between time 0 and day 69.

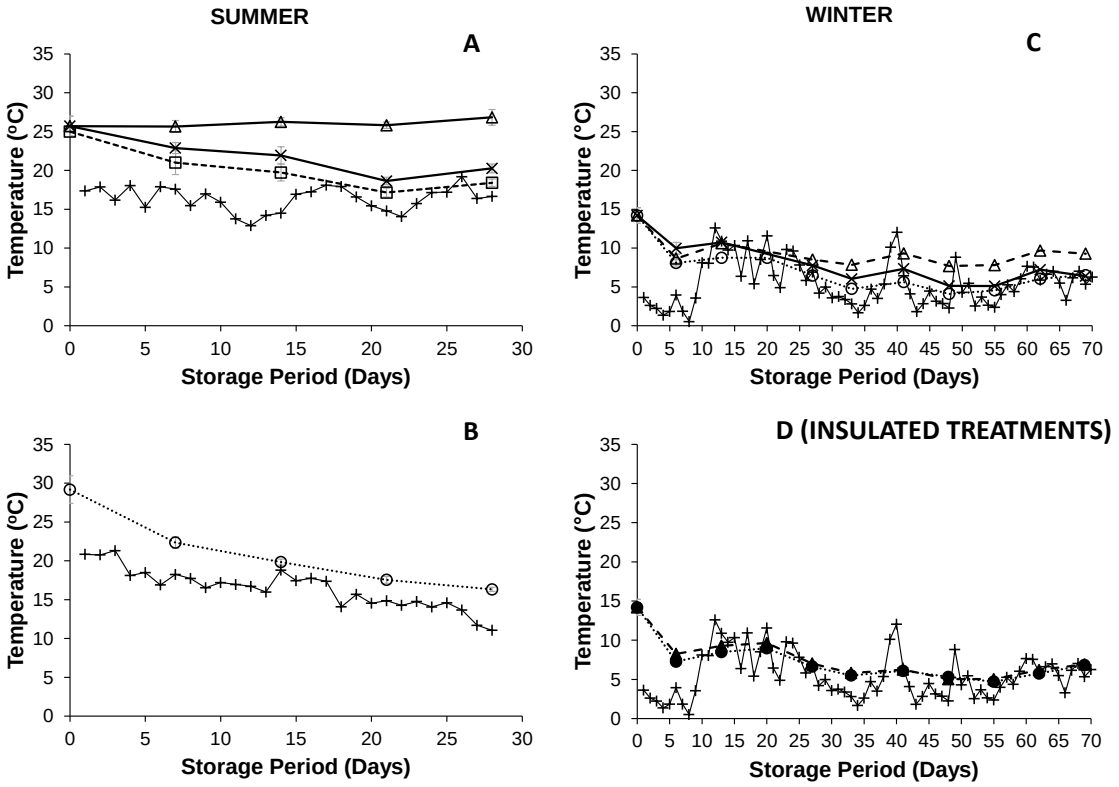

Figure 5.5 Temperature results from summer (28 day) and winter (69 day) pilot trials. Summer results in graph B display data from the sealed-bag treatment which started with a 28 day lag from the other tested treatments. Winter trial tested 2 additional insulated treatments shown separately in winter graph D. Key: open control, barn storage, membrane covered treatment, sealed-bag technology. Additional insulated treatments included: insulated membrane covered treatment and insulated sealed-bag technology. Ambient temperature data ( ) was recorded at 30 minute intervals during the pilot trial tests and results here show the average temperature over 24 hours. Error bars represent the standard deviation of 3 repeats (n= 3).

A 0.5 unit reduction in pH was observed between the summer and winter pilot trials at time 0 (Figure 5.6). In the summer trial the pH remained stable (8.1 pH average) and showed no difference between the treatments tested. During the winter trial the pH probe was damaged and remained with the manufacturers for repair over several weeks. Therefore pH data is limited, but showed a change between time 0 and day 7 of storage as the pH reduced from 8.8 to an average of 7.7 in the control, cover and bagged conditions (Figure 5.6A). Between day 42 and day 69 the pH level in the sealed-bag and control treatments varied marginally. Under the covered treatment the pH displayed a steady decline between days 42 and 69 reducing from 8.0 to 7.5 pH units. In the insulated

treatments (Figure 5.6B) a decline in pH to 7.4 units was observed in the first 7 days of storage. Over the following 62 days the pH reduced to 7.4 units in the insulated covered and insulated bagged conditions. ANCOVA analysis showed no significant effect of pH on *E. coli* concentrations.

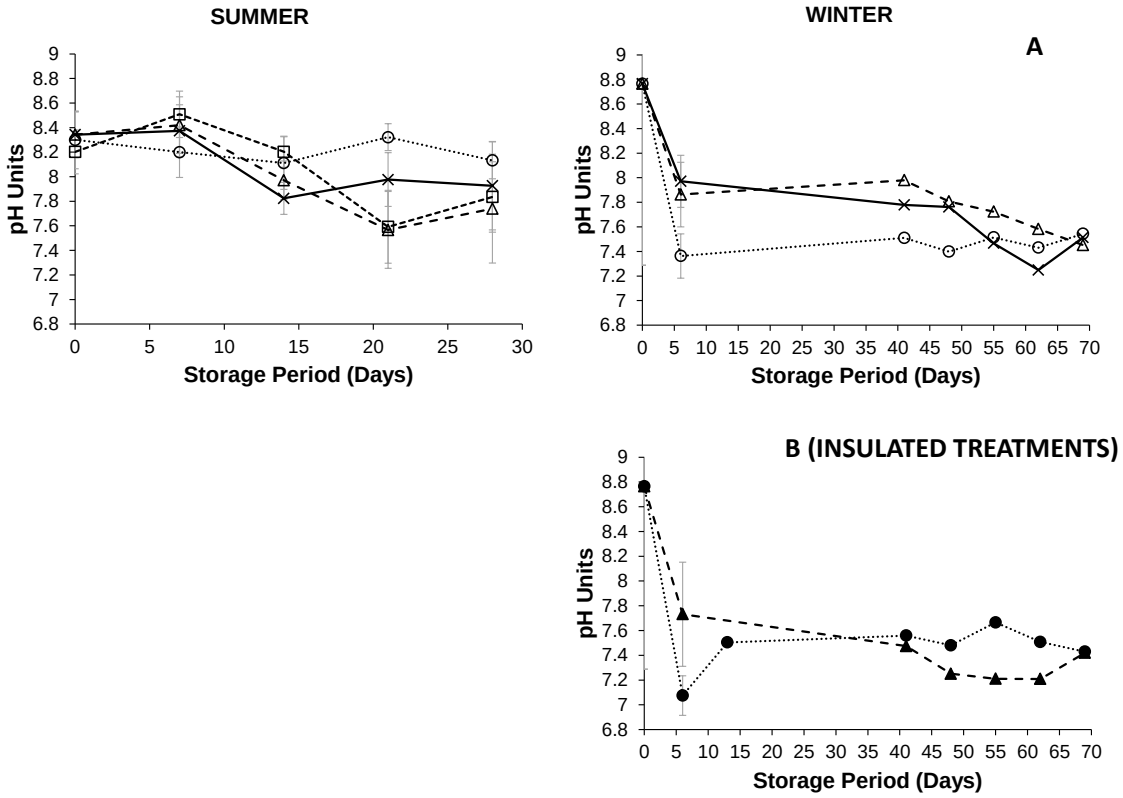

Figure 5.6 Results from pH monitoring during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: open control, barn storage, membrane covered treatment, sealed-bag technology. Additional insulated treatments included: insulated membrane covered treatment and insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3).

Stockpile moisture content changed between the summer and winter trials for the common conditions tested (control, cover and bagged treatments) and was significantly lower by 0.03 m^3m^{-3} at time 0 in the summer trial (Figure 5.7). Significantly higher stockpile moisture content in the covered condition averaging at 0.48 m^3m^{-3} was maintained over the 28 day summer trial (Figure 5.7A). Moisture content significantly change under the covered condition between time 0 and day 28 with a significantly higher moisture content on day 14 (0.5 m^3m^{-3}) in comparison to the other treatments tested. The control, barn and sealed-bag

conditions remained at approximately 0.46 m^3m^{-3} with no significant change for the duration of the storage period. Throughout the summer storage period the total rainfall during the control, barn and covered conditions was 60.6 mm with the peak precipitation event in week 2 where 20.7 mm fell over one 24 hour period. The level of precipitation during the summer sealed-bag trial (Figure 5.7B) increased and the total rainfall amounted to 152.4 mm (an increase of 91.8 mm between the two summer test periods). Rainfall over the winter trial amounted to 223.2 mm and the peak precipitation event was in week 3 with 40.8 mm recorded over one 24 hour period. Stockpile moisture content showed no significant difference between each of the treatments tested during the winter trial (treatment average 0.43 m^3m^{-3}) (Figures 5.7C and 5.7D). A significantly higher moisture content (0.03 m^3m^{-3}) was measured for the insulated treatments on day 49 of storage (Figure 5.7D). ANCOVA analysis showed stockpile moisture content to be significantly ($p < 0.05$) affected by rainfall ($p = 0.01$), the ambient temperature range over 24 hours and the stockpile temperature for both winter and summer trials.

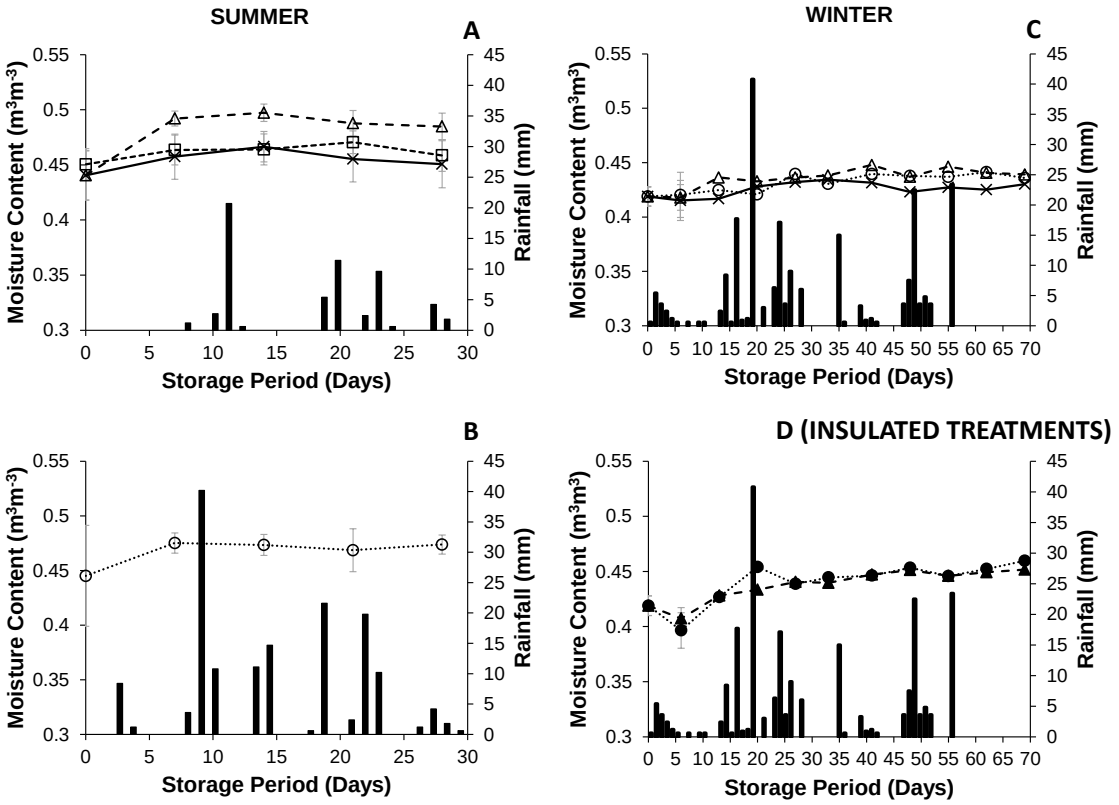

Figure 5.7 Moisture content and rainfall results from summer (28 day) and winter (69 day) pilot trials. Summer results in graph B display data from the sealed-bag treatment which started with a 28 day lag from the other tested treatments. Winter trial tested 2 additional insulated treatments shown separately in winter graph D (please note: rainfall data has been repeated in graphs C and D). Key: open control, barn storage, membrane covered treatment, sealed-bag technology. Additional insulated treatments included: insulated membrane covered treatment and insulated sealed-bag technology. Rainfall data is displayed in bars ( ) and results show the total rainfall recorded over 24 hours. Error bars represent the standard deviation of 3 repeats (n= 3).

Redox data recorded over the summer trial displayed no significant treatment effect or significant change over the 28 day storage period (Figure 5.8). The average redox level throughout the summer pilot trial was -382 mV. A significant difference in the redox potential was observed between the summer and winter pilot trials with an increase to -309.6 mV displayed. Overall no treatment effect was identified between the different storage conditions in the winter trial (Figures 5.8A and 8B). The insulated treatments (Figure 5.8B) and the sealed-bag condition displayed no difference in the redox potential when compared with the control.

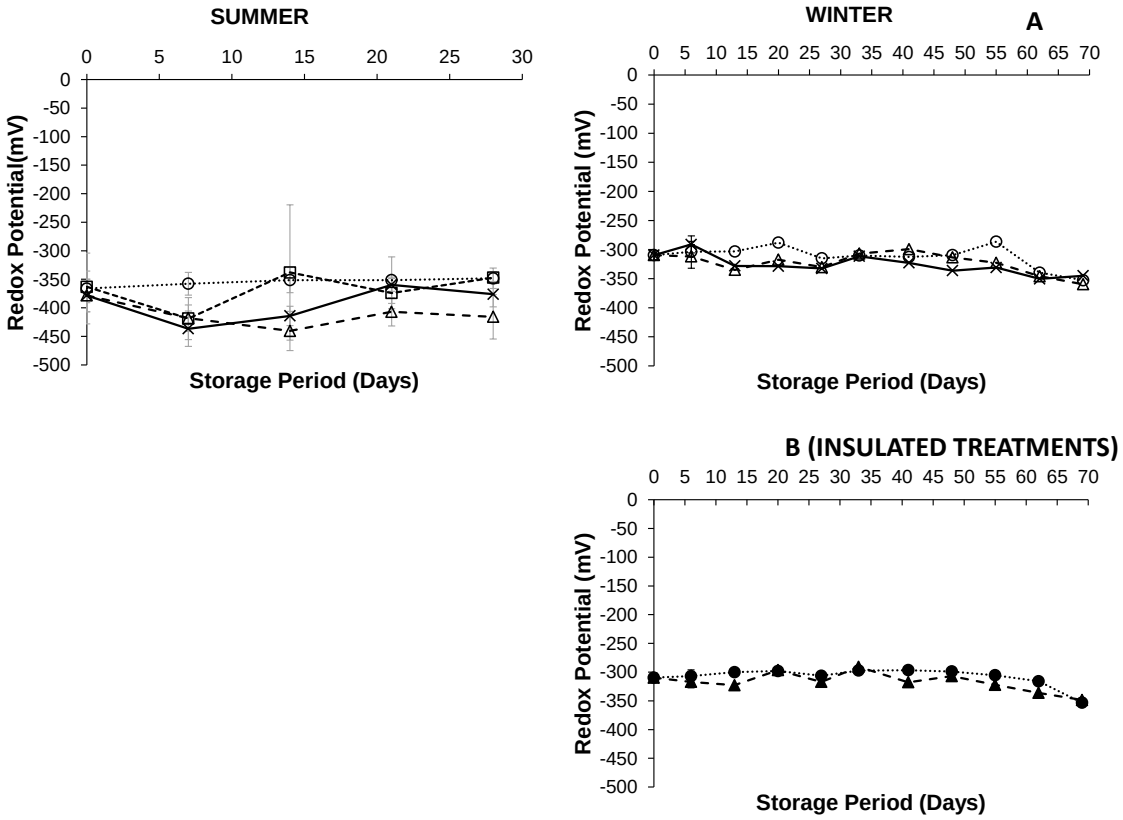

Figure 5.8 Redox potential results recorded during summer (28 day) and winter (69 day) pilot trials. Winter trial tested 2 additional insulated treatments shown separately in winter graph B. Key: open control, barn storage, membrane covered treatment, sealed-bag technology. Additional insulated treatments included: insulated membrane covered treatment and insulated sealed-bag technology. Error bars represent the standard deviation of 3 repeats (n= 3).

5.5 Discussion

5.5.1 Dominant environmental parameter affecting *E. coli* death rate

Temperature is the environmental parameter which has the most significant effect on *E. coli* concentrations in the stored biosolids material. Stockpile temperature directly impacts on the rate of *E. coli* decay whilst analysis of ambient temperature highlighted a significant influence on stockpile temperature fluctuations. Temperature influences on indicator bacteria dynamics have been observed in previous research on organic wastes, soils and biosolids-amended soils (Plachá *et al* 2001, Cools *et al* 2001, Iranpour *et al* 2005, Holley *et al* 2006, Lang *et al* 2007, Oliver *et al* 2016). Stockpile temperature and ambient temperature was

significantly lower in the winter trial compared to summer. This lower temperature coincided with a 2.9 Log increase in levels of *E. coli* bacteria at time 0 and is similar to results from Plachá *et al* (2001) who identified approximately a 1 Log increase in the levels of faecal coliforms between summer and winter/ spring seasons in solid fraction pig slurry. A study by Holley *et al* (2006) also found an increase in levels of *Salmonella* in winter test periods at time 0 of experimental study for control soils (without manure addition) which showed an increase of 0.1 Log (CFU/g). The change in starting *E. coli* concentration observed in Figure 5.4 is thought to be indicative of the high variability within the biosolids system. The changeability of *E. coli* levels in the fresh biosolids material (sampled from the centrifuge outlet) is shown in Appendix A and suggests that factors affecting microbial levels in biosolids may be a result of up-stream sludge treatment processes rather than seasonal trends in *E. coli* growth and death.

A steady decline in *E. coli* concentrations was observed in both trials but with a greater die-off rate in the summer storage trial. An increase in storage temperature coincided with higher die-off and in a study by Semenov *et al* (2007) a greater decline in *E. coli 0157:H7* was observed for storage temperatures changing from 7 to 33°C. For oscillating temperatures at 23°C (±4°C) final densities dropped to 5.67 (±0.27) Log CFU g DS^{-1} after 2 weeks of storage. In contrast, treatments at 7°C only decreased to 7.44 (±0.1) Log CFU g DS^{-1} (Semenov *et al* 2007), which is comparable with the winter pilot results. The authors suggest that the reduced survival at higher temperatures may be a consequence of greater stress and energy expenditure for a particular organism (Semenov *et al* 2007). Under warmer conditions microbial metabolic activity and increased competition for substrates is expected, accelerating cell nutrient exhaustion (Cools *et al* 2001, Jiang *et al* 2002, Holley *et al* 2006). This effect was observed in the covered treatment where a higher temperature was maintained above 25°C throughout the 28 day storage trial. In this condition *E. coli* reduced by 3.7 Log highlighting the decrease in survival times at elevated temperatures. During the winter trial *E. coli* concentrations always remained above 6 Log emphasising the reduced antagonistic activity and competition from indigenous microorganisms at lower temperatures (Cools *et al* 2001). Interestingly the

covered treatment displayed a significantly higher reduction in *E. coli* bacteria during the winter trial to a concentration of 6.1 Log after 69 days of storage. This was matched with a significantly higher stockpile temperature of 9.3°C (2.4°C higher than any other condition tested).

The insulated treatments in the winter pilot did not maintain the 15°C time 0 stockpile temperatures as originally intended. This may have been due to the greater surface area of the bagged sludge surrounding the tested treatments (Figures 5.2 and 5.3). Although the insulated treatments were covered with an additional dark coloured cover, likely to benefit from potential sunlight heating, the cover did not rest flat on the stockpile and therefore voids between stockpiles and insulating bags were easily filled with water during rainfall events as the cover increased pooling on the surface. These pools frequently froze during frosts adding a cooling effect to these treatments.

The results show that increasing the stockpile temperature to a level above 25°C, which achieved the greatest level of *E. coli* die-off, is challenging in the winter months. It therefore seems plausible that sites challenged by compliance limits should consider applications such as electrical heat tracing, as tested by Iranpour *et al* (2005), which highlighted the benefit of silo modifications to reduce the drop in biosolids temperature after digestion and prevent indicator bacteria regrowth. The results demonstrate that increased *E. coli* die-off can be achieved through the manipulation of storage environments where stockpile temperature is elevated and therefore the first hypothesis tested can be accepted.

5.5.2 Changes in moisture content

In previous research findings, the impact of moisture content on bacterial dynamics in biosolids and analogous environments is varied with bacterial survival recorded across a large range of moisture conditions (Jiang *et al* 2002, Zaleski *et al* 2005b, Lang and Smith 2007, Rouch *et al* 2011). ANCOVA tests confirmed no significant effect of stockpile moisture content on *E. coli* survival. The moisture content across both trials remained relatively stable with a change of only 0.03 m^3m^{-3} between the summer and winter trials. Studies on moisture content in stored biosolids have observed large fluctuations with dry solids

measurements ranging between 10 and 90% (Zaleski *et al* 2005b) and corresponding with a 6 order of magnitude variability in concentrations of faecal coliforms (MPN/ gram). The change in moisture content during both the summer and winter trial was limited and therefore the range measured was much narrower than in other research suggesting the moisture content changes in the tested biosolids were not sufficient to cause a significant shift in *E. coli* concentration.

The covered treatment in the summer months displayed the greatest change in stockpile moisture content increasing to 0.48 m^3m^{-3} between time 0 and day 7 of storage. Sampling observations identified condensation forming on the underside of the membrane cover which indicated a localised movement of water within the covered stockpiles. This may have caused the top 0.5 m of the covered stockpiles (where the probe recordings were taken) to have an increased moisture content, causing the change observed.

Findings are important for operational sites as stockpile moisture content is a measured parameter of biosolids quality (DS%). Ability to maintain the sludge matrix integrity for stockpiling on treatment sites and agricultural land is necessary to prevent pollution incidents (Environment Agency 2012). As results have identified a significant effect of rainfall on stockpile moisture content the stability achieved under covered treatments may provide a suitable avenue to prevent stockpile collapse commonly experienced during prolonged periods of heavy rainfall (Operational Data, Severn Trent Water Plc.).

5.5.3 Influence of treatments to suppress oxygen availability

Oxygen depletion has been attributed to bacterial die-off in anaerobic digestion (Smith *et al* 2005, Rosenblum *et al* 2014) and food preservation (Floros and Matsos 2005, Garrido *et al* 2016). Therefore limiting oxygen availability in biosolids storage environments was expected to have a detrimental effect on *E. coli* indicator bacteria. The storage study incorporated a sealed-bag technology which was anticipated to significantly reduce the amount of available oxygen in stockpiles for cell respiration and consequential growth (Alberts *et al* 2010). Test results demonstrated all stockpiles, including open treatments, to be anaerobic and within a redox potential range dominated by methanogenic bacteria, sulphate

reducing bacteria and heterotrophic anaerobic bacteria at the pH level measured in the biosolids (7- 8.8 pH units) (Sigma Aldrich 2016). Consequentially, the second hypothesis tested must be rejected as stored biosolids are anaerobic and therefore efforts to reduce oxygen availability have a limited impact on *E. coli* die-off rates.

5.6 Conclusions

1. Temperature is a critical environmental factor affecting *E. coli* cell death rates.
2. With increased storage time *E. coli* concentrations reduced in all treatments tested for both summer and winter trials.
3. Covered treatments, accelerated the suppression of *E. coli* bacteria in summer months through the maintenance of elevated stockpile temperatures >25°C. A reduction >2 Log was observed in covered treatments within 7 days of storage.
4. Ambient temperature (24 hour average) significantly influences stockpile temperatures and consequently indicator bacterial die-off.
5. Stored biosolids stockpiles are anaerobic and therefore efforts to reduce oxygen diffusion have limited impact on *E. coli* concentrations.
6. Understanding of the parameters in biosolids storage environments has been advanced through the trials completed and results have shown the stability of biosolids pH, moisture and redox potential levels which displayed no significant effect on *E. coli* dynamics.

5.7 Acknowledgements

The authors would like to thank the Natural Environment Research Council (NERC) and Severn Trent Water Plc. for their financial support. Thanks also go to WatStech Ltd., Wolverhampton, UK and ALS Environmental, Coventry, UK for their sampling and microbial analysis services in the winter pilot trial.

5.8 References

ADAS, (2001) THE SAFE SLUDGE MATRIX: Guidelines for the Application of Sewage Sludge to Agricultural Land. Gleadthorpe Research Centre.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Berry, E. D. and Miller, D. N. (2005) Cattle Feedlot Soil Moisture and Manure Content: II Impact on *Escherichia coli* 0157 *Journal of Environmental Quality* Vol. 34 pp. 656- 663

Carter M.R and Gregorich E. G., (2008) '*Soil Sampling and Methods of Analysis 2nd Edn.'* CRC Press, Taylor and Francis Group, Raton USA.

Centre for Ecology and Hydrology (CEH) (2016) Evaporation http://www.waterandclimatechange.eu/evaporation [accessed on 17/10/2016]

Chen, Y., Higgins, M. J, Beightol, S. M, Araujo, G. G., Murthy, S. N., Barben, E. J., Toffey, W. E. (2007) Odour Generation and Pathogen Indicator Regrowth after Dewatering: Are They Related? In: Proceedings of the Mid-Atlantic Biosolids Assocation Conference

Chen, Y. C., Higgins, M. J., Beightol, S. M., Murthy, S. N., & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research,* Vol. 45, pp. 2616- 2626.

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., (2001) Survival of *E. coli* and *Enterococcus spp*. derived from pig slurry in soils of different texture. *Appl. Soil Ecol.* Vol. 17, pp. 53–62.

DEFRA (2006) Code of Practice for Agricultural Use of Sewage Sludge sludgehttp://adlib.everysite.co.uk/adlib/defra/content.aspx?id=000IL3890W.16NTC17O1X82UF [accessed on 31/08/2016]

Dentel, S. K., Qi, Y., & Herson, D. S. (2008). Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration, and assessment. *Water Science and Technology,* Vol. 57 No. 2.

Environment Agency, (2003) The Microbiology of Sewage Sludge- Part 3- Methods for the isolation and enumeration of *Escherichia coli*, including verocytoxigenic *Escherichia coli*. Bristol.

Environment Agency (2012) Waste exemption: S3 storing sludge https://www.gov.uk/guidance/waste-exemption-s3-storing-sludge [Accessed on 01/09/2016]

European Union, (2000) Working Document on Sludge, 3rd Draft. ENV.E.3/LM. Brussels.

Floros, J. D and Matsos, K. I. (2005) Introduction to modified atmosphere packaging. In Han, J. H. (Ed.), *Innovations in Food Packaging* (pp. 159- 184). Elsvier Ltd.

Garrido, M. D., Hernández, M. D., Espinosa, M. C., López, M. B. (2016) Enhanced quality characteristics of refrigerated Seabream (Sparus aurata) fillets packed under different systems (modified atmosphere Vs. vacuum) *Journal of Aquatic Food Product Technology* Vol. 25, No. 2, pp. 156-168

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., Charnov, E. L. (2001) Effects of size and temperature on metabolic rate *Science* Vol. 293 pp.2248-2251

Gujarati, D. (2011) 'Econometrics by Example' PALGRAVE MACMILLAN, Hampshire, UK

Herrmann, C., Heiermann, M., Idler, C., (2011) Effects of ensiling, silage additives and storage period on methane formation of biogas crops. *Bioresour. Technol.* Vol. 102, pp. 5153–5161.

Henkel, S.G., Ter Beek, A., Steinsiek, S., Stagge, S., Bettenbrock, K., Teixeira de Mattos, M.J., Sauter, T., Sawodny, O., Ederer, M., (2014) Basic Regulatory

Principles of *Escherichia coli's* Electron Transport Chain for Varying Oxygen Conditions. *PLOS one* Vol. 9 No. 9 e107640

Higgins, M. J., Chen, Y. C., Murthy, S. N., & Hendrickson, D. (2007a). Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering. *Procedings on moving forward wastewater biosolids sustainability: Technical, managerial and public synergy*, pp. 204-19.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Henderickson, D., Farrel, J., & Schafer, P. (2007b). Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Research*, Vol. 41 pp. 665-673.

Holley, R.A., Arrus, K.M., Ominski, K.H., Tenuta, M., Blamk, G. (2006) *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure *Journal of Environmental Quality* Vol 35 pp. 1170- 1180

Hutchison, M.L., Nicholson, F.A., Smith, K.A., Keevil, C.W., Chambers, B.J., Moore, A., (2000) Study on Farm Manure Applications To Agricultural Land and an Assessment of the Risks of Pathogen Transfer Into the Food Chain FS2526.

Iranpour, R., Palacios, R., Cox, H. H. J., Abkain, V. (2005) Solving fecal coliform growth/ reactivation in biosolids during full-scale post-digestion processes *Water Science and Technology* Vol 52, No. 1-2, pp. 283- 288

Jiang, X., Morgan, J. and Doyle, M. P. (2002) Fate of *Escherichia coli 0157:H7* in manure amended soil *Applied Environmental Microbiology* Vol. 68, pp.2605-2609

Lang, N. L. and Smith, S. R. (2007) Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions *Journal of Applied Microbiology* Vol. 103, pp. 2122- 2131

Lang, N. L., Bellett-Travers, M. D. and Smith, S. R. (2007) Field Investigations on the survival of *Escherichia coli* and presence of other enteric micro-organisms in

biosolids-amended agricultural soil *Journal of Applied Microbiology* Vol. 103, pp. 1868- 1882

Le, M.S., Mayhew, M.E., Back, P.A., (2002) Effectiveness of Secondary Digesters as a Pathogen Controller in Winter. *J. CIWEM* Vol. 16, pp. 292–295.

Marques de Sa, J.P., (2007) Applied Statistics using SPSS, STATISTICA, MATLAB and R. Springer- Verlag, Berlin.

Monteleone, M. C., Furness, D., Jefferson, B., & Cartmell, E. (2004). Fate of *E. coli* Across Mechanical Dewatering Processes. *Environmental Technology*, Vol. 25 No.7 pp. 825-831.

Mowla, D., Tran, H.N., Grant Allen, D. (2013) A review of the properties of biosludge and its relevance to enhanced dewatering processes *Biomass and Bioenergy* Vol. 58 pp. 365-378

Oliver, D. M., Bird, C., Burd, E., Wyman, M. (2016) Quantitative PCR profiling of *Escherichia coli* in livestock feces reveals increased population resilience relative to culturable counts under temperature extremes *Environmental Science and Technology* Vol. 50 pp. 9497- 9505

Placha, I., Venglousky, J., Sasokova, N., Svoboda, I.F., (2001) The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solids of pig slurry. *J. Appl. Microbiol.* Vol. 91, pp. 1036–1043.

Qi, Y., Dentel, S. K., Herson, D. S. (2008) Effect of total solids on fecal coliform regrowth in anaerobically digested biosolids *Water Research* Vol. 42 pp. 3817-3825

Qi, Y., Dentel, S. K., & Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. 41, pp. 571- 580.

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and

chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Rouch, D. A., Mandal, T., Pai, S., Glauche, F., Fleming, V. A., Thurbon, N., *et al*. (2011). Microbial safety of air dried and re-wetted biosolids. *Journal of Water and Health*, Vol. 9.2 pp. 403-414.

Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M., Adani, F., (2014) Sanitation ability of anaerobic digestion performed at different temperature on sewage sludge. *Sci. Total Environ*. No. 466-467, pp. 888–897.

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M., (2007) Influence of temperature fluctuations on *Escherichia coli O157:H7* and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol.* Vol. 60, pp. 419–428.

Sigma Aldrich (2016) Microbology Introduction http://www.sigmaaldrich.com/technical-documents/articles/microbiology/microbiology-introduction.html [Accessed on 05/09/2016]

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manag*. Vol. 25, pp. 417–425.

Sprigings, A. E., & Le, M. S. (2011). Growth Potential- A New Method For Estimation of the Risk of *E. coli* Regrowth in Digested Sludge Cake. *16th European Biosolids and Organic Resources Conference.*

Vorenhout M., van der Geest H.G., and Hunting E. R. (2011) 'An Improved Datalogger and Novel Probes for Continuous Redox Measurements in Wetlands' *International Journal of Environmental Analytical Chemistry* Vol. 91 No. 7-8, pp. 801-810

Water UK. (2010). Recycling of Biosolids to Agricultural Land. *(3)*. London: Water UK

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005a). Survival, growth and Regrowth of Enteric Indicator and Pathogenic Bacteria in Biosolids, Compost, Soil, and Land Applied Biosolids *Journal of Residuals Science and Technology* Vol. 2, No. 1 pp. 49- 63

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005b). Potential Regrowth and Recolonisation of Salmonellae and Indicators in Biosolids and Biosolids-Amended Soil *Applied and Environmental Microbiology* Vol. 71, No. 7, pp. 3701- 3708

Chapter 6

Disruption of Cells in Biosolids Affects *E. coli* Dynamics in Stored Cake

Statement of author contributions:

S. Fane: Principal investigator, completion of experimental design and laboratory work, data analysis and chapter write up

D. Madureira: 6 month placement student supporting preliminary trials on thermal disruption treatments

Dr. A. Nocker: Provided advice on flow cytometry measurement and supported decisions on experimental design

A. Wilson: Industrial supervisor for Severn Trent Water Plc.

Dr. M. Rivas Casado: Statistical support

Dr. Y. Bajon-Fernandez: Industrial supervisor for Severn Trent Water Plc.

Professor J. Harris: Supported evaluation of experimental data

Professor E. Cartmell: Developed overall concept of work with Severn Trent Water Plc.

Professor S. Tyrrel: Principal supervisor supporting research concept development, guidance on experimental design, data analysis and evaluation of final chapter write up

6 Disruption of Cells in Biosolids Affects *E. coli* Dynamics in Stored Cake

S. Fane[1], D. Madureira[2], A. Nocker[3], A. Wilson[4], M. Rivas Casado[1], Y. Bajon-Fernandez[1], J. Harris[1], E. Cartmell[5], S. Tyrrel[1]

1 Water Science Institute, School of Water, Energy and Environment, Cranfield University, UK

2 Faculdade de Engenharia de Universidade do Porto, Portugal

3 IWW Rheinisch-Westfälisches Institut für Wasserforschung gemeinnützige GmbH, Germany

4 Severn Trent Water Plc., Coventry, UK

5 Scottish Water, Dunfermline, Scotland

6.1 Abstract

Achieving stipulated microbial compliance levels in biosolids storage is complicated by the unpredictable increase of *Escherichia coli* (*E. coli*), which serves as the most important indicator for pathogen presence. The effects of sludge dewatering operations may be a significant factor contributing to indicator survival as a result of shear stresses on the sludge matrix. Shear forces present during centrifuge dewatering may increase cell damage and lysis. The effect of cell damage on *E. coli* population survival was assessed in laboratory scale thermal and physical disruption experiments. *E. coli* growth curves for disrupted treatments were compared to undisrupted (control) conditions over a minimum 10 day storage period. Measurements of intact and damaged cell concentrations were recorded using flow cytometry and *E. coli* bacterial concentrations were monitored using traditional membrane filtration techniques. A significant difference ($p < 0.05$) in the level of damaged cells between undisrupted and disrupted conditions was shown. For thermal disruption treatments the peak *E. coli* concentration increased significantly by 1.8 Log (CFU g^{-1} DS) compared with control treatments. In addition results from physical disruption tests displayed a significant 2.4 Log (CFU g^{-1} DS) increase from control conditions. A greater degree of *E. coli* growth was observed in treatments achieving more cell

disruption. In addition to growth, *E. coli* death rates were studied after thermal disruption treatments in modified storage environments to test whether enhanced die-off could be achieved. For disrupted samples levels of *E. coli* never returned to pre-disrupted concentrations. Die-off rates were accelerated under higher storage temperatures (>25°C). For undisrupted treatments the combination of higher storage temperature and modified atmosphere increased the *E. coli* die-off rate, although concentrations only fell below disrupted treatment levels at 37°C storage (undetectable levels of *E. coli* after 7 days of storage at 37°C). The research findings contribute to the understanding of bacterial growth and death dynamics in dewatered biosolids. These studies suggest that cell lysis may support remaining *E. coli* populations. Storage trials have shown benefits of disruption treatment in combination with elevated temperature limiting bacterial population recovery and maintaining a lower, compliant concentration of *E. coli* indicator bacteria in stored biosolids.

6.2 Introduction

Monitoring levels of pathogenic indicators in biosolids validates the effectiveness of treatment processes and ensures the safe recycling of biosolids to agricultural land (European Commission 2015). Concerns of pathogen removal in biosolids treatment surface during storage where, post dewatering, levels of microbial indicators can exceed compliance levels (Chen *et al* 2011, Higgins *et al* 2007a, Monteleone *et al* 2004). In the UK, biosolids compliance levels are stipulated in the 'Safe Sludge Matrix' (ADAS 2001) and failure to comply with guidelines can have costly implications.

On sludge treatment sites centrifuge dewatering is a common method used to remove the liquid fraction of sludge and achieve a targeted ~25% dry solids (DS) product from the digested liquid sludge (~4% DS) (Operational data, Severn Trent Water Plc.). Mechanical dewatering provides an economical benefit to sludge handling and transportation costs. Prior to dewatering *E. coli* indicator concentrations in digested liquid sludge are approximately 4 Log (CFU g^{-1} DS) (Operational data, Severn Trent Water Plc.). However after dewatering operations, particularly in the first 48 hours of storage, indicator concentrations

have been observed to increase rapidly, reaching levels of approximately 7 Log (CFU g^{-1} DS) (Operational data, Severn Trent Water Plc.). This observation is not isolated and has been observed on other treatment sites by researchers (Monteleone *et al* 2004, Qi *et al* 2007, Higgins *et al* 2007a, Chen *et al* 2011). This rapid increase in *E. coli* levels may indicate the presence of pathogenic bacteria within the biosolids product and is a concern for utility operators.

The reasons for indicator growth in stored biosolids post-dewatering have not yet been clearly identified (Dentel, Qi and Herson 2008). One possible explanation is the release readily bioavailable nutrients during mechanical dewatering processes (Higgins *et al* 2007a, Chen *et al* 2011, Sun *et al* 2015). Nutrients are required for cell energy generation and biosynthesis (Todar 2012) and form the basic elemental composition of a cell. Previous studies have identified large amounts of bioavailable protein and polysaccharide in centrifuge dewatered sludge cake (Higgins *et al* 2006) that may provide substrates for bacterial growth. The pre-conditioning of digested sludge with polyelectrolyte to form flocs aggregates the sludge organic matter which could favour the growth of bacteria cells held within the floc matrix. In addition, the floc structure can provide protection from environmental stressors, prolonging cell survival (Mahendran *et al* 2012, Lin *et al* 2014).

Although the mechanical dewatering process is conditioned to support aggregation and the formation of the cake product (Higgins *et al* 2007b), researchers suggest that the effects of mechanical shear during dewatering treatments can cause floc disruption and even bacterial dispersal, elevating indicator concentrations. A strong argument for this is put forward by Chen *et al* (2011) who simulated both belt filter press and centrifuge dewatering in a shearing test. The effects of belt filter press were replicated with 0 passes and then pressed cakes were subject to 5 and 10 conveying passes to represent the internal conveying scroll of a decanter centrifuge at 2 levels of shearing force. Results indicated a greater faecal coliform density in cakes exposed to more shearing. With 10 passes faecal coliform concentrations increased from original values by 5 Log units after 2 days of storage (Chen *et al* 2011). Reasons for

bacterial increase were explained by the release of soluble proteins and other organics during shearing which serve as substrate sources for microbial organisms (Chen *et al* 2011). Higgins *et al* (2007b) suggests that shear force exerted on the sludge during centrifuge dewatering may release bioavailable nutrients which could support the culturability of bacterial cells previously held in a viable but non-culturable (VBNC) state. Sun *et al* (2015) investigated dewatering processes on cyanobacteria-containing sludge and attributed cell lysis to flocculation turbulence and pressure from mechanical operations on flocs.

The effects of cell lysis resulting from centrifuge dewatering are studied by Chen *et al* (2005) who identified negative impacts on methanogenic activity when comparing high and medium solids centrifuges. Results found a 3.7 times reduction in methane production rate from high solids centrifuge samples. This difference was attributed to the effect of high solids centrifuge shearing on methanogen cells. Researchers suggested that a higher level of shearing would lead to greater cell lysis inhibiting methanogenesis (Chen *et al* 2005). The sludge AD environment contains a complex community of bacteria, many of which are obligate anaerobes (Town *et al* 2014, Shah *et al* 2014). During dewatering oxygen exposure may be increased in the sludge matrix (Chen *et al* 2011) which could additionally have lethal effects on cells preferentially adapted for the anaerobic environment. The growth of *E. coli* bacteria may therefore be enhanced as obligate anaerobes decay and provide a food source for the viable *E. coli* bacteria to grow.

In response to other stressors such as heat, Noor (2015) identified lysis of viable but non-culturable (VBNC) *E. coli* cells in the early stationary phase, as a response to heat stress. Findings suggested a possible survival output from the cell lysis, contributing to the removal of damaged cells able to serve as nutrients for the remaining population. Research by Higgins *et al* (2007b) suggests that the effects of AD cause *E. coli* cells to enter a VBNC state which is reversed during mechanical dewatering as the environment favours the growth of indicator bacteria. Higgins *et al* (2007b) attributes the VBNC mechanism to the increased *E. coli* density observed in stored biosolids. Based on research by Noor (2015) it

may be feasible to suggest that a proportion of the VBNC cells (resulting from unfavourable conditions in AD) lyse and provide cellular nutrients for the remaining reactivated bacteria. Confirmation of cellular nutrients supporting viable cell survival is highlighted in Murate *et al* (2012) who studied the release of cytoplasmic materials into a culture medium. Investigations into the activity of β-galactosidase (cytoplasmic enzyme) in incubated culture medium fractions showed that cytoplasmic materials are released into the culture medium as a result of cell lysis and may act as a nutrient supplement for remaining cell population survival. Murate *et al* (2012) argues that further research is required to clarify whether cell lysis increases available nutrients able to support remaining cells survival. In Section 3.4.1 the additions of sludge extract to cake enhanced *E. coli* replication and the possible recovery of dormant cells. The disruption of cells may have a similar effect as cellular nutrients are released and added to the sludge matrix. This may provide a substrate source for viable cells to grow. Therefore the first hypothesis tested in this study is that:

1. Cell disruption provides a substrate source able to support the growth of *E. coli* bacteria in sewage sludge cake stored under favourable conditions.

Research has shown that effects from mechanical dewatering and other environmental parameters such as heat stress are able to cause cell lysis which, in favourable conditions may support *E. coli* growth. Following growth, the effect of disruption treatment and the release of cellular nutrients may have a long term effect on *E. coli* survival. Sludge cake will contain a finite amount of nutrients available for cell growth. The increase in nutrients after disruption treatments are likely to be rapidly assimilated by the viable *E. coli* bacteria. The elevated concentrations of bioavailable substrates may be exhausted quickly under increased storage temperatures leading to cell starvation over time as the metabolic rate of cells and biosynthesis is increased under higher temperature regimes (Todar 2012).

It might therefore be possible to achieve a greater *E. coli* death rate if treatments causing cell lysis are coupled with unfavourable biosolids storage conditions such as those investigated in Section 3.4.3. An evaluation of up-stream sludge

treatment processes in Section 3.2 identified the inhibitory effects of AD contributing to *E. coli* die-off. The anaerobic environment and elevated reactor temperature of AD causes a significant reduction of pathogenic indicator bacteria (Smith *et al* 2005, Alberts *et al* 2010, Scaglia *et al* 2014, Rosenblum *et al* 2014). When similar conditions are applied to biosolids storage environments greater cell die-off is observed for *E. coli* indicator bacteria (Figure 3.5). Following disruption treatment it may be possible to initiate a rapid growth phase proceeded by greater cell death as nutrient exhaustion occurs more rapidly under inhibitory storage environments The second hypothesis tested in this work is that:

2. Following cell disruption, enhanced *E. coli* die-off is achieved in controlled biosolids storage environments with higher temperatures and a gas evacuated or modified atmosphere.

6.3 Methodology

6.3.1 Sludge sampling and storage

Samples of sludge cake, which had been both mesophilic digested and centrifuge dewatered were collected from a wastewater treatment works serving a population equivalent (PE) of 440,000. Representative sub-samples were taken to form a bulk sample of approximately 26 kg and were collected at a depth of 0.5 m using a spade or ‘corer’ device from central locations on the sludge stockpile after the 0.5 m surface ‘crust’ had been discarded. From this bulk (26 kg) sample sub-samples were taken for the subsequent experimental treatments. The sludge cake samples collected from the treatment site were less than 24 hours old from the point of centrifuge output and enabled laboratory tested storage environments to begin from a ‘day 1’ assumption. Samples were placed in screw lid sampling pots (Nalgene, Rochester, USA.) and transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2-8°C to suppress biological activity. Samples were processed within 24 hours of sampling**.**

Samples collected from the treatment site were then used in the three sets of experiments described below.

6.3.2 Experimental treatments

Hypothesis 1: Cell disruption provides a substrate source able to support the growth of E. coli bacteria in sewage sludge cake stored under favourable conditions.

6.3.2.1 Thermal disruption

Samples of 50 g x 6 were subjected to treatment at 62°C (± 2°C) for 20, 80 and 120 minutes (Gallenkamp, A. Gallenkamp and Company Ltd. London, England). An additional comparative treatment included 50 g x 4 samples subject to autoclaving at 121°C for 15 minutes at 1.03 Bar (± 0.03 bar (Priorclave, London, UK). For experimental set up; 100 g (50 g x 2) of each thermally treated sludge cake sample was placed into a glass beaker (FisherBrand, Loughborough, UK). One (100 g) autoclaved sample was mixed with an additional 0.5 g of undisrupted sludge cake to reintroduce a community of sludge derived *E. coli*. Alongside the thermal treatments, an undisrupted sample of sewage sludge cake (100 g) was placed into a glass beaker (FisherBrand, Loughborough, UK) and tested as a control condition. All samples were well mixed, covered with Parafilm M™ (FisherScientific, Loughborough, UK) and stored at 22°C (± 2°C) for 10 days. Flow cytometry analysis distinguished intact and damaged cell concentrations and membrane filtration determined *E. coli* bacterial levels. All sample conditions were repeated in triplicate.

6.3.2.2 Physical disruption

Microcosms of 3 g fresh sewage sludge cake sample were mixed with 15 mL de-ionised water and sonicated for 7 minutes at 33 W (MicroTip, Virsonic 600, VirTis, USA). Additional microcosms were set up and not subjected to sonication treatment, remaining as control conditions. All samples were covered with Parafilm M™ (FisherScientific, Loughborough, UK) and stored at 22°C (± 2°C) for 10 days. Flow cytometry analysis distinguished intact and damaged cell concentrations and membrane filtration determined *E. coli* bacterial levels. Dissolved Organic Carbon (DOC) analysis was conducted on samples before and after physical disruption treatment. All sample conditions were replicated in triplicate and each microcosm was used sacrificially on the day of testing.

6.3.2.3 Thermal disruption combined with controlled storage

Hypothesis 2: Following cell disruption, enhanced E. coli die-off is achieved in controlled biosolids storage environments with higher temperatures and a gas evacuated or modified atmosphere.

Sub-samples of 50 g were subjected to thermal disruption treatment at 62°C (± 2°C) for 80 minutes (Gallenkamp, A. Gallenkamp and Company Ltd. London, England). Following disruption DOC, Total Oxides of Nitrogen (TON) and flow cytometry analysis was completed to measure the level of disruption achieved. For condition set up; thermally treated samples of 100 g (50 g x 2) were placed into plastic, 'zip seal' bags (18 x 18 cm). The bags were then left open, purged with nitrogen gas, or had a gas evacuated treatment using a vacuum pump (KNF Neuberger N035.1.2AN.18, Witney, UK). The gas evacuated condition was double bagged to reduce risk of a breached seal allowing gas to re-enter the sample bag. Alongside the thermal disruption samples, 100 g undisrupted samples were also set up as control conditions. In addition to different storage atmospheres, storage temperature was also manipulated. Samples were either stored at 20°C (± 2°C), 25°C (± 1°C) or 37°C (± 0.5°C). *E. coli* concentrations, pH and TON levels were monitored at intervals of 0, 7, 14 and 21 days. Each 100 g sample bag was sacrificially used for one time point only. Three replications of each sample condition were incorporated into the experimental design giving a total of 216 sample bags. See Figure 6.1 below for the experimental set up.

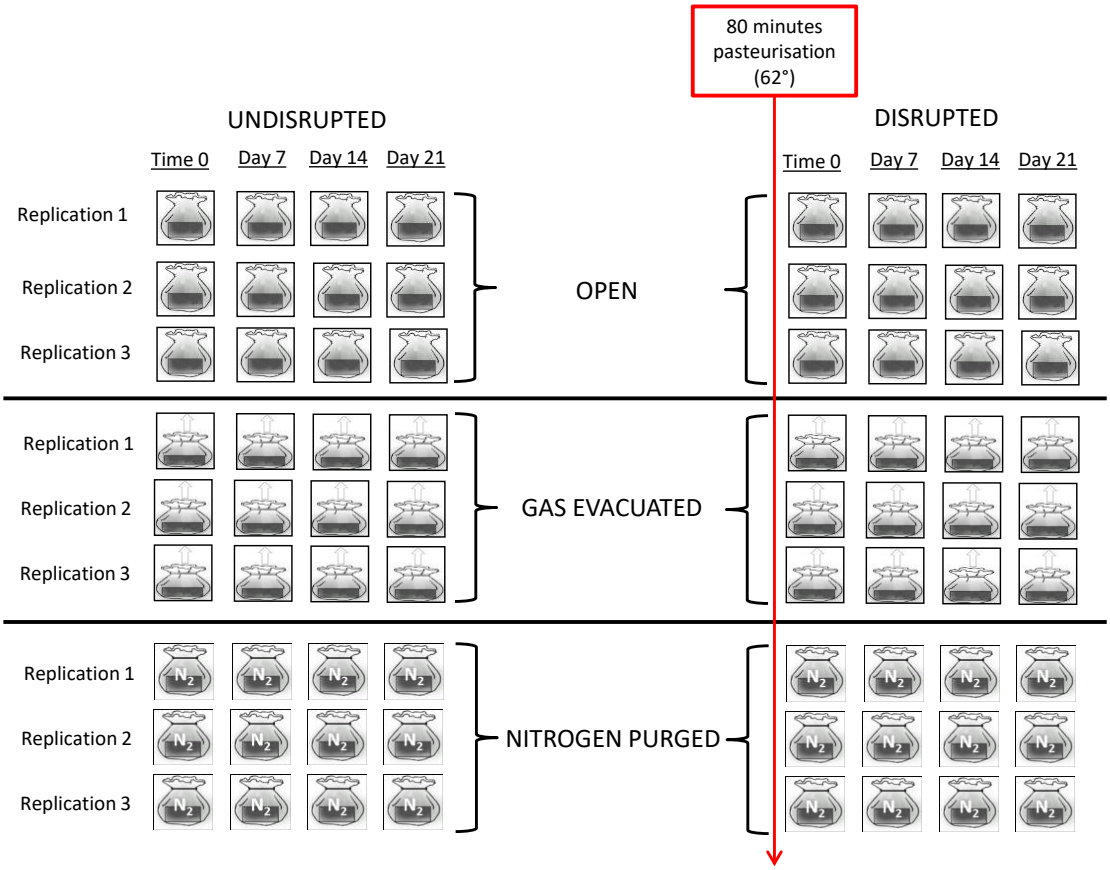

Figure 6.1 Set up for experiments testing the effects of thermal disruption followed by storage under controlled temperature conditions and modified atmosphere treatments. Diagram represents the number of samples held under one of three temperature conditions tested.

6.3.3 Analytical methods

6.3.3.1 Microbial enumeration

Membrane filtration (Environment Agency 2003b) was used for bacterial enumeration using Membrane Lactose Glucuronide Agar (MLGA) (OXOID, Basingstoke, UK) plates to distinguish *E. coli* from coliforms. From each sample condition, 3 g of material were removed and mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube. To ensure effective homogenization, samples were subjected to vortexing for 1 minute (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA.) prior to settling for a further 20 seconds. The supernatant was then removed for serial dilution. Samples were serial diluted with MRD to ensure colony forming units (CFU) were <80/plate. Samples were passed through 0.45 µm filters (Millipore S-PAK® 47

mm, Watford, UK) using a 3-way vacuum manifold (CombiSart®, Sartorius UK Ltd., Surry, UK). Filters were placed on MLGA plates and incubated at 30°C (± 1°C) for 4 (± 1) hours and 37°C (± 0.5°C) for 18 (± 2) hours as described by the Environment Agency (Environment Agency 2003b). Arising green colonies were counted as presumptive *E. coli*, yellow colonies were counted as coliforms and pink colonies were recorded as non-coliforms. All *E. coli* enumeration results were normalized against percentage dry solids (DS %). A 3 g sludge sample was used for DS determination and analysis was performed as per Environment Agency (2003b) guidance.

6.3.3.2 Flow Cytometry Measurement (FCM)

For flow cytometric analysis 1 g of sample was vortexed for 30 seconds with 9 g MRD to form a liquid-sludge mix. Samples were sonicated for 2 x 2 minutes (Grant Ultrasonic Bath XUBA1, Grant Instruments Ltd. Cambridgeshire, England) to enhance the disaggregation of sludge flocs within the sample and disperse bacteria from sludge particle attachment. Once sonicated, samples were passed through a 20 µm filter (Cat No: 1004-070, Whatman™, GE Healthcare, Buckinghamshire, England) using a vacuum pump (No35.1.2AN.18, KNF Neuberger Ltd., Witney, UK) to remove sludge particles from the sample, preventing interference with FCM (Foladori *et al* 2010).

Serial dilution was completed on prepared samples using filtered, cell-free (0.1 µm; Millex, Merck Millipore Ltd., Tullagreen, Ireland) bottled mineral water (EVIAN, EVIAN-les-Baines, France) so that the concentration measured with the flow cytometer was always less than 10^6 counts mL^{-1}. Evian water tends to be biologically stable with little variation between bottles (Gillespie *et al* 2014, Lipphaus *et al* 2013).

FCM was completed as described by Gillespie *et al* (2014), Foladori *et al* (2007) and Foladori *et al* (2010); SYBR Green I (10,000 x stock, cat no: S-7567, Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (DMSO, Fisher Scientific, Fair Lawn, NJ) forming a working stock solution of 100 x. For measurement of intact and damaged cell concentrations, a dye mix containing

five parts of 100 x SYBR Green I and one part of Propidium Iodide (PI) (Cat No:P3566, Life Technologies Ltd., Paisley, UK) was prepared. 2.76 µL of this dye mix were added to 230 µL of sample. Following the addition of dye, samples were placed in an incubator shaker (Grant-Bio PHMP, Grant Instruments Ltd. Cambridgeshire, England) set at 620 rpm, 37°C for 13 minutes with the lid closed to ensure homogeneous temperature conditions and to avoid light exposure (Gillespie *et al* 2014). Following incubation defined sample volumes of 50 µL were analysed using a BD Accurri C6 flow cytometer equipped with a 488 nm solid state laser (Becton Dickinson UK. Ltd., Oxford, UK).

Green fluorescence was collected in the 'FL1' machine channel setting at 533 nm and red fluorescence was collected in the 'FL3' machine channel setting at 670 nm with the trigger set on green fluorescence (FL1). No compensation was used. A fixed gate, previously defined by Gatza *et al* (2013) was used as a template with the corresponding instrument settings and determined the intact cell concentration (ICC). Data was processed using the Accurri C6 software (Becton Dickinson UK. Ltd., Oxford, UK). Damaged cell concentrations were defined by a second fixed gate using guidance from Foladori *et al* (2007), Foladori *et al* (2010) and work published by Harry *et al* (2016). Figure 6.2 shows the gate positions used.

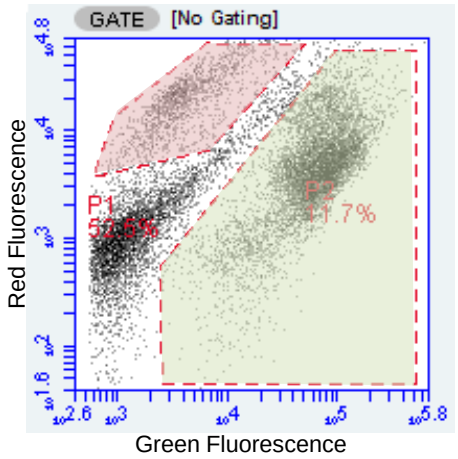

Figure 6.2 Representative FCM cytogram showing distribution of microbial clusters in samples of prepared sewage sludge cake. Signals from intact cells are within the P1 (green) gated region (Gatza *et al* 2013) and signals from damaged bacteria (Propidium Iodide stained cells) are shown in gate P2 (red shaded region) (Folardori *et al* 2007, Foladori *et al* 2010, Harry *et al* 2016). Other signals are caused by organic and inorganic background (Harry *et al* 2016).

All measurements were performed in triplicate and FCM analysis at time 0 was always taken pre and post-disruption treatment.

For normalized results in both membrane filtration and flow cytometry analysis, sludge samples were analysed for percentage dry solids (DS%) content (as described in Section 6.3.3.1) based on Environment Agency (2003b) methods.

6.3.3.3 pH measurement

Measurements of pH were conducted following recommended soil pH analysis techniques (GEB 2007), a 3 g sub-sample was removed and mixed with approximately 15 mL de-ionised water in a universal tube. The solution was homogenised by vortexing (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA) for 1 minute followed by resting for 30 minutes to allow stabilisation. A pH probe (FisherBrand Hydrus 300, Loughborough, UK) was used to record pH fluctuations.

6.3.3.4 Dissolved Organic Carbon (DOC) analysis

DOC determination was conducted using a Series 2000 autosampler (Burkard, Uxbridge, UK) following methods supplied by Burkard Scientific. For sample

preparation, 1 g sludge cake was vortexed for 30 seconds with 9 g de-ionised water to form a liquid-sludge mix. Samples were sonicated for 2 x 2 minutes (Grant Ultrasonic Bath XUBA1, Grant Instruments Ltd. Cambridgeshire, England) to enhance the disaggregation of sludge flocs within the sample. Once sonicated, samples were passed through a 20 µm filter (Cat No: 1004-070, Whatman™, GE Healthcare, Buckinghamshire, England) using a vacuum pump (No35.1.2AN.18, KNF Neuberger Ltd., Witney, UK) to remove sludge particles. The Persulphate-Ultraviolet Oxidation method was used to measure DOC concentrations in the prepared sample (Standard Methods for the Examination of Water and Wastewater, 1995).

6.3.3.5 Total Oxides of Nitrogen (TON) analysis

TON determination was conducted using a Series 2000 auto-sampler (Burkard, Uxbridge, UK) following methods supplied by Burkard Scientific and Method 53 of the Ministry of Fisheries and Food (MAFF), Reference Book 427 (1986). Nitrate extraction utilised 20 g of tested sludge cake material placed in a screw lid container (Nalgene, Rochester, USA) and mixed with 100 mL of 2 M potassium chloride solution. Samples were subsequently placed on a reciprocating shaker (Stuart SSL2, Bibby Scientific, Stone, UK) set at 220 rpm for 2 hours (±10 minutes). The automated hydrazine reduction method was used to measure TON (Standard Methods for the Examination of Water and Wastewater, 1995).

6.3.4 Statistical analysis

Using STATISTICA (Version 12, Tulsa, USA.) a normality assessment was conducted on the dataset using the histogram and normal probability plot program function. Variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá, 2007). A repeated measures ANOVA was performed to identify statistical significant difference ($p < 0.05$) between and within the conditions tested. In individual tests the dependent variable was set as either ‘damaged cell concentration’, ‘*E. coli’,* ‘DOC’, ‘TON’ or ‘pH’. Treatment conditions such as ‘undisrupted’ and ‘disrupted’ were set as the categorical factors. The validity of the ANOVA assumptions, including normality of residuals (Gujuarti 2011), were tested through residual analysis. A post-hoc analysis

(Fisher LSD) was carried out to assess the distinct differences between experimental conditions (Marques de Sá, 2007).

6.4 Results

6.4.1 Thermal disruption

A significant difference was found between levels of damaged bacteria in undisrupted (control) and disrupted treatments (Figure 6.3). For thermal treatments at 62°C, results show a significant increase of 48.2% in the concentration of damaged cells. A gradual increase in damaged cells is observed up to 80 minutes of exposure. Between 80 and 120 minutes exposure at 62°C a marginal increase of 5.6% in damaged cells was observed.

Autoclave treatments showed a significant increase of 176.4% in damaged cell concentration when compared to undisrupted samples. In comparison with samples exposed to 62°C for 120 minutes, levels of damaged cells were 128.2% higher in autoclaved treatments. The reintroduction of 0.5 g undisrupted sludge into autoclaved samples, increased the proportion of intact cells by 3%.

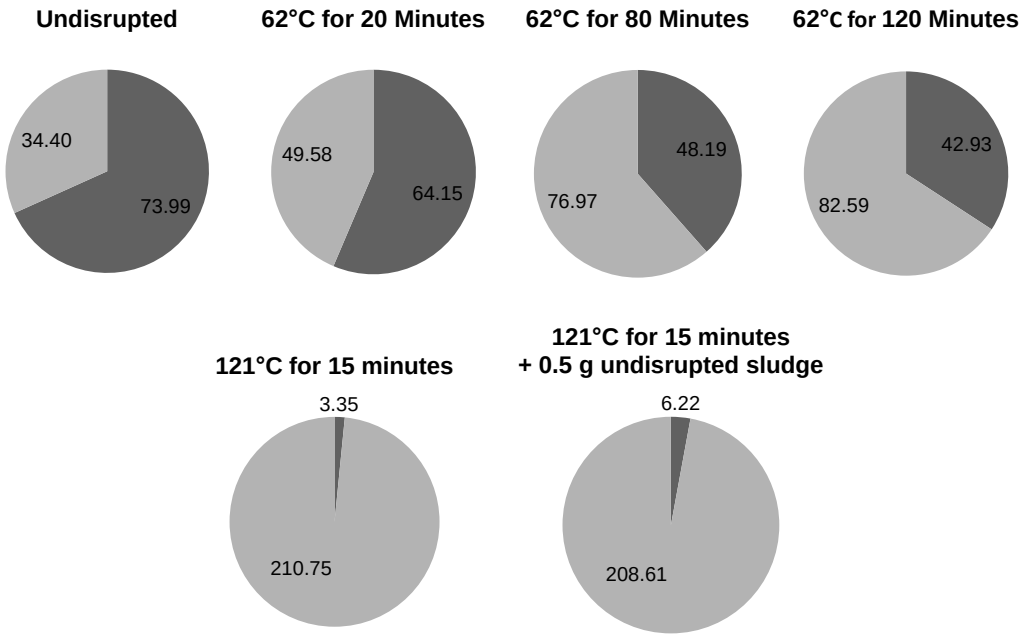

Figure 6.3 Proportions of intact and damaged cells in samples after thermal disruption treatments. Samples thermally disrupted at 62°C for 20, 80 and 120 minutes or autoclaved at 121°C for 15 minutes at 1.03 Bar (± 0.03 bar). Second autoclave sample included 0.5 g undisrupted sludge

addition to reintroduced sludge derived *E. coli* after autoclaving sample. Control condition represents an undisrupted sample. Key: ■ % Intact, ■ % Damaged.

Thermal disruption treatments significantly reduced levels of *E. coli* bacteria. The greatest reduction was observed after 80 minutes exposure where *E. coli* concentrations fell to 0.8 Log and were significantly different from other treatments at day 1 (Figure 6.4B). In contrast, after 120 minutes exposure *E. coli* concentrations only showed a reduction to 2.8 Log (Figure 6.4C). For samples exposed to a greater degree of thermal disruption a longer recovery time was necessary for *E. coli* populations to return back to pre-disrupted levels (or above). Undisrupted samples retained a relatively stable concentration of *E. coli*, averaging 5.9 Log throughout the 10 day test period.

Thermal disruption of samples consistently resulted in an increase in peak *E. coli* concentration when compared to undisrupted treatments. A disruption time of 20 minutes peaked at 7.7 Log, 80 minutes at 7.2 Log, and 120 minutes showed a peak *E. coli* concentration of 7.7 Log. Peak *E. coli* concentrations for each respective treatment were significantly different ($p < 0.05$) from undisrupted samples. By day 10 observations of the 20 and 80 minute disruption treatments showed a plateau in *E. coli* concentrations at 7.3 and 7 Log (respectively). This result was significantly higher from the undisrupted sample concentration. For 120 minutes exposure, no significant difference was shown with undisrupted samples at day 10.

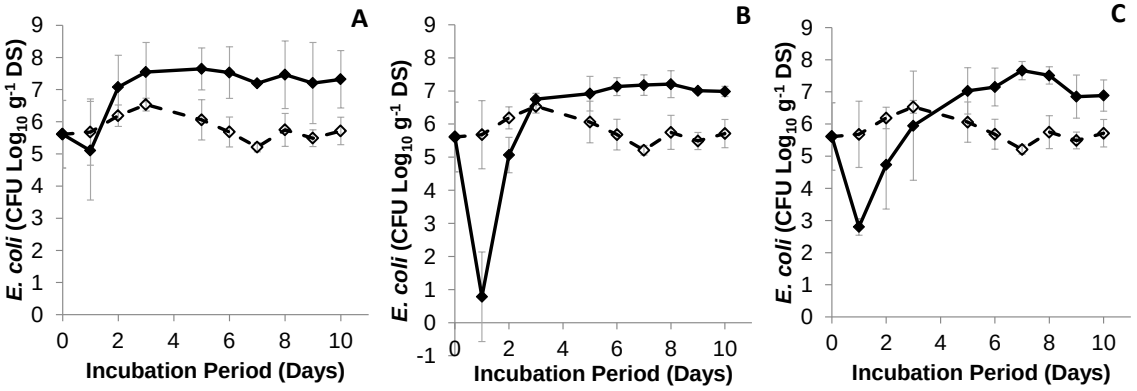

Figure 6.4 *E. coli* concentration during controlled storage at 22°C for 10 days after thermal disruption treatment at 62°C for (A) 20 minutes (—◆—), (B) 80 minutes (—◆—) and (C) 120 minutes

(—◆—). All plots include *E. coli* concentrations recorded from undisrupted control condition (– ◇ –). Error bars represent the standard deviation of three repeats (n=3).

E. coli concentrations fell significantly to undetectable levels after autoclave disruption (Figure 6.5A). For samples inoculated with 0.5 g undisrupted sample after autoclaving *E. coli* concentrations were raised to 2.1 Log at day 1. A significant difference ($p < 0.05$) in peak *E. coli* concentrations was observed between the two autoclaved treatments. A longer recovery time of 6 days was necessary for autoclaved *E. coli* populations to return to pre-disrupted levels. Autoclaved treatments showed a steady increase in *E. coli* concentrations over the test period reaching 6 Log on day 10. For samples inoculated with 0.5 g undisrupted sludge, *E. coli* recovery time was faster and peaked after 3 days at 7.3 Log followed by a steady decline in bacterial concentrations. A comparison with undisrupted samples showed only the autoclaved treatment with the additional 0.5 g undisrupted sample had a significantly higher peak *E. coli* concentration (Figure 6.5B).

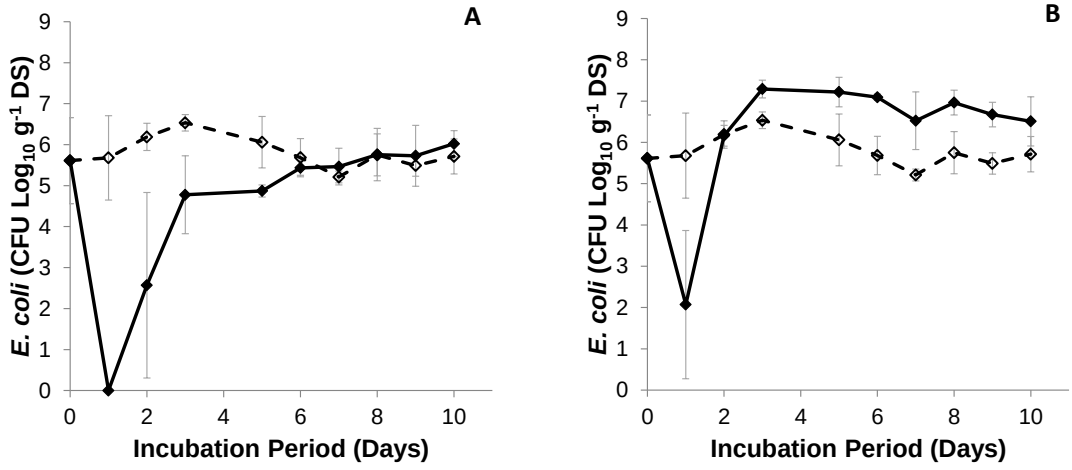

Figure 6.5 *E. coli* concentration during controlled storage at 22°C for 10 days after samples thermally disrupted through autoclaving for 15 minutes at 121°C (pressure = 1.03 Bar ± 0.03). Key: (A) —◆— Autoclaved sample, (B) —◆— Autoclaved sample inoculated with 0.5 g undisrupted sludge sample to reintroduce sludge derived *E. coli*. Both conditions plotted against undisrupted control conditions (– ◇ –). Error bars represent the standard deviation of 3 repeats (n=3).

6.4.2 Physical disruption

Physical disruption significantly increased the proportion of damaged cells in the sludge sample by 51.6% (Figure 6.6).

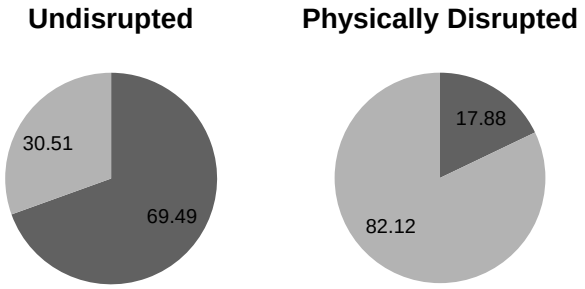

Figure 6.6 Proportions of intact and damaged cells in samples after physical disruption treatment and in undisrupted control condition. Key: ■ % Intact, ■ % Damaged.

A significant increase of 44484 mg/kg DS in levels of DOC after physical disruption treatment was observed (Figure 6.7).

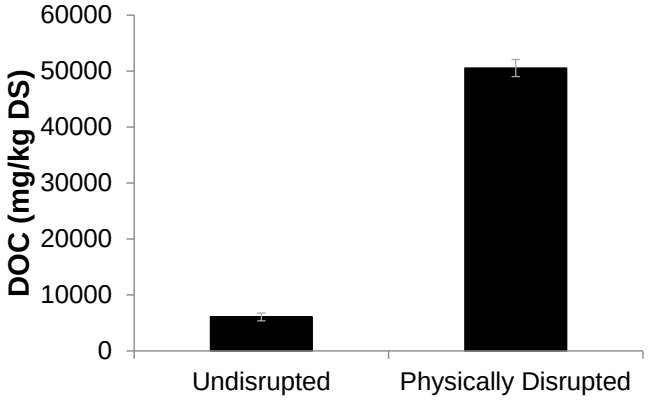

Figure 6.7 Dissolved Organic Carbon concentration in control (undisrupted) and samples exposed to physical disruption. Error bars represent the standard deviation of three replicates (n= 3).

A significant increase of 3 Log was observed in the levels of *E. coli* bacteria between time 0 and day 3 of storage for physically disrupted samples (Figure 6.8). Undisrupted samples remained at a consistent level of 6.6 Log over the 10 day test period. *E. coli* concentrations in physical disruption treatments increased steadily, peaking at 9 Log on day 4 of storage. The peak *E. coli* concentration in disrupted samples was significantly different from undisrupted treatments ($p < 0.05$). Between day 4 and day 10 *E. coli* concentrations in disrupted samples

reduced steadily by 1 Log, although remaining above control treatment conditions.

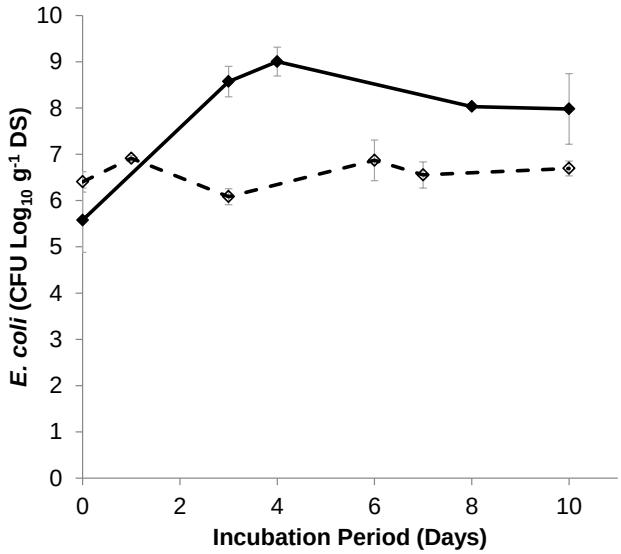

Figure 6.8 Effect of physical disruption treatment on *E. coli* bacteria concentration plotted against control (undisrupted) sample. Key: undisrupted control, physically disrupted sample. Error bars represent the standard deviation of 3 replicates (n=3).

6.4.3 Thermal disruption combined with controlled storage

Thermal disruption treatment at 62°C for 80 minutes showed a significant 67.7% increase in proportions of damaged cells (Figure 6.9).

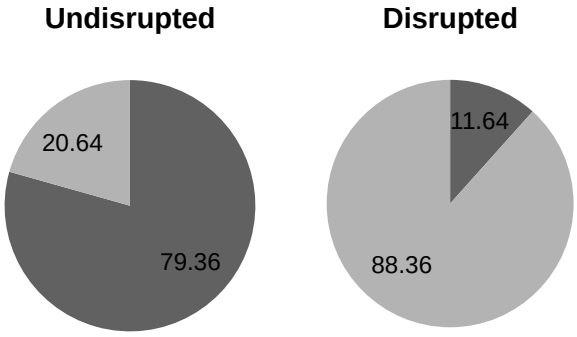

Figure 6.9 Proportions of intact and damaged cells in samples after thermal disruption treatment at 62°C for 80 minutes and undisrupted control condition. Key: % Intact, % Damaged.

A significant increase in levels of DOC and TON was observed after disruption treatment. Disruption resulted in a 55475 mg/kg DS increase in the concentration

of DOC (Figure 6.10). Levels of TON (Figure 6.11) showed a significant increase of 1.85 mg/kg DS between undisrupted and disrupted samples.

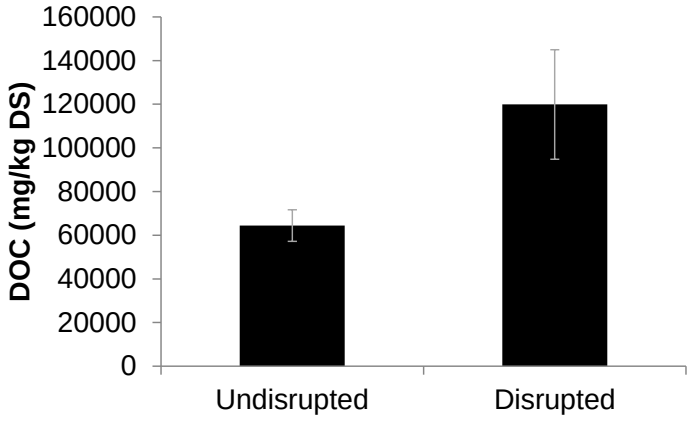

Figure 6.10 Dissolved Organic Carbon concentration in samples exposed to thermal disruption treatment at 62°C for 80 minutes and undisrupted control condition. Error bars represent the standard deviation of three replicates (n= 3).

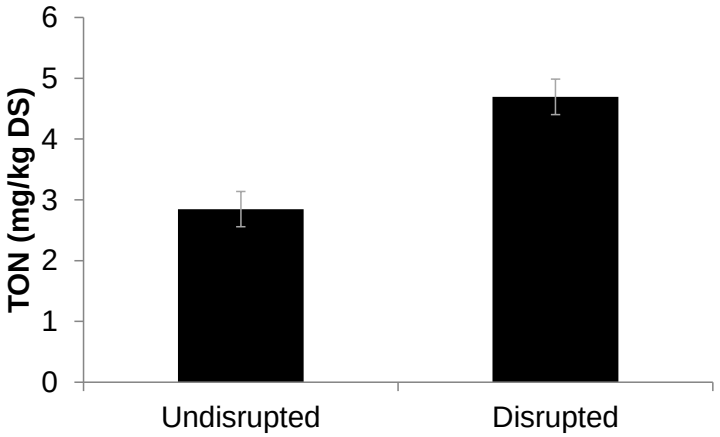

Figure 6.11 Total Oxides of Nitrogen concentration in samples exposed to thermal disruption treatment at 62°C for 80 minutes and in undisrupted control condition. Error bars represent the standard deviation of three replicates (n= 3).

All undisrupted conditions showed a significant reduction in *E. coli* concentration over the 21 day storage period from initial levels of 7.4 Log (Figure 6.12). Storage under 20°C showed the lowest decrease in *E. coli* with approximately a 2 Log reduction observed. No significant difference was observed between the three

modified atmosphere treatments when stored at 20°C. Levels of *E. coli* reduced to a greater extend at 25°C than in the 20°C storage condition. A significant reduction of 5.2 Log was shown under nitrogen purged treatment over the 21 day test period. In comparison to *E. coli* concentrations in other treatments stored at 25°C no significant difference was identified for the nitrogen purged treatment. The greatest decrease in *E. coli* concentrations for undisrupted treatments was at 37°C where concentrations fell by a minimum of 3.8 Log within 7 days. Under the higher temperature treatment both nitrogen purged and gas evacuated treatments reduced to undetectable levels within 14 days and conditions under open storage showed a 5.1 Log reduction by day 21. At temperatures of 37°C there was a significant difference ($p < 0.05$) between open conditions versus the nitrogen purged and gas evacuated treatments on days 14 and 21 of storage. Statistical analysis showed differences between concentrations of *E. coli* at 20°C and 37°C after 7 days of storage for open, nitrogen purged and gas evacuated treatments.

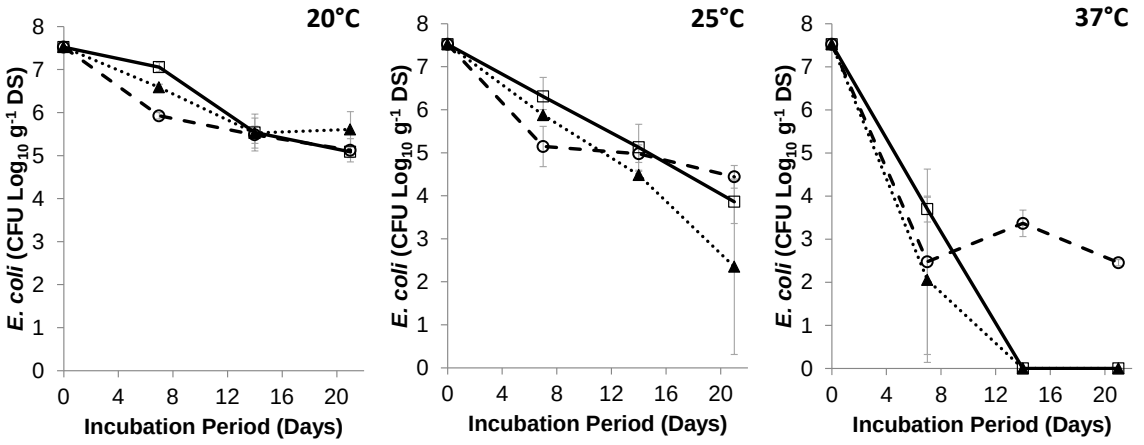

Figure 6.12 Storage temperature and storage condition impact on *E. coli* growth and death over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

Samples exposed to thermal disruption (Figure 6.13) displayed a lower *E. coli* concentration of 3.6 Log at time 0. A significant difference in the *E. coli* concentration between undisrupted and disrupted treatments ($p < 0.05$) was shown. The concentration of *E. coli* never returned to the pre-disrupted level (7.4

Log) over the 21 day storage period. Variability in *E. coli* results after disruption treatment was greater than in undisrupted, particularly at the lower storage temperatures of 20°C and 25°C where *E. coli* growth in nitrogen purged (0.8 Log) and gas evacuated storage (0.4 Log) was identified at day 7. At 20°C storage open and gas evacuated treatments showed a significant decrease to undetectable levels of *E. coli* by day 14. *E. coli* values for nitrogen purged treatments at 20°C showed high levels of variation with concentrations falling by 0.3 Log over the 21 days of storage. No significant differences were shown between the modified atmosphere treatments and open conditions when stored at 20°C. A high degree of variation was also displayed at 25°C between treatments. Open conditions showed a steady reduction to undetectable levels of *E. coli* by day 21. In gas evacuation treatments, an overall reduction of 1.5 Log was observed over the tested storage period. Nitrogen purged treatments fell rapidly to undetectable levels after 7 days of storage and remained at this level until day 21 when enumeration results showed an increase of 4.5 Log to levels higher than initial time 0 concentrations. No significant difference was identified between the storage conditions at each of the time points tested at 25°C. The higher storage temperature of 37°C showed a significant reduction in levels of *E. coli* bacteria between time 0 and day 7 for open and gas evacuated conditions which reduced to undetectable levels. After 14 days all treatment conditions, including samples purged with nitrogen, had reduced to undetectable *E. coli* concentrations.

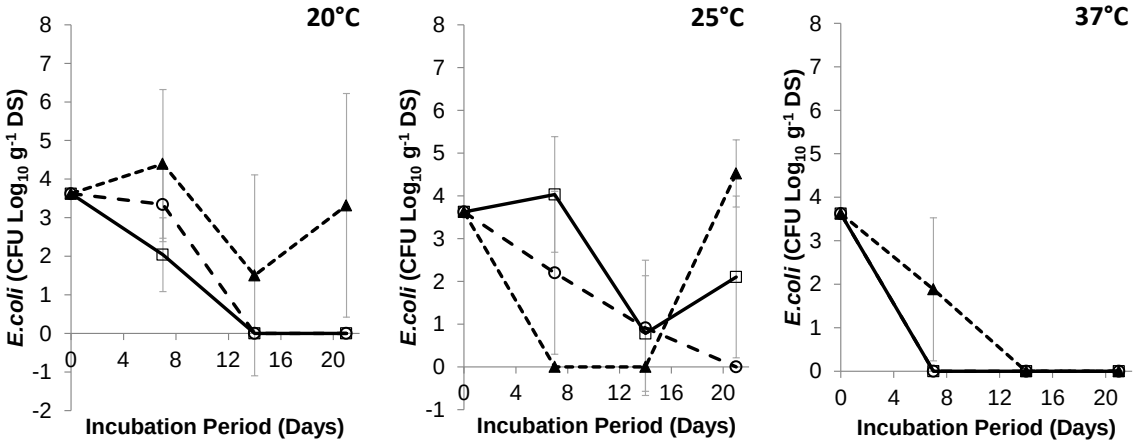

Figure 6.13 Storage temperature and storage condition impact on *E. coli* growth and death over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

The open storage condition showed a consistent pH reduction over the 21 day test period under all storage temperatures tested (Figure 6.14). At the storage temperature of 20°C a small, significant reduction in the pH level for open and nitrogen purged treatments was shown with results indicating a drop of 0.9 pH units by day 21. Gas evacuated treatments remained relatively stable with no significant difference displayed from the initial time 0 value (8 pH units) over the storage period. For 25°C storage, a similar trend was shown, open and nitrogen purged conditions reduced significantly, by approximately 1 pH unit, over the 21 days of storage. Gas evacuated storage at 25°C remained stable with no significant change in the pH value. At 37°C findings showed a greater shift in the pH levels and a statistically significant difference between time 0, day 7 and day 14 for each of the modified atmosphere storage conditions. At the higher storage temperature the pH in the open condition reduced to 6.8 pH units by day 14. This result was significantly different from the nitrogen purged and gas evacuated treatments ($p < 0.05$) which showed an elevated pH level over the 21 day storage period, reaching 8.4 and 8.7 respectively.

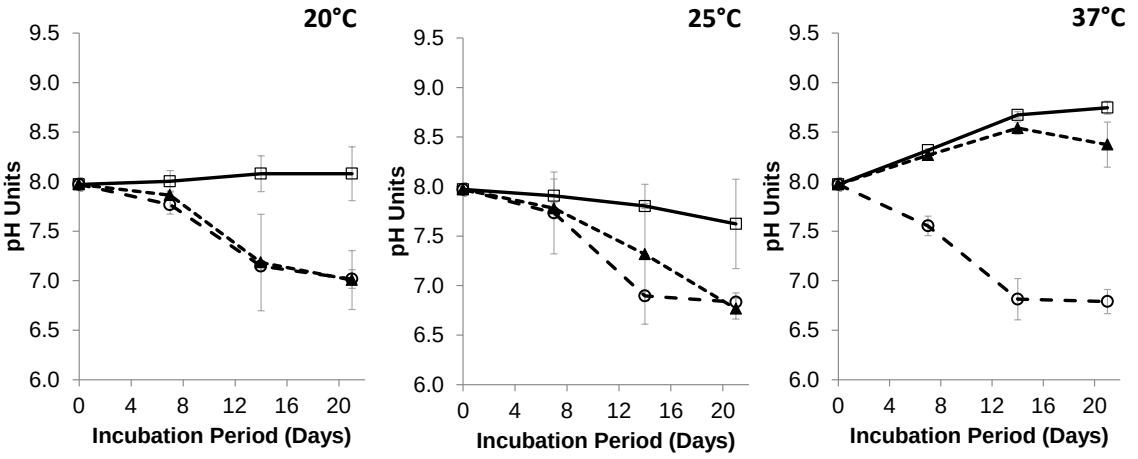

Figure 6.14 Storage temperature and storage condition impact on sample pH over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

For thermally disrupted treatments the initial pH level recorded for time 0 was 7.8 units (Figure 6.15). A comparison between the disrupted and undisrupted treatments showed no significant difference between the results and a similar trend was displayed for each of the three temperatures tested. Open storage treatments show a reduced pH level over the 21 days with significant differences shown in the 20°C storage condition which reduced by 0.5 pH units over the 21 day test period. Gas evacuated and nitrogen purged treatments maintained a stable pH at 20°C with no significant change from time 0 levels. Under 25°C storage pH levels in all treatments remained stable over the 21 day period and show no significant difference between the three modified atmosphere treatments. For 37°C a greater, and more pronounced, shift in the levels of pH was displayed. Gas evacuated and nitrogen purged treatments showed a significant increase of 1 and 0.6 pH units by day 21 (respectively). For the open condition held at 37°C, a significant reduction in pH level was observed between time 0, day 7, and day 14 where the pH level was recorded at 6.7 units.

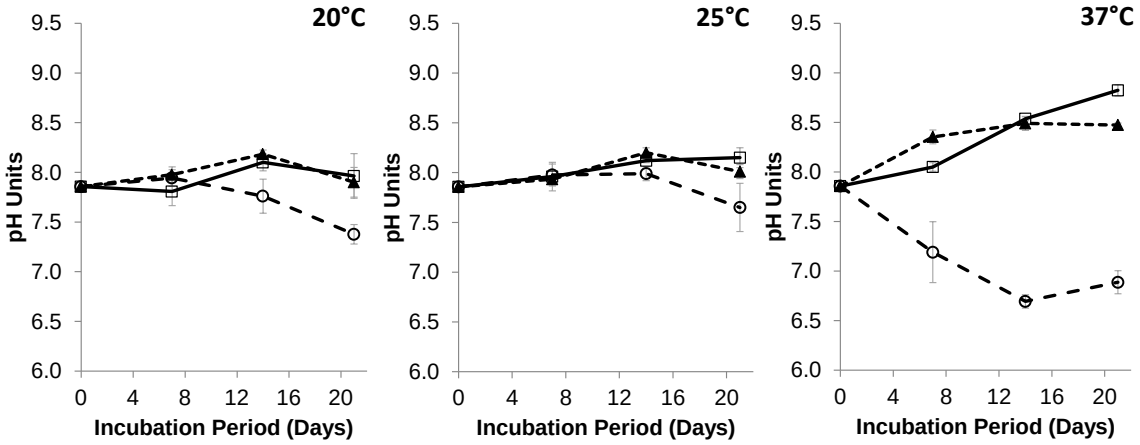

Figure 6.15 Storage temperature and storage condition impact on sample pH over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

In undisrupted treatments, TON showed a gradual increase, particularly at the lower storage temperatures (20 and 25°C) under open and nitrogen purged conditions (Figure 6.16). For open treatments held at 20°C levels of TON increased significantly from the gas evacuated treatments by 207.7 mg/kg DS over the 21 day period. Under the 25°C condition this increase was less and TON levels rose only to 92.6 mg/kg DS, peaking at day 14, where a significant difference from gas evacuated treatments was shown. The nitrogen purged treatment showed the greatest concentration of TON at 25°C and increased significantly to 210.6 mg/kg DS at day 14. The gas evacuated treatment maintained a very low level of TON at 20°C and 25°C with a small increase after 14 and 21 days storage in the 25°C condition to 47.9 mg/kg DS. Treatments stored at 37°C showed very low TON concentrations and displayed no significant change from initial time 0 concentrations (2.8 mg/kg DS).

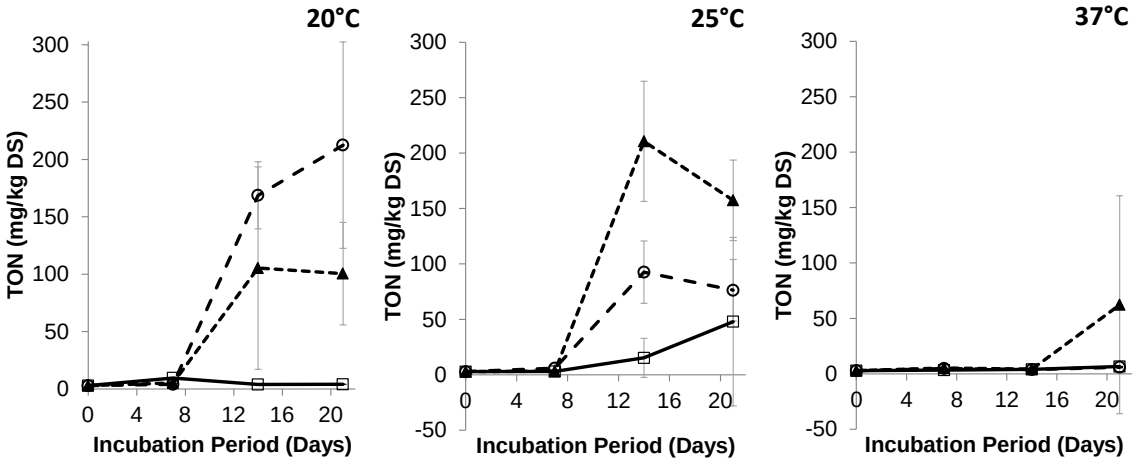

Figure 6.16 Storage temperature and storage condition impact on Total Oxides of Nitrogen (TON) in samples over 21 days (no disruption treatment). Samples stored at; 20°C, 25°C or 37°C storage. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

Low concentrations of TON were observed across all treatment conditions tested after disruption (Figure 6.17). TON concentrations at time 0 were marginally higher than undisrupted treatments at 4.7 mg/kg DS, however no change in TON concentration was observed over the following 21 days of storage for all treatments tested.

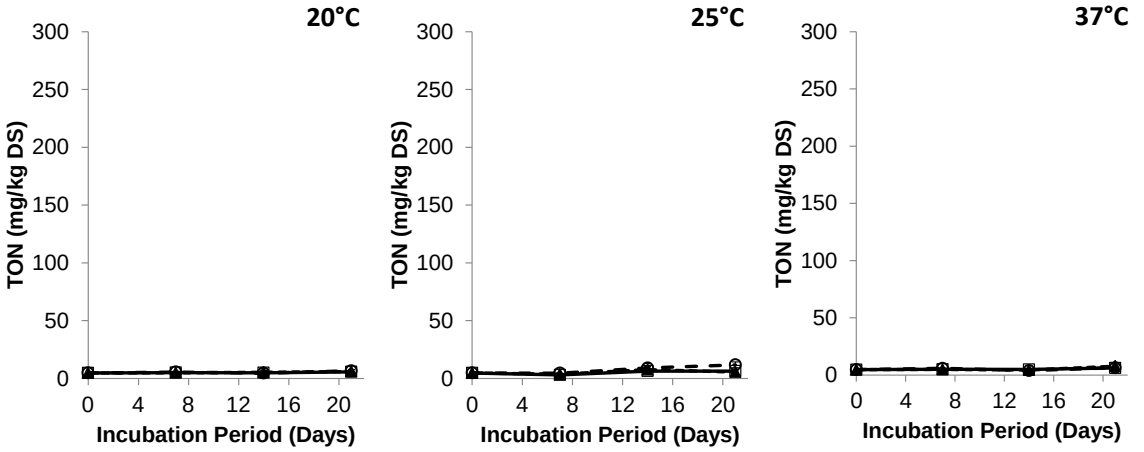

Figure 6.17 Storage temperature and storage condition impact on Total Oxides of Nitrogen (TON) in samples over 21 days after disruption treatment (62°C for 80 minutes). Samples disrupted and stored at; 20°C, 25°C or 37°C. Key: open treatment condition, gas evacuated storage, nitrogen purged condition. Error bars represent the standard deviation of 3 replicates (n=3).

6.5 Discussion

6.5.1 Microbial and nutrient response to effects of disruption treatments and growth in subsequent storage

Indicator bacteria increase in stored biosolids has been attributed to effects of mechanical dewatering. Researchers highlight possible effects of shear impacting upon the integrity of the sludge matrix and causing the release of nutrients which may support bacterial growth (Higgins *et al* 2007b, Chen *et al* 2011, Sun *et al* 2015). Flocculation turbulence and pressure from mechanical operations on flocs can cause cell damage (Sun *et al* 2015). Further to this, researchers have identified cell lysis as a response to mechanical shear and also heat stress factors (Chen *et al* 2005, Noor 2015). This research work assessed the impact of thermal and physical disruption on *E. coli* behaviour in stored biosolids after initial on-site centrifuge dewatering treatments. Laboratory treatments were conducted as a proof of principle to study the effect of cell disruption on *E. coli* dynamics and therefore were not intended to replicate on-site biosolids production processes. In order to clarify whether cell disruption increased available nutrients able to support remaining cell survival (Murate *et al* 2012), measurements of DOC and TON were undertaken and *E. coli* enumeration completed in the subsequent days of storage to determine peak concentrations.

Results showed a significant effect of disruption treatment, both thermal and physical, on levels of damaged cells within test samples. Longer exposure to temperature treatments (62°C) and the combination of high temperature and pressure (autoclaving) caused intact cell numbers to fall by up to 70.6%. Flow cytometry results were compared with *E. coli* enumeration data and reflected similar trends with a reduction in levels of culturable *E. coli* bacteria after thermal disruption treatments. A methodological limitation identified from the thermal disruption treatments was the lower level of *E. coli* bacteria inactivation identified after 120 minutes exposure to 62°C where *E. coli* concentration was only reduced to 2.8 Log. This result was not comparable to the elevated level of cell damage identified in FCM data for 120 minutes and may have been due to sludge flocs protecting *E. coli* bacteria and reducing effects from thermal stress (Chang *et al*

2001, Qi *et al* 2007). Presence of highly aggregated structures or flocs in the tested sludge cake where bacteria may be embedded (Orruño *et al* 2013) could protect cells from the 62°C disruption treatment and enable them to remain in a viable state and grow when returned to a lower, more favourable storage temperature (such as the 22°C incubation temperature). Therefore a pre-treatment to disrupt flocs may have been a beneficial stage of sample preparation.

Physical disruption was likely to be more similar to actions experienced in the sludge matrix during mechanical dewatering. Although effects of physical disruption treatments on *E. coli* were less prominent, FCM data showed damaged cells to increase by 51.6% in tested samples, indicating that other, perhaps more sensitive, sludge-derived bacteria were affected by the treatment. For *E. coli* concentrations, the reductions displayed after disruption treatments emphasised the inhibition of culturable cells. Though *E. coli* colonies were reduced on the culture media (in some cases to undetectable levels), this cannot be attributed to cell death and most likely reflects a state of VBNC in cells exposed to disruption treatments. Oliver (2010) suggests that incubation outside of the normal temperature range of growth can induce the VBNC state and is a common response to stress in bacterial cells. We know this may be true from the *E. coli* growth curves generated in the following days of storage at 22°C where, in all disrupted treatments, concentrations recovered to pre-disrupted levels. Oliver (2010) studied the effects of temperature on culturability in three strains of *V. vulnificus* incubated at 11°C to induce a VBNC state (*V. vulnificus* enters the VBNC state when exposed to temperatures <13°C). After a temperature upshift to 22°C for 24 hours all three strains tested resuscitated to levels above 10^4 CFU mL^{-1} (Oliver 2010). The incubation temperature of 22°C in tested samples for the 10 days of storage may have provided an adequate environment for cell resuscitation and growth where viable cells could utilise the nutrients released from cells damaged during the disruption treatments. Likely evidence of the VBNC mechanism may be identified in autoclave treatments where CFU's reached undetectable levels after treatment but recovered to 2.6 Log within 24 hours of incubated storage. A longer storage period may have allowed the peak

E. coli concentration to be reached in autoclaved treatments as cell recovery time was slower. However, the significant change in concentration between time 0 to 24 hours suggests cells were able to recover and grow rapidly after such an extreme thermal treatment. Higgins *et al* (2007a) highlighted that after dewatering, conditions develop that encourage rapid growth of coliforms with concentrations peaking in 1-3 days of storage. They attribute reactivation of VBNC cells to shear in mechanical dewatering releasing growth factors which resuscitate bacteria to a culturable state (Higgins *et al* 2007b). In our experiments, growth factors such as cellular nutrients have been quantified through the surrogate measure of DOC concentrations. Data showed a significant rise in levels of DOC present in the sludge matrix after disruption treatments (DOC increased by 4448 mg/kg DS after physical disruption of samples) which coincided with elevated concentrations of *E. coli* bacteria. This finding is similar to a study by Franz *et al* (2008) who found microbial activity per unit of biomass to be positively correlated with concentrations of DOC in manure-amended soils. Natural sources of carbon in sewage sludge will be present in organic compounds (Sigma Aldrich 2016) however this may not be in a bioavailable form. Carbon is an important component of cellular material (Todar 2012) and therefore a critical element required for microbial growth. Tang *et al* (2012) observed growth stimulation of *E. coli* bacteria in assays with DOC-rich samples. In biologically treated sewage effluent, Shon *et al* (2007) highlighted the constituents of DOC which included amino acid, polysaccharide and protein. It is likely that the constituents of DOC identified by Shon *et al* (2007) are also present (perhaps to a more limited extent) in sewage sludge and therefore the increase in DOC observed after laboratory disruption treatments is a relevant measure of increasing nutrient levels in the biosolids tested. As sewage sludge cake is known to be nutrient limiting after stabilising up-stream AD processes (Smith *et al* 2005) it is likely that any increase in nutrient availability will create a favourable effect on the growth of microorganisms.

In these experiments physical and thermal stress was imposed on the biosolids material with the aim of increasing cell disruption (lysis) which was quantified using FCM of damaged cells. Although FCM data shows damaged cell increase

in post-disruption treatments, methods for identification are based upon cell membrane integrity. Cells with a compromised membrane will allow the PI dye to enter and bind to the DNA, displacing the SYBR Green I dye (Gillespie *et al* 2014) and classifying the cell as 'damaged'. It should be considered that a cell, if still metabolically active, may be able to repair a partially damaged membrane (Camper and McFeters 1979, Gillespie *et al* 2014) and therefore recover to an 'intact cell' status based on the FCM methods applied. This presents concern as 'damaged' cells have been taken as a surrogate measure of disrupted cells whose cellular material is able to be utilised by the remaining cell population. As Shon *et al* (2007) shows DOC to include cell fragment, extracellular enzyme, DNA, RNA and other components of cellular material it is plausible to assume that in the experiments conducted, cell lysis was achieved from the disruption treatments imposed and is verified by the increased DOC levels in disrupted samples. To add to this, the growth of *E. coli* bacteria during incubated storage of the disrupted samples consistently showed a significantly higher peak *E. coli* concentration when compared to undisrupted treatments, indicating an increase in available nutrients for growth.

These findings suggest that cell disruption does provide a substrate source able to support the growth of *E. coli* bacteria in sewage sludge cake stored under favourable conditions and therefore the first hypothesis tested can be accepted.

6.5.2 Disruption treatment combined with environmental parameter modification of storage environments negatively impact upon *E. coli* dynamics

It has been highlighted by Higgins *et al* (2007b) that storage environments post-dewatering may favour the reactivation and growth of bacteria. Manipulating the physical parameters of the storage environment to create inhibitory growth conditions, such as those observed in AD, Modified Atmosphere Packaging (MAP) and ensiling, may enhance cell die-off (Smith *et al* 2005, Herrmann *et al* 2011, Rosenblum *et al* 2014, Garrido *et al* 2016). The second phase of this work tested whether following cell disruption, enhanced *E. coli* die-off was achieved in

controlled biosolids storage environments with higher temperatures and a gas evacuated or nitrogen purged atmosphere.

For undisrupted samples, the significant difference between the 20°C and 37°C storage temperatures tested emphasise the death rate of *E. coli* bacteria increasing with higher storage temperature. These results verify previous laboratory outputs generated in Section 3.4.3 and confirm temperature to be a critical environmental factor promoting *E. coli* reduction in biosolids storage. For disrupted treatments the effect of temperature was the dominant factor influencing *E. coli* death rates as treatment effects from modified atmosphere conditions were variable, particularly at the lower storage temperatures tested. Following disruption, *E. coli* levels were greatly reduced and showed no significant recovery to pre-disruption levels. This observation was not anticipated as the level of growth displayed in results from thermal and physical treatments (Sections 6.4.1 and 6.4.2) showed *E. coli* bacteria after disruption to increase significantly above pre-disruption concentrations. The reduced growth observed in the thermal disruption combined with controlled storage experiments may have been due to the restricted sampling times over the 28 day study as only weekly enumerations were completed. In previous disruption experiments daily sampling was undertaken and peak *E. coli* concentrations were typically identified in the first 4 days of storage. Under increased temperature regimes *E. coli* growth may have occurred more rapidly followed by a reduction in concentration due to the unfavourable storage conditions present. Therefore daily sampling in the first week of storage may have shown a higher peak *E. coli* concentration resulting from cell disruption nutrient release and consequential growth.

Storage at 37°C reflected the greatest reduction in *E. coli* bacterial for disrupted samples with *E. coli* reaching undetectable levels by day 7 of storage in all treatment conditions tested. Initial expectations were that disruption, followed by storage in unfavourable conditions would encourage a faster rate of *E. coli* die-off. The results do not show this for all conditions tested however and only at the 37°C storage temperature was an enhanced level of die-off observed. A possible explanation for this may be that at low temperatures the metabolic rate of *E. coli*

bacteria is slowed and therefore the assimilation of nutrients and consequential growth is reduced. The nutrient reserves generated from disruption treatments may have provided the bacteria with a sufficient substrate reservoir to prolong survival and therefore a longer storage time may have been necessary for disrupted treatments to reach undetectable levels. Findings on elevated storage temperature enhancing die-off rates are consistent with results from Semenov *et al* (2007) who demonstrated a significant decline in pathogens (*Salmonella* and *E. coli* 0157:H7) with increasing mean temperatures (7 to 33°C) in cow manure microcosms. Pathogen decline was attributed to greater stress and energy expenditure for organism survival (Semenov *et al* 2007) and was reflected by *E. coli 0157:H7* declining to undetectable levels in samples exposure to a constant temperature of 33°C after just 7 days. In comparison, at 23°C *E. coli 0157:H7* only reduced to 6.09 (± 0.05) g dw^{-1} after 2 weeks of storage. The greater level of die-off experienced in samples stored at 37°C could also be attributed to the higher antagonistic activity and competition from other indigenous microorganisms (Cools *et al* 2001). This literature supports experimental results as storage at 20ºC caused less cell suppression and more variability between treatments. Although the disruption treatment reduced concentrations to below the critical 10^5 (MAC 5) threshold (Environment Agency 2003a), only under the 37°C storage condition was almost complete inhibition observed. Although the optimal temperature for *E. coli* bacterial growth is 37°C (Alberts *et al* 2010) heat tolerance might be reduced under hostile environmental conditions causing physiological stress (Lang and Smith 2008).

The substantial drop in concentrations stored under gas evacuated and nitrogen purged treatments at 37°C shows the enhanced inactivation efficiency achieved when higher temperatures are coupled with modified atmospheres in undisrupted samples. In nitrogen purged treatments oxygen availability was assumed to have been removed or greatly diluted in the sealed sample bags. The physical effects of gas evacuated storage will have reduced pore spaces in the sludge cake sample and caused mild compression effects in the material. Containment in a sealed bag for both nitrogen purged and gas evacuated samples would have additionally prevented oxygen diffusion. Whether the conditions tested achieved

complete anoxia is uncertain and methods would have benefitted from a measure of the oxygen concentration in sealed samples. As such, it is difficult to attribute the enhanced die-off observed in samples stored under modified atmosphere treatments to oxygen depletion. The variability shown in *E. coli* concentration for disrupted samples under the gas evacuated and nitrogen purged treatments may be a result of inconsistencies with the level of oxygen available to cells. However, a counter argument to this is the effects of atmosphere control shown in food preservation methods such as MAP (Floros and Matsos 2005). Investigations into modified atmosphere treatments to partially dilute oxygen, found significant inhibition of viable bacteria in modified atmosphere treatments of 40% carbon dioxide, 30% nitrogen and 30% oxygen compared to control conditions where full oxygen exchange was available through a permeable packing film (Garrido *et al* 2016). This evidence supports results showing greater cell die-off in both gas evacuated and nitrogen purged treatments where oxygen availability would be restricted in comparison to the aerobic (open) control condition. In MAP the delay in bacterial growth is also attributed to the level of carbon dioxide in samples as this gas can have a bacteriostatic effect which is related to the breakdown of cell membranes and changes in the physiochemical properties of proteins (Sivertsvik *et al* 2002). Although *E. coil* are a facultative bacteria, aerobic respiration will generate more energy (Alberts *et al* 2010) and therefore under oxygen depleted conditions such as those tested, inactivation of the bacteria is likely to occur as stress on cell functions are increased (Lang and Smith 2008).

The results show that following cell disruption enhanced *E. coli* die-off is achieved under elevated storage temperatures of 37°C. However, the combination of higher storage temperature and gas evacuated or nitrogen purged treatment was not able to significantly accelerate *E. coli* death rate over the tested 21 day storage period and therefore the second hypothesis tested can only be partially accepted.

6.5.3 Chemical response to disruption treatment and subsequent storage environments

Chemical changes to the sludge matrix may give an indication of the mechanisms impacting upon indicator bacteria within the modified storage conditions. For pH levels, no significant difference was identified between undisrupted and disrupted treatments. Results showed pH to be affected to a greater extent by the atmosphere condition tested, with more pronounced shifts in the pH trend displayed under 37°C storage. A process causing acidification of the sludge material could be nitrification which is based on the release of hydrogen ions when ammonium is oxidised to nitrite and nitrate (Rowell 1994). In open treatments the pH level declined across all storage temperatures tested. As nitrifying bacteria are strict aerobes (Gerardi 2002, Rowell 1994) this presents a possible mechanism causing the observed pH reduction. In contrast, for gas evacuated treatments the aerobic nitrifying bacteria were unable to grow and a stable pH was maintained over the 21 day storage period. Temperature will affect rates of nitrification (Geradi 2002) as the optimal temperature range for the growth of nitrifying bacteria is between 28 and 32°C (Geradi 2002). Nitrogen purged treatments showed a reduced pH level at temperatures of 20 and 25°C but maintained a stable pH when stored at 37°C. This result indicates that only partial dilution of oxygen was achieved in the nitrogen purge treatment which, at lower temperatures, provided nitrifying bacteria with sufficient oxygen availability to remain active. The stable pH for nitrogen purged treatments at 37°C suggests that oxygen depletion coupled with elevated temperature prevented nitrification. TON results provide evidence of the nitrification process occurring in undisrupted open and nitrogen purged treatments at 20 and 25°C as TON increased up to 212 mg/kg DS under these conditions after 7 days of storage. Interestingly, the TON levels remained very low (<11 mg/kg DS) in undisrupted samples at 37°C and in all storage conditions exposed to disruption treatment. As pH showed no significant difference between undisrupted and disrupted samples another mechanism may be influencing the pH decline in the storage environments exposed to thermal stress. Nitrifying bacteria are known to grow slowly (Bougard *et al* 2006) and it is therefore likely that a long recovery period would be necessary

after exposure to the 62°C disruption treatment and under higher storage temperatures of 37°C. Concentrations of NH_4-N in undisrupted and disrupted samples were similar and it therefore seems likely that the nitrification process has potential to influence the pH change displayed in disrupted samples. Further investigation into the affect disruption treatment may have on nitrifying bacteria and their recovery may provide indication of why TON levels remained low. For undisrupted samples however, results show the modified storage environments to influence biochemical changes in the sludge matrix. The data suggest that the difference in *E. coli* die-off was a direct result of the environmental factors occurring in the modified storage conditions. This provides evidence of the biological processes present in the treatments tested and identifies the influence physical changes may have on bacterial communities in the stored biosolids. Mechanisms of *E. coli* growth and death will be interrelated with indigenous bacteria behaviour and therefore identifying the inhibition of nitrifying bacteria as a consequence of disruption treatments may suggest that these bacteria cells provide substrate sources for *E. coli* growth, supporting the first hypothesis tested.

6.6 Conclusions

1. Thermal and physical disruption treatments were a successful method to increase levels of cell damage within a sample
2. An increase in damaged cells coincided with higher DOC concentrations indicating cell lysis and an increase in available nutrients for the viable cells remaining
3. Peak *E. coli* concentration was significantly higher for disrupted samples in comparison to undisrupted treatments, suggesting the utilisation of cellular nutrients for growth when samples are placed in favourable growth conditions (22°C).
4. Modified storage conditions for undisrupted samples highlighted the importance of a combined effect from elevated temperature and modified atmosphere achieving greater die-off of *E. coli* bacteria.

5. After disruption *E. coli* concentrations were greatly reduced and further die-off was achieved under higher temperature storage with no treatment effect displayed from modified atmosphere environments. This result indicates that temperature is a critical environmental factor promoting *E. coli* reduction in biosolids storage.
6. The likely release of cellular nutrients from cells damaged during mechanical dewatering may be a contributory factor elevating *E. coli* concentrations in stored biosolids. Results have shown that the modification of environmental parameters within biosolids storage can achieve significant indicator bacteria die-off.

6.7 Acknowledgements

The authors would like to thank the Natural Environment Research Council (NERC) and Severn Trent Water Plc. for their financial support.

6.8 References

ADAS, (2001) THE SAFE SLUDGE MATRIX: Guidelines for the Application of Sewage Sludge to Agricultural Land. Gleadthorpe Research Centre.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Bougard, D., Bernet, N., Chèneby, D., Delgenès, J.P. (2006) Nitrification of a high-strength wastewater in an inverse turbulent bed reactor: Effect of temperature on nitrite accumulation. *Process Biochem*. Vol. 41, pp. 106–113.

Camper, A. K and McFeters, G (1979) Chlorine injury and the enumeration of waterborne coliform bacteria *Applied Environmental Microbiology* Vol. 37 pp. 633- 641

Chang, L.L., Raudenbush, D.L., Dentel, S. K., (2001) Aerobic and anaerobic biodegradability of a flocculant polymer *Water Science and Technology* Vol. 44 No. 2-3, pp. 461- 468

Chen, Y., Higgins, M. J., Maas, N. A., Murthy, S. N., Toffey, W. E., Foster, D. J., (2005) Roles of methanogens on volatile organic sulfur compound production in anaerobically digested wastewater biosolids *Water Science and Technology* Vol 52 No. 1-2 pp. 67- 72

Chen, Y.C., Higgins, M.J., Beightol, S.M., Murthy, S.N., Toffey, W.E., (2011) Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Res.* Vol. 45, pp. 2616–2626.

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., 2001. Survival of *E. coli* and Enterococcus spp. derived from pig slurry in soils of different texture. *Appl. Soil Ecol*. Vol. 17, pp. 53–62.

Dentel, S.K., Qi, Y., Herson, D.S., (2008) Improving the assessment of risk from pathogens in biosolids: fecal coliform regrowth, survival, enumeration and assessment. *Water Sci. Technol*. Vol. 57, pp. 189–193.

Environment Agency, (2003a) The Microbiology of Sewage Sludge-Part 1- An overview of the treatment and use in agriculature of sewage sludge in relation to its impact on the environment and public health. Bristol.

Environment Agency, (2003b) The Microbiology of Sewage Sludge- Part 3- Methods for the isolation and enumeration of *Escherichia coli*, including verocytoxigenic *Escherichia coli*. Bristol.

European Commission (2015) '*Evaluation of sludge treatments for pathogen reduction*' http://ec.europa.eu/environment/waste/studies/sludge_eval.htm [04/01/2016]

Floros, J. D and Matsos, K. I. (2005) Introduction to modified atmosphere packaging. In Han, J. H. (Ed.), *Innovations in Food Packaging* (pp. 159- 184). Elsvier Ltd.

Foladoria, P., Bruni, L., Gianni, A., Ziglio, G. (2007) Effects of sonication on bacteria viability in wastewater treatment plants evaluated by flow cytometry- Fecal indicators, wastewater and activated sludge *Water Research* Vol. 41 pp. 235- 243

Foladori, P., Bruni, L., Tamburini, S., Ziglio, G. (2010) Direct quantification of bacterial biomass in influent, effluent and activated sludge of wastewater treatment plants by using flow cytometry *Water Research* Vol. 44 pp. 3807- 3818

Franz, E., Semenov, A.V., Termorshulzen, A.J., de Vos, O.J., Bokhorst, J.G., van Bruggen, H.C. (2008) Manure-amended soil characteristics affecting the survival of *E. coli 0157:H7* in 36 Dutch soils *Environmental Microbiology* Vol. 10 No. 2 pp. 313- 327

Gatza, E., Hammes, F., Prest, E. (2013) 'Assessing water quality with the BD Accurri™ C6 flow cytometer'. BD Biosciences White Paper

Gerardi, M.H., 2002. Nitrification and Denitrification in the Activated Sludge Process. John Wiley and Sons Inc., New York.

Garrido, M. D., Hernández, M. D., Espinosa, M. C., López, M. B. (2016) Enhanced quality characteristics of refrigerated Seabream (Sparus aurata) fillets packed under different systems (modified atmosphere Vs. vacuum) *Journal of Aquatic Food Product Technology* Vol. 25, No. 2, pp. 156-168

Geotechnical Engineering Bureau (GEB), 2007. Geotechnical Engineering Bureau: Test method for determination of pH value of water or soil.

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Wier, P., Juskowiak, K., Jefferson, B., Jarvis, P., Nocker, A. (2014) 'Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry'. *Water Research* Vol. 65 pp. 224- 234

Harry, I.S.K., Ameh, E., Coulon, F., Nocker, A. (2016) Impact of treated sewage effluent on the microbiology of a small brook using flow cytometry as a diagnostic tool *Water Air Soil Pollution Vol.* 227 No. 57 DOI 10.1007/s11270-015-2723-9

Herrmann, C., Heiermann, M., Idler, C., 2011. Effects of ensiling, silage additives and storage period on methane formation of biogas crops. Bioresour. Technol. Vol. 102, pp. 5153–5161.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Hendrickson, D., Schafer, P., & Farrell, J. (2006). WERF Phase 2: The Impact of Digestion and Dewatering on Reactivation and Regrowth of Viable but Non-Culturable Indicator Bacteria. WEFTEC.

Higgins, M. J., Chen, Y. C., Murthy, S. N., & Hendrickson, D. (2007a). Latest Developments on the Emerging Issue of *E. coli* and Fecal Coliform Reactivation and Regrowth after Dewatering. *Procedings on moving forward wastewater biosolids sustainability: Technical, managerial and public synergy*, pp. 204-19.

Higgins, M.J., Chen, Y.C., Murthy, S.N., Hendrickson, D., Farrel, J., Schafer, P., (2007b) Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Res*. Vol. 41, pp. 665–673.

Lang, N.L. and Smith, S.R. (2008) Time and temperature inactivation kinetics of enteric bacteria relevant to sewage sludge treatment processes for agricultural use. *Water Res*. Vol. 42, pp. 2229–2241.

Lin, H., Zhang, M., Wang, F., Meng, F., Liao, B-Q., Hong, H., Chen, J., Gao, W. (2014) A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: Characteristics, roles in membrane fouling and control strategies. *Journal of Membrane Science* Vol 460 pp. 110- 125

Lipphaus, P., Hammes, F., Kötzsch, S., Green, J., Gillespie, S., Nocker, A. (2013) 'Microbiological tap water profile of a medium-sized building and effect of water stagnation' *Environmental Technology* Vol. 35 No. 5 pp. 620

Mahendran, B., Lishman, L., Liss, S. N. (2012) Structural, physiochemical and microbial properties of flocs and biofilms in integrated fixed-film activated sludge (IFFAS) systems *Water Research* Vol. 46 pp. 5085- 5101

Marques de Sá, J.P., (2007) Applied Statistics using SPSS, STATISTICA, MATLAB and R. Springer- Verlag, Berlin.

Ministry of Fisheries and Food (MAFF), (1986) The Analysis of Agricultural Materials, RB427 ed. Her Majesty's Stationary Office, London.

Monteleone, M.C., Furness, D., Jefferson, B., Cartmell, E., (2004) Fate of *E. coli* across mechanical dewatering processes. *Environ. Technol*. Vol. 25, pp. 825–831.

Murate, M., Noor, R., Nagamitsu, H., Tanaka, S and Yamada, M. (2012) Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*. *Genes to Cells* Vol. 17 pp. 234-247

Noor, R (2015) Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. *SpringerPlus* Vol. 4 pp. 599

Oliver, J. D. (2010) 'Recent findings on the viable but nonculturable state in pathogenic bacteria' *FEMS Microbiology Reviews* Vol. 34 pp. 415-425

Orruño, M., Garaizabal, I., Arana, I., Barcina, I. (2013) 'Validation of a sonication-based method for bacterial dislodgement from flocs' *Journal of Microbiology Research* Vol. 3 No. 6 pp. 208-212

Qi, Y., Dentel, S. K., & Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. *41*, pp. 571- 580.

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Rowell, D.L., 1994. Soil Science: Methods and Applicatons. Longman Scientific and Technical, Harlow, UK.

Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M., Adani, F., 2014. Sanitation ability of anaerobic digestion performed at different temperature on sewage sludge. *Sci. Total Environ.* 466-467, pp. 888–897.

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M. (2007) Influence of temperature fluctuations on *Escherichia coli*

O157:H7 and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol*. Vol 60, pp. 419–428.

Shah, F. A., Mahmood, Q., Shah, M. M., Pervez, A., Asad, S. A. (2014) Microbial Ecology of Anaerobic Digesters: The Key Players of Anaerobiosis *The Science World Journal* Vol. 2014 pp. 1- 21

Shon, H. K. and Vigneswarn, S. (2006) Effluent Organic Matter (EfOM) in Wastewater: Constituents, Effects, and Treatment *Critical Reviews in Environmental Science and Technology* Vol. 36 pp. 327- 374

Sigma Aldrich (2016) Microbology Introduction http://www.sigmaaldrich.com/technical-documents/articles/microbiology/microbiology-introduction.html [Accessed on 05/09/2016]

Sivertsvik, M., Jeksrud, W. K., Rosnes, J. T. (2002). A review of modified atmosphere packaging of fish and fishery products- Significance of microbial growth, activities and safety. *International Journal of Food Science and Technology* Vol. 37 pp. 107- 127

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manag*. Vol. 25, pp. 417–425.

Standard Methods for the Examination of Water and Wastewater, (1995) Automated Hydrazine Reduction Method (19th Edn). Washington DC, USA.

Sun, F., Hu, W., Pei, H., Li, X., Xu, X., Ma, C. (2015) Evaluation on the dewatering process of cyanobacteria-containing $AICI_3$ and PACI drinking water sludge. *Separation and Purification Technology* Vol 150 pp. 52- 62

Tang, J. Y. M., Glenn, E., Thoen, H., Escher, B. I. (2012) *In vitro* bioassay for reactive toxicity towards proteins implemented for water quality monitoring *Journal of Environmental Monitoring* Vol. 14 pp. 1073- 1081

Todar, K (2012) Online Textbook of Bacteriology: Nutrition and Growth of Bacteria http://textbookofbacteriology.net/nutgro.html [Accessed on 05/09/2016]

Town, J.R., Links, M. G., Fonstad, T. A., Dumonceaux, T. J. (2004) Molecular characterisation of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. *BioResource Technology* Vol. 151 pp. 249- 257

Chapter 7

Final Summary

7 Final Summary

7.1 Conclusions of research and implications for practice

7.1.1 E. coli die-off in biosolids is significantly increased at storage temperatures greater than 25°C

An understanding of the temperature regimes effective for the inhibition of *E. coli* growth and to reduce cell survival can support the achievement of microbial compliance targets in stored biosolids. Temperature was identified as a critical parameter controlling cell death rates in the literature review (Section 2.5.1). Assessment of anaerobic digestion (AD) in up-stream sludge treatment processes highlighted the importance of elevated temperature on bacterial inactivation. Temperature regulates cell metabolism and the microbial competition for substrates which will cause microbial inactivation (Smith *et al* 2005, Rosenblum *et al* 2014). In laboratory scale experiments at conditions of elevated temperature (25°C, 30°C and 37°C), a 7 Log decline in concentrations of *E. coli* bacteria was observed at 37°C within 21 days of storage (Sections 3.4.3 and 6.4.3). Elevated temperature increasing *E. coli* die-off was confirmed at an operational scale in pilot trials (Chapter 5). Covered biosolids storage during summer months (June to July 2014) maintained a core stockpile temperature above 25°C and consequentially *E. coli* levels reduced by 3.7 Log over the 28 day test period (Section 5.4), verifying previous laboratory findings in Section 3.4.3. Similarly, an assessment of stockpile temperatures and ambient temperature during the summer months of June to September 2016 in the long term monitoring study (Chapter 4, Figure 4.5) showed a significant effect ($p < 0.05$) on *E. coli* die-off. Survey of literature on analogous environments (Section 2.5.1) such as cow manure showed concentrations of *Salmonella* and *E. coli* to reduce significantly under increasing temperature conditions up to 33°C (Semenov *et al* 2007). The rapid inactivation can be attributed to higher energy expenditure, as elevated metabolic rates increase nutrient demands and cell stress as substrate depletion is increased in the immediate environment (Semenov *et al* 2007, Alberts *et al* 2010). However, it must be accepted that, from these experiments, we cannot conclude whether it is nutrient depletion or another

mechanism that leads to enhanced die-off at elevated temperature. *E. coli* survival in cooler temperatures, as highlighted by Plachá *et al* (2001) and Lang *et al* (2007), was verified in Chapters 4 and 5 during winter study months where temperatures ranged between -4°C and 18°C. A prolonged survival at approximately 7.5 Log *E. coli* concentration was observed for the duration of the first 5 months of storage from October 2015 to March 2016 (Figure 4.4) and in the 69 day winter pilot trial from January to March 2016 (Figure 5.4 A and B).

In the literature survey (Section 2.5.1) lower temperatures were linked with reduced antagonistic activity and competition from indigenous microorganisms (Cools *et al* 2001). Chapters 4 and 5 provide evidence of prolonged survival at lower temperatures in the biosolids environment and the risk of increased compliance failure under these conditions. Pilot trials testing insulated conditions failed to achieve elevated stockpile temperatures and therefore further investigation into the operational feasibility of biosolids stockpile heating would be advantageous to support decisions for the implementation of controlled storage practices able to meet compliance levels.

7.1.2 *Sludge-derived nutrient addition to biosolids matrix increases E. coli growth*

The addition of sludge-derived nutrients to recently dewatered biosolids samples led to a significant 0.5 Log elevation in *E. coli* concentrations within 24 hours (Section 3.4.1). Up-stream sludge treatment processes, such as AD, are designed to stabilise the sludge product (Metcalf and Eddy 2003) and therefore biosolids are considered relatively nutrient limiting in substances required for cell energy generation and growth. The increase in nutrient availability derived from raw (undigested) sludge (Section 3.3.2.1) and the significant change in *E. coli* levels shows the ability of this bacterium to respond quickly to chemical changes in the immediate environment. In Chapter 6 cell disruption was investigated based on literature from Chen *et al* (2011), Sun *et al* (2015) and Murate *et al* (2012) (Section 2.4). Shear forces acting upon the sludge matrix during dewatering can be a possible cause of cell lysis due to flocculation turbulence and pressure from mechanical operations on flocs (Sun *et al* 2015). Thermal and

physical cell disruption increased the concentration of dissolved organic carbon (DOC), indicating cell lysis and release of cytoplasm (Shon *et al* 2007). The experiments were conducted as a proof of the principle that cell disruption leads to increasing levels of cellular derived nutrients which in turn causes a higher peak *E. coli* concentration when compared to undisrupted treatments. Section 6.4 confirmed a significant increase in levels of DOC for samples exposed to disruption treatments and a peak *E. coli* concentration significantly higher than undisrupted conditions. This result suggests that nutrients may be a controlling factor of the *E. coli* growth observed immediately after mechanical dewatering (Higgins *et al* 2007).

It had been anticipated that a rise in available nutrients, following disruption treatments, would increase the rate of *E. coli* die-off when samples were held under unfavourable conditions at elevated storage temperatures (Section 6.2). The highest level of die-off in disrupted treatments was observed at 37°C, with a 4 Log reduction in *E. coli* concentration (Figure 6.13). The nutrient reserves generated by disruption treatments may have prolonged survival at the lower storage temperatures tested (20°C, 25°C) as the additional substrates were utilised at a slower rate. In an open environment, such as biosolids, Van Elsas *et al* (2011) argues that *E. coli* bacteria conserve key genomic adaptations to aid survival in conditions of fluctuating nutrient availability. The mechanisms of adaptation are likely to increase persistence of the bacterium slowing the rate of die-off.

The work was limited to laboratory scale disruption treatments using elevated temperature and sonication, therefore an assessment of treatments relative to sludge dewatering would have been beneficial to understand the degree of disruption that may occur on treatment sites. In Section 6.4.3 the effect of thermal disruption combined with controlled storage conditions such as elevated temperature and gas evacuated storage would have benefitted from a longer storage time with increased sampling frequency for *E. coli* enumeration to improve the resolution of results and study the die-off rates that may have been slower at lower storage temperatures.

7.1.3 *Laboratory scale experiments and field trials confirm oxygen availability is not the dominant factor affecting E. coli die-off rates in stored biosolids*

In Chapters 3 and 6 the effect of elevated temperature combined with gas evacuated and nitrogen purged storage was investigated based upon the success of methods to achieve microbial inhibition applied in agricultural silage making and modified atmosphere packaging (MAP) in food preservation (Section 2.5.3). A combination of elevated temperature and gas evacuated treatment showed up to a 7 Log reduction in levels of *E. coli* bacteria (Section 3.4.3) with undisrupted nitrogen purged treatments in Section 6.4.3 showing similar results within the 21 day test period at temperatures above 25°C. The control of oxygen availability was attributed to the increased inactivation efficiency and results on chemical changes in the storage conditions suggested that *E. coli* die-off was a direct result of the modification of environmental factors occurring in the sludge matrix. In Chapter 6 modified atmosphere treatments had less effect on thermally disrupted samples with some nitrogen purged and gas evacuated conditions showing levels of *E. coli* growth at lower storage temperatures (Figure 6.13). The effect of gas evacuated treatment was tested in pilot trials as described in Chapter 5 with a sealed-bag technology. As biosolids were forced into bags with a hydraulic ram and fully sealed it was anticipated that this treatment would lead to reduced oxygen exposure and a lower redox potential in the tested material. Redox measurements (Figure 5.8) confirmed no treatment effect and showed all stockpiles to be of an anoxic state. No increased *E. coli* die-off was observed in sealed-bag treatments and therefore the pilot trials indicated that oxygen exposure may not be a critical factor for the reduction of *E. coli* indicator bacteria. Similarly analysis of redox potential results from the long term monitoring study (Chapter 4) showed no significant effect ($p > 0.05$) on the concentration of *E. coli* bacteria in the stored material. Although this finding is in contrast to results in Chapter 3 and for undisrupted samples in Chapter 6, no measurement of oxygen availability in the modified atmosphere, sealed bag conditions was undertaken in the laboratory scale experiments. Therefore oxygen depletion cannot be dismissed as a contributory factor to die-off but results suggest that it is not a

dominant parameter, due to the anoxic state of the stored biosolids identified in up-scaled trials.

7.1.4 Moisture content within the ranges tested has no effect on E. coli survival in stored biosolids

Within the operational range of biosolids moisture contents identified on treatment sites (Operational data, Severn Trent Water Plc.) experiments at a laboratory scale investigated whether higher moisture content would cause *E. coli* numbers to increase. The water content of sludge can be an influential factor for the control of indicator organisms (Berry and Miller 2005, Rouch *et al* 2011) as identified in Section 2.5.2. However, experiments testing a dry solids (DS) % between 17 and 35% showed no significant treatment effect on *E. coli* concentration (Figure 3.8). Moisture has been shown to significantly affect *E. coli* levels in Lang *et al* (2007) who studied biosolids-amended soils in long term field trials. In the long term monitoring trials (Chapter 4), statistical analysis confirmed no significant ($p > 0.05$) effect on *E. coli* concentrations (Figure 4.8). Similarly results from pilot trials in Chapter 5 showed relatively stable moisture content throughout both winter and summer test periods (Figure 5.7). Therefore within the range measured for stored biosoilds, moisture content changes are not sufficient to cause a significant shift in *E. coli* concentration.

7.1.5 Polyelectrolyte addition does not cause E. coli concentrations to increase in dewatered sewage sludge

Studies identified in Chapter 2 suggest the effects of polyelectrolyte addition during mechanical dewatering are an important consideration when explaining *E. coli* increase in biosolids products (Monteleone *et al* 2004, Qi *et al* 2007). In Section 3.4.2 the impacts of polyelectrolyte on *E. coli* dynamics were investigated. Observations on polyelectrolyte addition compared with control treatments (no polyelectrolyte dosing) and polyelectrolyte type (FLOPAM and ZETAG) demonstrated no significant difference ($p > 0.05$) between the conditions tested. Following dewatering, all treatments showed a rapid increase of *E. coli* concentrations in the first 48 hours of storage. This result dispels arguments of substrate effects from polyelectrolyte degradation or flocculent influences (Chang

et al 2001). In contrast, the investigation into polyelectrolyte dosing concentration found a higher level of bacterial die-off in samples dosed with 0.5 g/L compared to 0.3 g/L polyelectrolyte solution (Figure 3.3). This observation supports previous literature identified in Section 2.4 suggesting a possible toxicity effect from the polyacrylamide based polyelectrolytes which can degrade to form acrylamide, a substance toxic to cells (Junqua *et al* 2015). The research finding supports operations of continued polyelectrolyte dosing during mechanical dewatering in order to achieve cake quality targets and DS% estimates.

7.2 Contribution to knowledge

Overall this research work has contributed to the identification of factors affecting the dynamics of *E. coli* growth and death in stored sewage sludge cake with a key conclusion on temperature as a critical environmental parameter controlling *E. coli* survival. The research has made an assessment of possible solutions to control indicator growth and enhanced levels of die-off have been achieved under elevated storage temperatures in combination with gas evacuated treatments at a laboratory scale. The significant effect of elevated storage temperature has been verified in up-scaled trials including a long term monitoring programme and seasonal pilot tests that enabled the characterisation of the biosolids environment to be profiled and assessed. Substantial die-off was achieved at temperatures above 25°C in the summer pilot trial under covered treatments indicating significant benefits from modified storage conditions that are able to control *E. coli* bacteria and confidently achieve compliance requirements.

The table below summarises the contributions to knowledge that this research presents:

Table 7.1 Contributions to knowledge presented by the research work

Domains of contribution	**Extent of contribution**		
	What has been confirmed	**What has been developed**	**What has advanced knowledge**
Theoretical knowledge		- Understanding of *E. coli* growth and death in biosolids (all chapters) - Identification of the physical and chemical properties of biosolids and their relative influence on *E. coli* dynamics (all chapters)	
Empirical evidence	- *E. coli* can survive for prolonged periods in stored biosolids at low temperatures (chapter 4 and 5) - Polyacrylamide based polyelectrolytes showed toxicity effects on *E. coli* bacteria (chapter 3) - Nutrient availability in stored biosolids limits microbial growth (chapters 3 and 6)	- Understanding the rates of *E. coli* die-off in biosolids exposed to elevated temperatures (>25°C) (chapters 3 and 5) - The stability of pH, moisture content and redox potential in stored biosolids have no significant effect on *E. coli* concentrations within the ranges observed (chapter 4 and 5)	- At a laboratory scale the combination of elevated temperature (>25°C) and gas evacuated storage increases *E. coli* death rates (chapter 3) - The environmental conditions prevailing in stored biosolids have been quantified for the first time (chapter 4) - Physical disruption of intact cells in dewatered cake leads to release of DOC which in turn promotes *E. coli* growth in stored biosolids (chapter 6)
Methodology		- *E. coli* regrowth following dewatering by filter press and centrifuge has been recreated under laboratory conditions (chapter 3)	- Flow cytometry measurements are a rapid and consistent method for the identification of intact and damaged bacteria in biosolids (chapter 6)
Knowledge of practice		- Significant die-off of *E. coli* bacteria occurs in the first month of storage. Efforts to meet compliance thresholds should target this period to enhance mechanisms which control cell death rates (chapter 4)	- Polyelectrolyte addition to the sludge matrix does not elevate *E. coli* concentrations (chapter 3) - Covered storage in summer presents a method to elevate biosolids temperatures and control *E. coli* survival (chapter 5) - Elevated storage temperatures (>25°C) increase *E. coli* die-off to achieve compliance limits (chapters 4 and 5)

7.3 Future work

7.3.1 Maintaining a stable and elevated stockpile temperature on treatment sites

- *Investigation into methods to increase stockpile temperature and insulate biosolids against the temperature drop typically observed on treatment sites after mechanical dewatering, particularly in cooler seasonal temperatures when prolonged E. coli survival is known to be more prolific.*

The winter pilot trial completed from January to March 2016 showed that the insulated treatments tested were unable to maintain elevated stockpile temperatures. The cooler winter temperature presents a challenging environment for the control of biosolids stockpile temperatures. Therefore further investigation into the operational feasibility of heating methods is necessary.

7.3.2 Cell disruption increasing rates of *E. coli* growth and death in an elevated temperature storage environment

- *An evaluation (at a laboratory scale) of mechanical dewatering including centrifuge and filter press on the level of cell disruption and the release of cellular nutrients able to support growth. Profiling the changes experienced over the dewatering process through flow cytometry measurement, E. coli enumeration and parameters such as DOC.*
- *Tests assessing the combination of disruption treatments with controlled storage environments should be developed. Experiments including an increased sampling frequency for E. coli enumeration and a prolonged storage period in order to study die-off rates in conditions known to suppress bacterial survival.*

Results in Chapter 6 suggest that cell disruption is able to support bacterial growth and potentially prolong survival. Understanding the effects of on-site dewatering processes on cell damage may quantify and develop appreciation of the factors controlling *E. coli* growth observed immediately after dewatering

(Higgins *et al* 2007). In addition, the possibility to enhance the utilisation of released nutrients through modified storage conditions, as suggested in Section 6.2, may increase die-off rates providing a suitable method to predictably achieve compliance levels.

7.3.3 Oxygen exposure providing a selective advantage for facultative *E. coli* bacterial growth during dewatering processes

- *Laboratory scale investigation into whether increased oxygen exposure to the dewatered biosolids causes higher levels of E. coli growth.*
- *Measurement of changes in redox potential throughout the sludge dewatering process in order to identify whether increased oxygen exposure may be a contributory factor to E. coli growth.*

Redox potential results recorded during the 12 month monitoring programme (Chapter 4, Figure 4.9) showed a significant decrease in redox potential levels in the first 48 hours after biosolids stockpiling for study quarters 1 and 4. This finding suggests increased oxygen exposure to the sludge matrix during dewatering (Chen *et al* 2011) and indicates that the maintenance of a higher oxygen availability in fresh stockpiled material may be attributed to the observed increase in *E. coli* density in the first 1- 3 days of storage (Higgins *et al* 2007). Understanding the changes in oxygen exposure during dewatering and whether *E. coli* bacteria are able to respond to these changes may provide indication of a controlling parameter influencing the rapid growth of *E. coli* and present possible strategies to minimise this phenomenon.

7.3.4 Indicator organism: Environmental strains of *E. coli* able to survive for prolonged periods at low temperature

- *Investigation to confirm whether E. coli bacteria have evolved to preferentially adapt and survive in both a host and open (biosolids) environment.*
- *Identify the presence of an environmental reservoir of E. coli bacteria in biosolids products which may present a secondary habitat outside of the normal host environment.*

This research has shown the stability of *E. coli* populations in stored biosolids at low temperatures (Chapters 4 and 5), a phenomenon previously observed in sludge and manure amended soils. In addition, the fate of *E. coli* survival in biosolids has been shown to be influenced by the environmental parameters of the storage environment. The relationships between microbial dynamics in the matrix and abiotic factors have been highlighted. The possibility of an environmental reservoir (Van Elsas *et al* 2011) of *E. coli* in biosolids is supported by this research. Further investigation to prove this theory may pose interesting challenges to the definition of *E. coli* as an indicator organism, presenting important considerations for environment and public health.

7.4 References

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2010) Essential Cell Biology, 3rd ed. Garland Science, Taylor and Francis Group, London.

Berry, E. D. and Miller, D. N. (2005) Cattle Feedlot Soil Moisture and Manure Content: II Impact on *Escherichia coli* 0157 *Journal of Environmental Quality* Vol. 34 pp. 656- 663

Chang, L.L., Raudenbush, D.L., Dentel, S. K., (2001) Aerobic and anaerobic biodegradability of a flocculant polymer *Water Science and Technology* Vol. 44 No. 2-3, pp. 461- 468

Chen, Y. C., Higgins, M. J., Beightol, S. M., Murthy, S. N., & Toffey, W. E. (2011). Anaerobically digested biosolids odor generation and pathogen indicator regrowth after dewatering. *Water Research,* Vol. *45*, pp. 2616- 2626.

Cools, D., Merckx, R., Vlassak, K., Verhaegen, J., (2001) Survival of *E. coli* and *Enterococcus spp*. derived from pig slurry in soils of different texture. Appl. Soil Ecol. Vol. 17, pp. 53–62.

Higgins, M. J., Chen, Y.-C., Murthy, S. N., Henderickson, D., Farrel, J., & Schafer, P. (2007). Reactivation and growth of non-culturable indicator bacteria in

anaerobically digested biosolids after centrifuge dewatering. *Water Research*, Vol. 41 pp. 665-673

Junqua, G., Spinelli, S., Gonzalez, C. (2015) Occurrence and fate of acrylamide in water-recycling systems and sludge in aggregate industries *Environ Sci Pollut Res* Vol. 22 pp. 6452- 6460

Lang, N. L., Bellett-Travers, M. D. and Smith, S. R. (2007) Field Investigations on the survival of *Escherichia coli* and presence of other enteric micro-organisms in biosolids-amended agricultural soil *Journal of Applied Microbiology* Vol. 103, pp. 1868- 1882

Metcalf & Eddy., Tchobanoglous, G., Burton, F. L & Stensel, H. D (2003). Wastewater Engineering: Treatment and Reuse (4th Ed.). Boston: McGraw-Hill

Monteleone, M. C., Furness, D., Jefferson, B., & Cartmell, E. (2004). Fate of *E. coli* Across Mechanical Dewatering Processes. *Environmental Technology*, Vol. 25 No. 7 pp. 825-831.

Murate, M., Noor, R., Nagamitsu, H., Tanaka, S and Yamada, M. (2012) Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*. *Genes to Cells* Vol. 17 pp. 234-247

Placha, I., Venglousky, J., Sasokova, N., Svoboda, I.F., (2001) The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solids of pig slurry. J. Appl. Microbiol. Vol. 91, pp. 1036–1043.

Qi, Y., Dentel, S. K., Herson, D. S. (2007). Increases in fecal coliform bacteria resulting from centrifugal dewatering of digested biosolids. *Water Research,* Vol. *41*, pp. 571- 580.

Rosenblum, J., Bisesi, M., Castano, J., Tamkin, A., Ciotola, R., Lee, J., Martin, J. (2014) Influence of seasonal fluctuation and loading rates on microbial and chemical indicators during semi-continuous anaerobic digestion *Environmental Technology* Vol. 30 No. 10 pp. 1308- 1318

Rouch, D. A., Mandal, T., Pai, S., Glauche, F., Fleming, V. A., Thurbon, N., *et al*. (2011). Microbial safety of air dried and re-wetted biosolids. *Journal of Water and Health*, Vol. 9.2 pp. 403-414.

Semenov, A. V., Van Bruggen, A.H.C., Van Overbeek, L., Termorshuizen, A.J., Semenov, A.M., (2007) Influence of temperature fluctuations on *Escherichia coli O157:H7* and *Salmonella enterica serovar Typhimurium* in cow manure. *FEMS Microbiol. Ecol*. Vol. 60, pp. 419–428.

Shon, H. K. and Vigneswarn, S. (2006) Effluent Organic Matter (EfOM) in Wastewater: Constituents, Effects, and Treatment *Critical Reviews in Environmental Science and Technology* Vol. 36 pp. 327- 374

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manag*. Vol. 25, pp. 417–425.

Sun, F., Hu, W., Pei, H., Li, X., Xu, X., Ma, C. (2015) Evaluation on the dewatering process of cyanobacteria-containing $AICl_3$ and PACI drinking water sludge. *Separation and Purification Technology* Vol. 150 pp. 52- 62

Van Elsas, J. D., Semenov, A. V., Costa, R., Trevors, J. T. (2011) Survival of *Escherichia coli* in the environment: fundamental and public health aspects *The ISME Journal* Vol. 5 pp. 173- 183

Appendices

APPENDICES

Appendix A *E. coli* variability in biosolids sampled from centrifuge output

From June 2013 to July 2016 regular biosolids sampling of mesophilic digested sludge has been undertaken from the centrifuge outlet on a Severn Trent Water Plc. wastewater treatment works. Samples of sewage sludge cake were taken immediately after centrifuge dewatering to ensure the product was less than 24 hours old when experimental trials began. Time 0 *E. coli* concentrations were determined within 24 hours of sample collection at Cranfield University and the variation observed is displayed in Figure A-1.

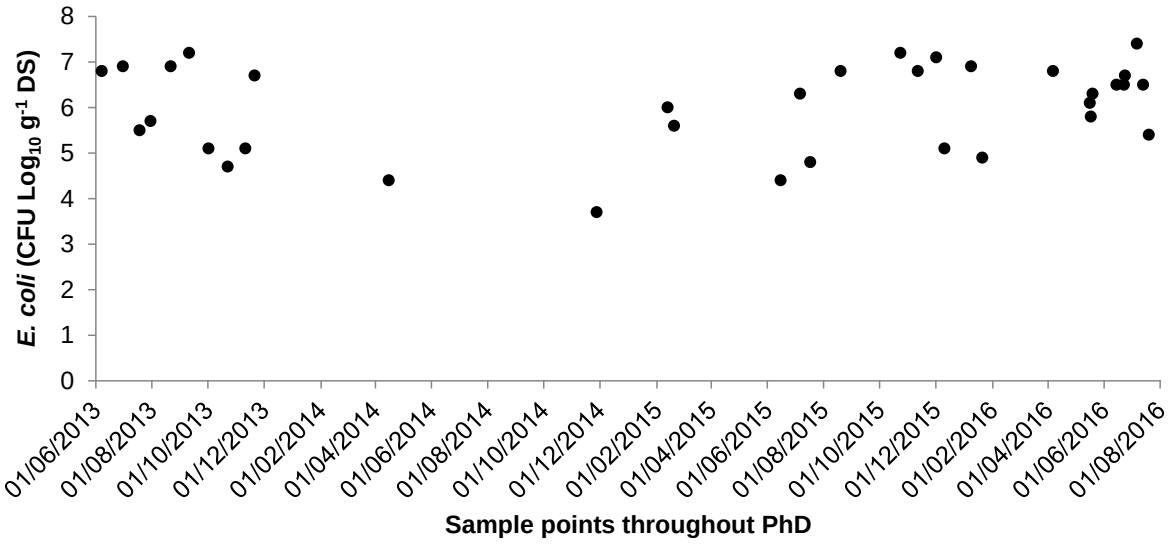

Figure A-1 *E. coli* (•) variation in the mesophilic digested biosolids from the centrifuge outlet at the sample source wastewater treatment site for experiments conducted between June 2013 and July 2016.