EARTHWORMS, FLOODING, AND SEWAGE SLUDGE.

Doctor of Philosophy

TAMSYN BIRGITTA WILSHIRE KISS

University of York

Environment and Geography

October 2019

Abstract

Earthworms are ecosystem engineers. Their burrowing increases soil water holding capacity, changes microbial populations, and improves soil nutrient content. In the UK, flooding is predicted to increase with climate change, and it is important to understand how increased flooding may affect earthworm populations.

Increases in flooding may also impact soils amended with sewage sludge. The use of sewage sludge as a soil amendment increases soil organic matter content, but the interaction between sludge amendment and flooding is not understood. Oxygen concentrations of flooded soils rapidly decrease as the soil microbial community breaks down soil organic matter. This thesis examined whether sewage sludge amendment of soil led to faster rates of oxygen depletion in flooded soils, and how this could affect earthworm populations.

Earthworm populations in pasture and crop fields known to regularly flood were surveyed every three months, and soil environmental factors measured. Populations subject to the stresses of both crop growth and flooding were lower than in pasture soils, with both fields dominated by the species *Allolobophora chlorotica*. Laboratory experiments investigating oxygen concentration depletion in flooded soil amended with sludge found little effect of sludge application on oxygen depletion; an unexpected result likely caused by the recalcitrant nature of sewage sludge. Experiments examining the oxygen requirements of some earthworm species found that the green morph of *A. chlorotica* appeared to aestivate in response to low oxygen conditions: a novel finding suggesting a mechanism for their survival in regularly flooded areas. Experiments examining how earthworms, flooding, and sewage sludge affected soil emissions of greenhouse gases found high emissions of N_2O from unflooded soils treated with both earthworms and sewage sludge.

The findings provide insight into earthworm responses to flooding at both the individual and population level, and increase understanding of interactions between earthworms, flooding, and sewage sludge.

Contents

Abstract 2

List of figures (short titles) 10

List of tables (short titles)..17

Acknowledgements...20

Author’s Declaration...21

1. Introduction...22

2 Earthworms, flooding, and sewage sludge: A literature review..27

2.1 Abstract...27

2.2 The scope of the review 27

2.3 Extreme weather and climate change..28

2.4 Types of flooding..28

2.5 The impact of flooding on soils 30

2.5.1 Soil dissolved oxygen content 30

2.5.2 Soil pH...31

2.5.3 Soil temperature...32

2.5.4 Soil structure...32

2.5.5 Soil organic matter 36

2.6 Earthworm ecology..36

2.7 Earthworm physiology and flooding 40

2.8 Sewage sludge usage...43

2.9 The impact of sewage sludge on soil 45

2.9.1 Soil physical properties 46

2.9.2 Soil pH...47

2.9.3 Soil fauna...47

2.10 Earthworms, flooding, and sewage sludge: The knowledge gaps 48

2.11 References 48

3. Seasonal differences in earthworm populations in pasture and arable fields with different localised flooding regimes 61

3.1. Abstract 61

3.2. The scope of the chapter 62

3.3. Introduction 62

3.4. Methods 65

3.4.1. Field sites 65

3.4.2. Earthworm sampling 69

3.4.3. Soil analysis 70

3.4.4. Data analysis 70

3.5. Data analyses and statistics 71

3.6. References 72

3A. Seasonal differences in earthworm populations in a pasture field with different localised flooding regimes 77

3A.1 Results 77

3A.1.1 Environmental factors across site and time 77

3A.1.2 Population metrics across site and time 86

3A.1.3 Population structure at the ecotype level across sites 90

3A.1.4 Differences in abundance and mass of mature individuals of species across sites 94

3A.1.5 Correlations between soil environmental factors and earthworm population metrics 98

3A.2 Discussion 99

3A.2.1 Soil environmental factors 99

3A.2.2 Earthworm Populations 104

3A.3 Conclusion 107

3A.4 References 109

3B. The long-term fluctuations of earthworm populations in an arable field with differing localised flooding effects 116

3B.1 Results 116

3B.1.1 Environmental factors across site and time 116

3B.1.2 Population metrics across site and time 128

3B.1.3 Population structure at the ecotype level across sites 133

3B.1.4 Differences in abundance and mass of species across sites 137

3B.1.5 Correlations between soil environmental factors and earthworm population metrics 142

3B.2 Discussion 148

3B.2.1 Soil environmental factors 148

3B.2.2 Earthworm populations 152

3B.3 Conclusion 160

3B.4 References 161

3C. Comparing and contrasting the effects of flooding on environmental factors and earthworm populations across an arable and pasture site 167

3C.1 Dataset combination 167

3C.2 Hypotheses 168

3C.3 Data analysis and statistical methods 168

3C.4 Results 170

3C.4.1 The effects of flooding and soil use on soil environmental factors 170

3C.4.2 The effects of flooding and soil use on earthworm population metrics 178

3C.4.3 The differences in the species composition of the adult earthworm population 183

3C.5 Discussion 185

3C.5.1 Interaction between soil environmental factors 185

3C.5.2 Earthworm populations 186

3C.6 Conclusion 187

3C.7 References 189

4 The availability of oxygen in flooded soil amended with sewage sludge 192

4.1 Abstract 192

4.2 Introduction 193

4.3 Methods 195

4.3.1 Sewage sludge 195

4.3.2 The soils 197

4.3.3 Experimental design 199

4.3.4 Measurements 200

4.3.5 Data analyses and statistics 200

4.4 Results 202

4.4.1 Relationships between soil characteristics 202

4.4.2 Twenty-four hour period 205

4.4.3 Depletion and plateau periods 214

4.5 Discussion 226

4.5.1 Intercorrelation of Soil Factors 226

4.5.2 The effect of sewage sludge application on soil oxygen concentration 226

4.5.3 Percentage increase of organic matter 228

4.5.4 The effect of soil characteristics 229

4.5.5 Further Experiments 231

4.6 Conclusion 232

4.7 References 233

4.8 Supplementary information 239

4.8.1 Untransformed oxygen depletion curves 239

4.8.2 Transformed oxygen depletion curves 243

5 Earthworm survival in water: the difference in oxygen requirements for an anecic (*Lumbricus terrestris*), an endogeic (*Allolobophora chlorotica*) and an epigeic (*Lumbricus castaneus*) species 247

5.1 Abstract 247

5.2 Introduction 247

5.3 Methods....249

5.3.1 Earthworm collection 249

5.3.2 Tube setup 250

5.3.3 Measurements 251

5.3.4 Data analyses and statistics....251

5.4 Results....252

5.4.1 Changes in oxygen concentration 252

5.4.2 Differences in oxygen concentration at which earthworms died 260

5.4.3 Earthworm mass gain 263

5.4.4 Changes in solution pH 265

5.5 Discussion 268

5.5.1 Control vs treatment deaths 268

5.5.2 Absolute oxygen concentration....270

5.5.3 Earthworm mass gain 271

5.5.4 Earthworm lifestyle 272

5.5.5 Field site context 276

5.5.6 Further work 277

5.6 Conclusion....277

5.7 References 278

6 The effect of flooding on greenhouse gas emissions from sewage sludge amended, earthworm populated mesocosms 283

6.1 Abstract 283

6.2 Introduction 284
6.3 Method 287
    6.3.1 Earthworms 287
    6.3.2 Sewage sludge 287
    6.3.3 Soil 288
    6.3.4 Oxygen sensor spots 288
    6.3.5 Experimental design 288
    6.3.6 Measurements 292
    6.3.7 Mesocosm deconstruction 295
    6.3.8 Data analysis and statistics 295
6.4 Results 296
    6.4.1 Earthworm activity 297
    6.4.2 Gas Fluxes 298
    6.4.3 Soil oxygen depletion 305
    6.4.4 Soil pH 309
6.5 Discussion 310
    6.5.1 Earthworms 310
    6.5.2 Gas fluxes and soil oxygen 311
    6.5.3 Soil pH 317
    6.5.4 Further work 319
6.6 Conclusion 321
6.7 References 322
7. Conclusions 330
7.1. Does regular flooding impact earthworm populations in arable and pasture soils differently? 330

7.2. Does increasing organic matter content, in the form of sewage sludge application, lead to faster rates of oxygen depletion when soils floods? 331

7.3. Are there species specific responses of earthworms to flooding? And, if so, how might these change with the application of sewage sludge to soil? 332

7.4. Concluding remarks 334

7.5. Limitations of the experiments 336

7.6. Further work 337

7.7. References 338

A1 Method Development 340

A1.1 Chapter 4: The availability of oxygen in flooded soil amended with sewage sludge 340

A1.2 Chapter 5: Earthworm survival in water; the difference in oxygen requirements for an anecic, an endogeic and an epigeic species. 343

A1.3 Chapter 6: The effect of flooding on greenhouse gas emissions from sewage sludge amended, earthworm populated mesocosms 351

A1.4 References 352

List of figures (short titles)

Figure 1-1. A conceptual diagram of the different thesis chapters, their relationship to the research questions posed at the end of the literature review, and their relationships to each other. 25

Figure 2-1. The Sewage Treatment Process (DEFRA, 2002) 43

Figure 3-1. A. Google earth image of the pasture field bordering the river Loddon. B. Ordnance survey map of the same field at the 1:25 000 scale 66

Figure 3-2. A. Google earth image of the arable field bordering the river Swale. B. Ordnance survey map of the same field at the 1:5 000 scales...68

Figure 3A-1. The mean soil temperature for the different sites over six sampling timepoints in the pasture site...80

Figure 3A-2. The mean soil bulk density (g cm^{-3}) for the different sites over six sampling timepoints in the pasture site 81

Figure 3A-3. The mean soil moisture content (%) for the different sites over six sampling timepoints in the pasture site 82

Figure 3A-4. The mean soil pH for the different sites over six sampling timepoints in the pasture site...83

Figure 3A-5. The mean soil carbon content for the different sites over six sampling timepoints in the pasture site 84

Figure 3A-6. The mean soil nitrogen content (%) for the different sites over six sampling timepoints in the pasture site. 85

Figure 3A-7. The mean total abundance of earthworms m^{-2} for the different sites over six sampling timepoints in the pasture site 87

Figure 3A-8. The mean total biomass of earthworms (g m^{-2}) for the different sites over six sampling timepoints in the pasture site 88

Figure 3A-9. The mean Shannon Diversity Index values of earthworms per pit for the different sites over six sampling timepoints in the pasture site. 89

Figure 3A-10. The proportions of the populations represented by adults and juveniles of the three earthworm ecotypes in the Loddon floodplain 93

Figure 3B-1. The mean soil temperature for the different sites over four sampling timepoints in the arable site. 122

Figure 3B-2. The mean soil bulk density (g cm^{-3}) for the different sites over four sampling timepoints in the arable site. 123

Figure 3B-3. The mean soil moisture content (%) for the different sites over four sampling timepoints in the arable site. 124

Figure 3B-4. The mean soil pH for the different sites over four sampling timepoints in the arable site. 125

Figure 3B-5. The mean soil carbon content (%) for the different sites over four sampling timepoints in the arable site. 126

Figure 3B-6. The mean soil nitrogen content (%) for the different sites over four sampling timepoints in the arable site. 127

Figure 3B-7. The mean total abundance of earthworms m^{-2} for the different sites sites over four sampling timepoints in the arable site. 130

Figure 3B-8. The mean total biomass of earthworms (g m^{-2}) for the different sites over four sampling timepoints in the arable site. 131

Figure 3B-9. The mean Shannon Diversity Index values of earthworms per pit for the different sites over four sampling timepoints in the arable site. 132

Figure 3B-10. The mean proportions of the population represented by adults and juveniles of the three earthworm ecotypes in the arable site..136

Figure 3C-1. A conceptual diagram of the changes in earthworm populations observed in this study with increased frequency of flooding in crop, field margin, and pasture sites. 167

Figure 3C-2. The mean soil bulk densities (g cm^{-3}) for the different flooding frequencies and soil uses across both field sites..173

Figure 3C-3. The mean soil moisture content (%) for the different flooding frequencies and soil uses across both field sites...174

Figure 3C-4. The mean soil pH values for the different flooding frequencies and soil uses across both field sites. 175

Figure 3C-5. The mean soil carbon content (%) for the different flooding frequencies and soil uses across both field sites 176

Figure 3C-6. The mean soil nitrogen content (%) for the different flooding frequencies and soil uses across both field sites 177

Figure 3C-7. The mean total earthworm abundance m^{-2} for the different flooding frequencies and soil uses across both field sites. 180

Figure 3C-8. The mean total earthworm biomass (g m^{-2}) for the different flooding frequencies and soil uses across both field sites. 181

Figure 3C-9. The mean Shannon diversity index values for the different flooding frequencies and soil uses across both field sites. 182

Figure 3C-10. The mean abundances of individuals m^{-2} of *A. caliginosa*, *A. chlorotica*, *A. rosea*, *L. castaneus*, *L. rubellus* and *L. terrestris* for different flooding frequencies and soil uses across both field sites. 184

Figure 4-1. The pH measures of all soils, grouped by soil uses of allotment, arable, and pasture 203

Figure 4-2. The percent organic matter measures of soils, grouped by soil uses of allotment, arable, and pasture 204

Figure 4-3. The gradients of oxygen concentration for all treatments of each soil across the twenty-four hour dataset. 206

Figure 4-4. The gradients of oxygen concentration for all treatments of the soil use categories of allotment, arable, and pasture across the twenty-four hour dataset. 207

Figure 4-5. The relationship between the gradient of oxygen concentration within the beakers over log time and the soil pH across the twenty-four hour dataset 209

Figure 4-6. The relationship between the gradient of oxygen concentration over log time and the soil organic matter percentage across the twenty-four hour dataset 210

Figure 4-7. The relationship between the gradient of oxygen concentration over log time and the percentage increase in soil organic matter content over the twenty-four hour dataset 211

Figure 4-8. The gradients of oxygen concentration for all treatments of each soil texture across the twenty-four hour dataset 213

Figure 4-9. The gradients of oxygen concentration over log time for the depletion and plateau phases of the exponential curve 215

Figure 4-10. The gradients of oxygen concentration over log time for the depletion phase of the exponential curve across the different soil origins and sludge loading percentages. 217

Figure 4-11. The gradients of oxygen concentration for each soil origin over log time for the plateau phase of the exponential curve...218

Figure 4-12. The gradients of oxygen concentration for the depletion phase of the exponential curve for all sludge loadings bulked across soil origins. 220

Figure 4-13. The gradients of oxygen concentration over log time for the plateau phase of the exponential curve for all soil uses..221

Figure 4-14. The relationship between the percentage increase in the organic matter content and the gradient of oxygen concentration over log time during the depletion phase of the oxygen depletion curve..222

Figure 4-15. The relationship between the logged percentage increase in the organic matter content and the gradient of oxygen concentration over log time during the depletion phase of the oxygen depletion curve 223

Figure 4-16. Gradients of oxygen depletion over log time for the plateau phase of the exponential curve for all soil uses...225

Figure 4-17. The oxygen concentration depletion curves over unlogged time for the A. Loddington and B. Spen Farm soil origin sites..239

Figure 4-18. The oxygen concentration depletion curves over unlogged time for the A. Cusgarne and B. Heslington East soil origin sites...240

Figure 4-19. The oxygen depletion curves over unlogged time for the A. Heslington and B. Mansfield soil origin sites 241

Figure 4-20. The oxygen depletion curves over unlogged time for the Newcastle soil origin site 242

Figure 4-21. The oxygen depletion curves over logged time for the A. Loddington and B. Spen Farm soil origin sites..243

Figure 4-22. The oxygen depletion curves over logged time for the A. Cusgarne and B. Heslington East soil origin sites 244

Figure 4-23. The oxygen depletion curves over logged time for the A. Heslington and B. Mansfield soil origin sites 245

Figure 4-24. The oxygen depletion curves over logged time for the Newcastle soil origin site 246

Figure 5-1. The changes in oxygen concentration in control and treatment tubes containing individuals of *L. terrestris* over time. 253

Figure 5-2. The changes in oxygen concentration in control and treatment tubes containing individuals of *A. chlorotica* over time.. 254

Figure 5-3. The changes in oxygen concentration in control and treatment tubes containing individuals of *L. castaneus* over time. 255

Figure 5-4. The differences in the absolute oxygen concentration in solution at which individuals of *L. castaneus* and *L. terrestris* were found dead. 260

Figure 5-5. The oxygen concentration normalised per gram biomass at which individuals of *L. castaneus* and *L. terrestris* were found dead. 261

Figure 5-6. The oxygen concentration normalised per mm^2 earthworm surface area at which individuals of *L. castaneus* and *L. terrestris* were found dead 262

Figure 5-7. The absolute oxygen concentration in solution at which individuals of *L. terrestris* and *L. castaneus* died, and from hour 144 for *A. chlorotica* 263

Figure 5-8. The mass gained by earthworms of *A. chlorotica* in the control and treatment tubes 264

Figure 5-9. The mass gained by alive and dead earthworms of *L. castaneus* across the control and treatment tubes 265

Figure 5-10. The pH values over the hours submerged in treatment and control tubes containing individuals of *L. terrestris*. 266

Figure 5-11. The pH values over the hours submerged in treatment and control tubes containing individuals of *A. chlorotica* 267

Figure 5-12. The pH values over the hours submerged in treatment and control tubes containing individuals of *L. castaneus*. 268

Figure 5-13. The same individual of species *A. chlorotica* before and after submergence in a treatment tube for 144 hours. 273

Figure 6-1. Experimental factorial design of the treatment combinations of flooding, earthworms, and sewage sludge………………………….. 289

Figure 6-2. A. Representative diagram of the placement of sensor spot tubes in the soil filled mesocosms and B. Photograph of the tubes of length 10cm and 15cm 290

Figure 6-3. Individual of *L. terrestris* on the surface of a flooded mesocosm at 2 weeks post flooding event. 292

Figure 6-4. Experimental setup for gas flux measurements. A. Sealed 'Lock and Lock' containers. B. Containers connected by flexible tubing to the White Dwarf EGA61 Multi Channel Gas Exchange System. C. LGR CH_4 (X) and CO_2 and N_2O (Y) analysers in the next laboratory. D. N_2O output.. 294

Figure 6-5. Mean fluxes of CH_4 (μg m^{-2} h^{-1}) for each soil treatment under flooded and unflooded conditions over the duration of the experiment. 300

Figure 6-6. Mean fluxes of CO_2 (μg m^{-2} h^{-1}) for each soil treatment under flooded and unflooded conditions over the duration of the experiment 302

Figure 6-7. Mean fluxes of N_2O (μg m^{-2} h^{-1}) for each soil treatment under flooded and unflooded conditions over the duration of the experiment 304

Figure 6-8. Mean dissolved oxygen content (mg O_2 L^{-1}) at a depth of 11cm for each soil treatment under flooded and unflooded conditions over the duration of the experiment. 306

Figure 6-9. Mean dissolved oxygen content (mg O_2 L^{-1}) at a depth of 5cm for each soil treatment under flooded and unflooded conditions over the duration of the experiment 308

Figure 6-10. The pH of the flooded and unflooded mesocosm soils in the different soil treatment categoriest. 310

Figure A1-1. Individuals of *L. rubellus* in 50ml reconstituted groundwater solution in 100ml beakers in a 15°C CT room.. 344

Figure A1-2. Recording the percent oxygen saturation (displayed), oxygen concentration (mg O_2 L^{-1}) and temperature of the reconstituted groundwater solution 345

Figure A1-3. 50ml centrifuge tubes, completely filled with reconstituted groundwater and an individual of *L. terrestris*, attached to a peristaltic pump in a 15°C CT room 350

List of tables (short titles)

Table 2-1. The maximum permissible ground pressure on soils of different values of bulk density and water content (Medvedev and Cybulko, 1995; Hamza and Anderson, 2005) ………………. 35

Table 2-2. A summary of earthworm ecotypes, their characteristics, and species belonging to each ecotype (Sims and Gerard, 1999)..37

Table 2-3. The mean weights of juveniles and adults of *L. rubellus* at frequently, moderately and seldom inundated floodplain sites in 2002 (Klok et al, 2006). 39

Table 2-4. Examples of effective sludge treatment processes (DEFRA, 2006; European Commission, 2010) 45

Table 3A-1. Correlations between soil environmental factors in the pasture site.......................77

Table 3A-2 - The outcomes of two way ANOVAs between soil environmental factors, sampling sites, and time of sampling in the pasture site. 78

Table 3A-3. The outcomes of GLMS and two way ANOVAs between population metrics, sampling sites, and time of sampling in the pasture site. 86

Table 3A-4. The outcomes of GLMS between the proportion of the population represented by adult and juveniles of the different ecotypes, sampling sites, and time in the pasture site……..91

Table 3A-5. The mean and range of the abundances of individuals m^{-2} of the six species present in the pasture site 95

Table 3A-6. The mean and range of the biomass (g) of adult individuals of the six species present in the pasture site. 97

Table 3A-7. Correlations between soil environmental factors and earthworm population metrics. 98

Table 3A-8. The average daily rainfall (mm) and total month's rainfall (mm) reported at Wokingham Weather Monitoring Station 100

Table 3B-1. Correlations between soil environmental factors within both the whole dataset and land use categories in the arable site 117

Table 3B-2. The outcomes of two way ANOVAs between soil environmental factors, sampling sites, and time of sampling in the arable site..120

Table 3B-3. The outcomes of the GLM and two way ANOVAs between population metrics, sampling sites, and time of sampling in the arable site...128

Table 3B-4. The outcomes of GLMs between the proportion of the population represented by adult and juveniles of the different ecotypes, sampling sites, and time in the arable site……..134

Table 3B-5. The mean and range of the biomass (g) of individuals of the six species present in statistically valid quantities in the in the arable site 138

Table 3B-6. Correlations between soil environmental factors in the arable site 143

Table 3B- 7. The key factors for each earthworm population metric, as selected by Stepwise Model Selection, across the whole dataset and soil uses in the arable site.. 147

Table 3B-8. The average daily rainfall (mm), total month's rainfall (mm), and mean and range of air temperatures at 9am and 9pm reported at Topcliffe Weather Monitoring Station 150

Table 3B-9. The mean and standard deviation of the total abundance of earthworms m^{-2} and the total biomass (g m^{-2}) of earthworms in the different flooding regimes and soil uses in the arable site...153

Table 3B-10. The mean, standard deviation and coefficient variation of the abundance of individuals of each of the seven species found in the arable site 155

Table 3B-11. The mean, standard deviation and coefficient variation of the soil environmental factors for the whole dataset and soil uses in the arable site 156

Table 3C- 1. The re-categorisation of the datasets from Chapters 3A and 3B for comparing the effects of flooding regimes and soil use across the whole dataset..169

Table 3C-2. The outcomes of GLMs between soil environmental factors, flooding frequency, and soil use categories...171

Table 3C-3. The outcomes of GLMs between earthworm population metrics, flooding frequency, and soil use categories 178

Table 4-1. Mean and standard deviation of the percent carbon, percent nitrogen, and pH values of sludge. 196

Table 4-2. The description, origin, soil type, pH and soil organic matter content of the experimental soils 198

Table 4-3. Summary of the statistical test outputs of intercorrelated soil characteristics 202

Table 4-4 – The number of replicates of each combination of soil use and sludge loading percent in the depletion phase. 219

Table 5-1. The mean and standard deviation oxygen concentration normalised by the gram biomass (mg O_2 L^{-1} g^{-1}) in control and treatment tubes containing individuals of *L. terrestris*, *A. chlorotica* and *L. castaneus* at each sampling time point..257

Table 5-2. The mean and standard deviation oxygen concentration normalised by the mm^2 surface area (mg O_2 L^{-1} mm^{-2}) in control and treatment tubes containing individuals of *L. terrestris*, *A. chlorotica* and *L. castaneus* at each sampling time point..259

Table 5-3. Key life cycle characteristics of an anecic, two epigeic, and an endogeic species, summarised from a number of studies 275

Table 6-1. The mean and range of changes in earthworm biomass, the wet mass of cast material, and the number of burrow openings counted on the soil surface across flooding and soil treatments. 298

Table 6-2. Ranges of gas fluxes of CH_4, CO_2, and N_2O reported in review papers and observed in this study 313

Acknowledgements

I would like to thank my two supervisors, Mark Hodson and Xiaohui Chen, for their support throughout this process. Their input has been invaluable in developing my skills as a researcher, and I must sincerely thank them for taking the chance on me and offering me this project.

I would also like to thank the members of the BIOchemical-physical-biological function of Sludge in Agricultural Soils (BIOSAS) research group, for their input and feedback over the past three years.

My thanks to the numerous people who aided with the fieldwork and experimental chapters. My thanks to Tom Sizmur and numerous research students and assistants at the University of Reading for access to the research site and assisting with earthworm sampling. My thanks to Mark Marley, for access to his crop field, and to my colleagues at the University of York for their assistance in sampling slightly closer to home. My thanks to the kind individuals who unquestioningly provided me with soil for experiments: Lisa Wilshire, from her vegetable patch, Bibiana Betancur and Colin Beale, from their research site, Mark Hodson, from a farm down the road, Ian and Sheila Crawford, from their allotment, George and Jillian Hodson, from their friend Derek Colley's allotment, Will Burn and Abby Mycroft, from their research site, and staff at the Spen Farm research site and within the University of York Estates team. Soil reached me by post, by bin bag, and by bucket, and it was greatly appreciated. My thanks to Sylvia Toet for the use of her experimental setup for taking measurements of greenhouse gas fluxes.

My thanks to my family and my friends. Near and far, scientists and non-scientists, your continuous support, confidence, and interest (genuine or well-feigned) has kept me going through the difficult stages of this process. Thank you for understanding as I complained about experiments going wrong, and for cheering as I described experiments going right.

Finally, saving the best until last, my thanks to my husband Robert for his unwavering support. For his endless patience. For occasional IT support. For all the cups of tea. And for a hundred other indescribable acts that helped make this PhD run a little smoother. With all my heart: thank you.

Author's Declaration

This work was funded by the White Rose University Consortium.

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references.

No papers have yet been published from this work at time of writing. Results from Chapters 3 and 5 have been presented internally in the department of Environment and Geography, University of York, at the British Ecological Society conference in December 2018, and the British Soil Science Society Early Career Researcher's Conference in April 2019.

1. Introduction

This PhD thesis consists of eight chapters, including one fieldwork chapter (Chapter 3) and three laboratory experiments (Chapters 4, 5, and 6). The contents of each chapter, excluding this introduction, are described briefly below.

Chapter 2 is a literature review contextualising the thesis. This chapter summarises different kinds of flooding known to occur in the UK, changes predicted to flooding with climate change, and its impact on soils and earthworm populations. The chapter also summarises the rules limiting and affecting applications of sewage sludge to UK agricultural soil, and the changes that sewage sludge application causes to soil physical properties. The literature review also places this thesis in the context of the work being performed by the BIOchemical-physical-biological function of Sludge in Agricultural Soils (BIOSAS) research group, and outlines the three key questions that this thesis aimed to answer.

Chapter 3 describes field work carried out at a pasture (Chapter 3A) and an arable site (Chapter 3B), both of which had a history of experiencing flooding in parts of the field. Sampling at both sites was carried out every three months for a duration of eighteen months (Chapter 3A) and twelve months (Chapter 3B). Earthworms were collected, weighed, and identified to the species level. Soil samples were analysed for bulk density, percent soil moisture, pH, percent carbon and percent nitrogen. Soil temperature was measured in the field. For both sites variation in environmental variables and earthworm communities and their interactions were compared across the different flooding regimes and sampling time points.

Chapter 3A describes the pasture field. The field was separated into three sites of different flooding regimes: regularly flooded, fast-draining, regularly flooded, slow-draining, and rarely flooded.

Chapter 3B describes the arable field. The field was separated into three regions of flooding: rarely flooded, occasionally flooded, and regularly flooded. Within each of these sites, the crop and the margin were compared, giving a total of six sampling sites within the field. The final two sites were the field and river sides of the riverbank, which were not accessed by farmland machinery.

Chapter 3C compares and contrasts the pasture and arable site (Chapters 3A and 3B), indicating where patterns of soil environmental factors and earthworm populations are similar, and where they differ.

Chapter 4 investigates the rates of oxygen depletion in flooded soil amended with sewage sludge. Seven of soils of different textures, pH values, and organic matter contents were obtained from around the UK. These soils were amended with 100%, 50%, 25% and 0% (control soils) of the maximum sludge loading rate according to UK guidelines. The soils were flooded, and the gradients of oxygen depletion over time were calculated. This experiment aimed to understand if the amendment of soils with anaerobically digested sewage sludge led to faster rates of oxygen depletion in flooded soil. The appendix for Chapter 4 contains the untransformed datasets of changes in oxygen concentrations over time in flooded soils amended with the different sludge loading percentages.

Chapter 5 investigates the oxygen concentrations required in solution for survival of three earthworm species: *Lumbricus terrestris*, *Allolobophora chlorotica*, and *Lumbricus castaneus*. These species represent the three different earthworm ecotypes, and so their oxygen requirements may be expected to differ based on the characteristic sizes and behaviours of these functional groups. Individuals of each earthworm species were sealed into 50ml centrifuge tubes, with control tubes continuously supplied with air by a peristaltic pump. The oxygen concentration of tubes was measured at discrete timepoints determined in preliminary studies detailed in Appendix 1, and the oxygen concentration at which earthworms were found dead compared across the three species.

Chapter 6 investigates the emissions of the greenhouse gases methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O) from flooded soil mesocosms containing combinations of earthworms and sewage sludge. Mesocosms were amended with 100% of the maximum sludge loading rate, earthworms, or a combination of earthworms and sewage sludge. The gas fluxes were recorded for two weeks before flooding, then the mesocosms were flooded for a period of four weeks, with a further two weeks of measurements following drainage of surface water. The gas fluxes from the flooding, sludge, and earthworm treatment combinations were compared. This experiment aimed to bring together the elements of Chapters 3, 4, and 5, and understand some of the potential interactions of earthworms, flooding, and sewage sludge.

Chapter 7 concludes by examining how the field work and experimental chapters have answered the questions posed at the end of Chapter 2. The chapter also addresses the limitations of the work performed and suggests future research questions that have arisen from the work that comprises the thesis.

Appendix 1 describes the method development process undertaken for each of the three experimental chapters (Chapters 4 – 6). As each experiment went through a number of iterations before the method described in the relevant chapter, this chapter outlines the previous versions of the experiments, and the changes that occurred between each version.

The detailed hypotheses of each experiment are given within the relevant chapters.

A conceptual diagram (Fig. 1-1) demonstrates the interrelation of the different chapters, and their relation to the research questions posed at the end of the literature review.

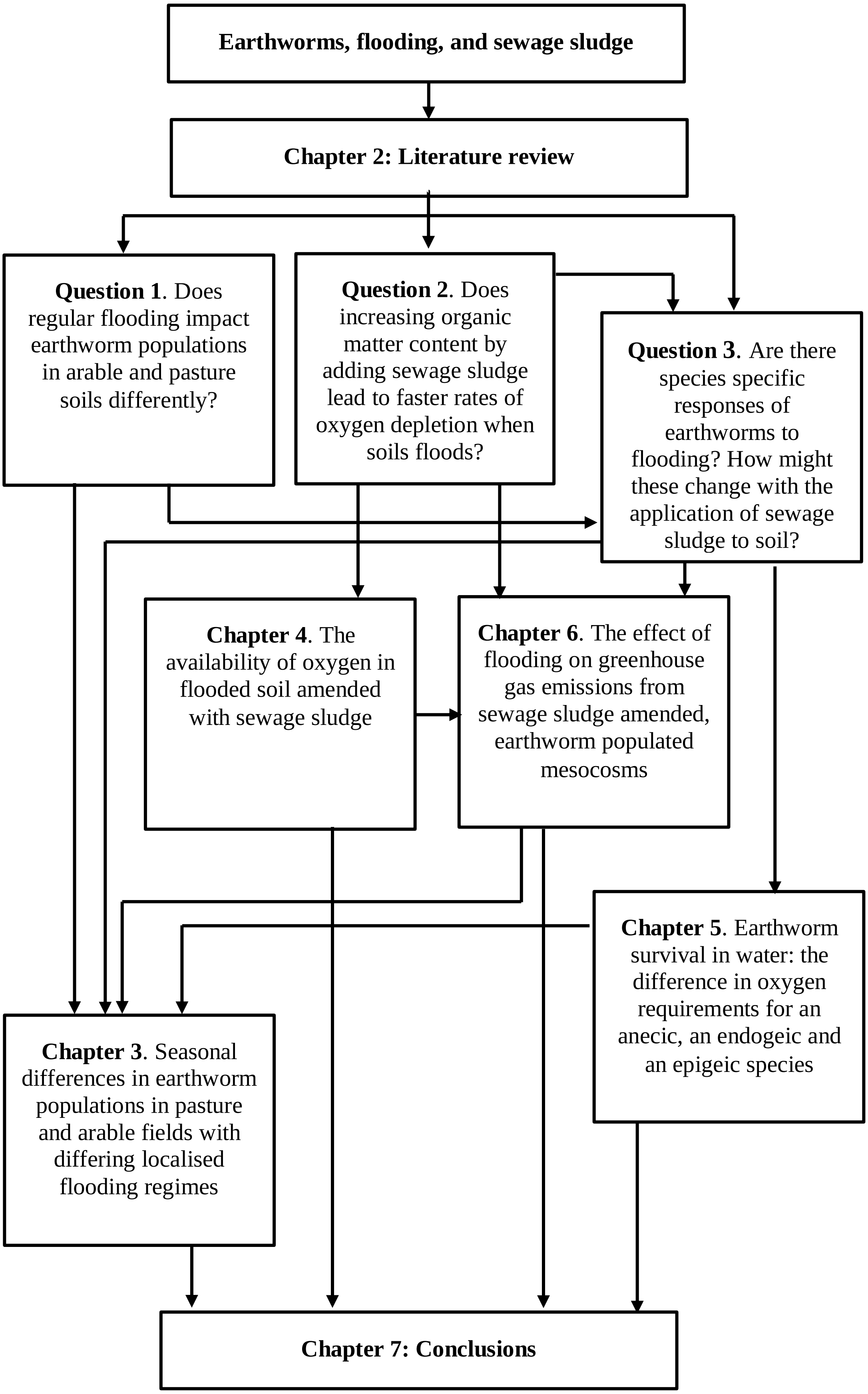

Figure 1-1. A conceptual diagram of the different thesis chapters, their relationship to the research questions posed at the end of the literature review, and their relationships to each other.

2 Earthworms, flooding, and sewage sludge: A literature review

2.1 Abstract

With increasing global temperatures contributing to changing weather patterns, the rate and intensity of flooding events on UK agricultural soils are increasing. Flooding induces an anaerobic soil environment driven by the rapid microbial consumption of available oxygen, as well as a number of other physical and chemical changes within the soil, such as reduction of soil bulk density, changes in soil carbon and nitrogen availability, and soil pH.

These changes in the soil chemical and physical environment drive earthworm population changes, with earthworm survival and population recovery highly variable between earthworm species and ecotypes. Epigeic species may recover faster due to their rapid maturation in comparison to other ecotypes. Endogeic species seem to be the most resistant to waterlogged conditions, surviving longer than endogeic and anecic species in flooded soils. Anecic species seem to be the least resistant to flooding events, with the highest rates of mortality.

The amendment of soils with sewage sludge increases the quantities of nutrients important for plant growth, increases the soil organic carbon content, and improves soil structure and water holding capacity.

No studies exist yet that examine the effects of flooding on sewage sludge amended soils, and while studies show the impact of flooding on earthworm populations, there is insufficient information yet regarding earthworm survival in the flooding of sewage sludge amended soils.

2.2 The scope of the review

This review aims to examine the known effects of flooding on earthworm populations, by first considering both the chemical and physical impacts of flooding on soil, then in turn how these chemical and physical changes may impact upon earthworm populations and their survival in flooding events. The review will then go on to consider the types of sewage sludge currently used in agriculture, and the changes that agricultural amendment with sewage sludge leads to in soil. This review aims to contextualise the project, which will consist of an in depth investigation into the potential interactions between flooding, earthworm populations, and the amendment of soils with sewage sludge.

2.3 Extreme weather and climate change

To place the review in context, it is vital to consider the current rates of flooding events in the UK, and how these are predicted to change in the future.

There is strong evidence that, as global temperatures increase, the frequency and intensity of extreme weather events are also increasing (Easterling et al., 2000). In the UK, for example, changes in precipitation patterns have been observed over the last 35 years, with precipitation becoming more intense in the winter and less intense in the summer (Maraun et al., 2008). As the intensity and time of occurrence of precipitation throughout the year changes, there is a strong likelihood that flooding patterns will also change. Throughout the 20th century, observed data already shows an increase in severe flooding events (Milly et al., 2002), with the mean annual floodwater discharge in the UK increasing by approximately 12% between 1960 and 2010 (Blöschl et al., 2019). While flooding can be defined as a "low probability, high-impact event" (McMichael et al., 2006), several models based on this observational data indicate that the probability of flooding events will continue to increase (Prudhomme et al., 2003).

When considering how this will impact on earthworm populations, it is important to consider that future flooding events will not only be occurring more regularly, but also increasing in severity. This increase in severity may also mean that there is an increased likelihood of historically unflooded areas now being subject to flooding events. The populations of earthworms in these previously unflooded regions may respond very differently to these flooding events than earthworms in areas that regularly flood, and the earthworm population dynamics in regions that regularly flood may still change with increased occurrence and severity of flooding.

2.4 Types of flooding

There are multiple types of flooding that can affect farmland soil, depending on both the geographic location of the farm and the recent local weather conditions. When examining the effects of flooding on soils, it is important to consider that the floodwater will have different characteristics.

Fluvial flooding is flooding associated with rivers overflowing their banks, generally following high rainfall events upstream. Fluvial flooding of agricultural soils can lead to extensive soil structural damage, such as the loss of topsoil through erosion, or the reduction of soil infiltration capacity (Holman et al., 2003), but the extent of the damage is likely dependent on the flow rate of the floodwater (Holman et al., 2003). For example, a faster flow rate of floodwater may provide a higher rate of oxygen replenishment in soil, but may also lead to an increased rate of soil erosion (Parsons et al., 1963).

Occurrences of coastal flooding are highly dependent on the topography of the land bordering the coast, with low lying land more at risk from inundation. With increased extreme weather events predicted with climate change, many of these coastal areas are at increased risk of flooding, with the contribution of coastal flooding to the UK's national flood risks predicted to increase from approximately 50% to between 60 and 70% from 2002 to 2080 (Hall et al., 2006). In the UK, both arable and agricultural regions can be found bordering coasts and estuaries, placing them at risk of flooding with salt water (Cave et al., 2003; Dawson et al., 2009), with agricultural growth of low salinity crops such as wheat (Akhtar et al., 1994) less resistant to flooding than grassland (Hoggart et al., 2014). However, it has also been suggested that the negative effects of coastal flooding on soil quality, including reduced fertility, may be simply due to the inundation with water rather than the salinity (Page and Williams, 1926). Page and Williams (1926) found the increase in soil salinity due to salt water flooding to be only 0.05%. While this low increase may have little impact on tolerant plants and ecosystems, it may still be sufficient to cause osmotic stress to earthworms. A study by Owojori et al. found a 100% mortality rate of the earthworm *Eisinea fetida* in soil amended with an increase of 0.008% NaCl (Owojori et al., 2008), while *Apporectodea caliginosa* also shows low survival rates in soils with between 80 and 110 nmol kg^{-1} Na content (Owojori et al., 2009). Later field experiments considering the effect of regular irrigation of arid soils with saline water found that salinity in the soil profile increased proportionally to the salinity of the irrigation water (Chen et al., 2010), while approximately 8.5% of the regularly flooded, saline soils in Bangladesh can be considered very saline (Haque, 2006), casting doubt on Page and Williams' assertion that salinity increases in soil are not severe. Although it must also be considered that Chen et al. (2010) and Haque (2006) focus on regular inundation with saline water, while Page and Williams (1926) considered a single extreme flooding event.

Groundwater flooding occurs following prolonged periods of heavy rainfall, which cause the water table to rise. Although it is rarer than fluvial and coastal flooding in the UK, it can be more disruptive, as the water can take longer to recede (Environment Agency, 2007), persisting for up to 8 months if effective rainfall continues after the initiation of flooding (Hughes et al., 2011). The composition of groundwater is highly dependent on the underlying geology and the groundwater residence time (Edmunds et al., 2003). This, along with potential contamination from agriculture, the local population, and saltwater in coastal areas, may potentially lead to contamination of soils and reduced soil quality. However, the majority of existing research seems to focus on the impacts of groundwater flooding in urban areas, rather than agricultural areas of the UK. Further information is needed in order to know how potential groundwater compositions and contamination may impact upon soil physical and chemical properties.

2.5 The impact of flooding on soils

Flooding greatly impacts several key physical and chemical characteristics of soil.

2.5.1 Soil dissolved oxygen content

Oxygen diffusion within water occurs 10^4 times slower than in air (Schlesinger, 2013). As a result, when soils are flooded, demand for oxygen can rapidly outstrip supply. Shortly after flooding, oxygen is consumed by microorganisms and plant roots, leaving only a narrow “zone” of available oxygen at the water-air interface (Ponnamperuma, 1984). This results in the soil becoming anoxic within 24 hours of flooding (Ponnamperuma, 1984), with the soil dissolved oxygen content dropping rapidly (Tanji et al., 2003) and remaining low throughout the inundation period, with some studies finding oxygen concentrations remaining as low as approximately 1 mg L^{-1} during a five week flooding period from a peak of c.10-15 mg L^{-1}(Unger et al., 2009).

2.5.2 Soil pH

The reduction in free oxygen through its use as an electron acceptor by soil microbes induces a change in the pH of flooded soil (Kögel-Knabner et al., 2010); in rice paddy soil, it has been suggested that flooding induces a move back towards pH neutrality. This occurs in acidic soils due to the electron acceptors that replace oxygen in anoxic soils neutralising acidity by consuming free H^+ ions (Brinkman, 1970). During the oxidation phase, the reaction reverses, releasing hydrogen ions and creating an acidic environment. (Van Ranst and De Coninck, 2002). As acidic soil becomes more and more anaerobic, a number of reducing chemical reactions occur, consuming free H^+ ions and increasing the pH level of the soil towards pH neutrality (Equations 2-1 to 2-4; Rowell, 1994).

Equation 2-1. Denitrification of nitrate to nitrogen gas

$$2NO_3^- + 12H^+ + 10e^- \leftrightarrow N_2 + 6H_2O$$

Equation 2-2. Fermentation of acetaldehyde to ethanol

$$CH_3CHO + 2H^+ + 2e^- \leftrightarrow CH_3CH_2OH$$

Equation 2-3. Reduction of ferric hydroxide to ferrous iron

$$Fe(OH)_3 + 3H^+ + e^- \leftrightarrow Fe^{2+} + 3H_2O$$

Equation 2-4. Production of methane from carbon dioxide

$$CO_2 + 8H^+ + 8e^- \leftrightarrow CH_4 + 2H_2O$$

Anoxic alkaline soils, in contrast, decrease in pH. This is due to the accumulation of carbon dioxide in the flooded soil, which occurs due to the reduced rates of gas exchange in waterlogged soils (Greenway et al., 2006). The accumulated carbon dioxide neutralises alkalinity through the following process (Equation 2-5 and 2-6, Sahrawat, 2005):

Equation 2-5. Accumulation of carbon dioxide in flooded soil, leading to production of carbonic acid

$$CO_2 + H_2O = H_2CO_3$$

Equation 2-6. Release of H^+ ions into alkaline soils from dissociation of carbonic acid

$$H_2CO_3 = H^+ + HCO_3^-$$

The dissociation of accumulated carbonic acid in flooded conditions increases the quantity of H^+ ions, neutralising the alkalinity of the soil, and decreases the pH level towards neutrality.

2.5.3 Soil temperature

The temperature of flooded soil strongly influences chemical reactions that occur, including governing redox conditions and microbial activity within the soil. When soil temperatures fall below 10°C, the rate of denitrification (Equation 2-1) rapidly decreases (Stanford et al., 1975). Likewise, processes later in the redox chain are also highly dependent on the ambient temperature, with methanogenesis (Equation 2-4) decreasing to almost nothing once temperatures drop to 15°C or below (Dunfield et al., 1993). However, very little information exists regarding how the temperature of the soil may be expected to change under flooding. Due to the low reflectance of water surfaces, and the darkened colour of flooded soil, the surface of flooded regions has a higher albedo (Ponnamperuma, 1984). This increased absorbency of solar radiation may be expected to increase temperatures beneath floodwater. However, as the specific heat capacity of water is greater than that of soil, the water insulates the soil against rapid and dramatic temperature changes. In wetland soils, for example, the air temperature has been observed to differ by only ± 0.3°C from flooded soil at a depth of 20 cm (Seybold et al., 2002), showing the same relationship with ambient air temperature as unflooded soil at the same depth (Tsilingridis and Papakostas, 2014). Given the strong relationship between temperature and respiration rates, this may mean that during winter flooding it takes longer for flooded soils to become completely anoxic; little exists in the literature discussing the rates of oxygen depletion in soils at different times of year. This may simply be because flooding or high soil moisture contents are less likely to occur in the summer in temperate climates.

2.5.4 Soil structure

The most damaging physical effect of flooding on soil is the breakdown of soil structure. Flooding dissolves biological films and other cementing agents and reduces cohesion between soil particles.

Flooding also disrupts soil structure due to pressure from air trapped within the soil, and disrupts soil aggregates by causing swelling of soil particles (Ponnamperuma, 1984). Soil colloids swell at different rates depending on the clay content of the soil, meaning that soils with higher clay content have a greater rate of colloidal swelling (Ponnamperuma, 1984). The shrink-swell process in soils consistently subject to wetting and drying cycles leads to a reduction in the soil shear strength, reducing the resistance of the soil to physical disruption (Ponnamperuma, 1984). This reduction in shear strength means that waterlogged soils are much more vulnerable to physical compaction; soil moisture is widely recognised as being one of the most important factors influencing soil compaction (Soane, 1990). While information about soil moisture content and compaction is primarily used to schedule and plan for farm traffic (Ohu and Folorunso, 1989; Hamza and Anderson, 2005; Taboada and Lavado, 1988), there is another element of compaction potentially at play in flooded soil that has not yet been considered; water induced compaction itself may occur under standing water during a flooding event. Because saturated soil already has a decreased shear strength, it is possible that the pressure exerted under a body of water (a weight of 1000 kg per 1 m^3) may have a significantly compressing effect. The pressure beneath one cubic metre of water can be calculated using the following equation:

Equation 2-7. The equation to determine the pressure exerted beneath a fluid

$$\boldsymbol{P_{fluid} = \rho \times g \times h}$$

Where P_{fluid} represents the pressure beneath a fluid in Pascals (Pa), ρ represents the density of the fluid in kg m^{-3}, *g* represents the acceleration due to gravity in m s^{-1}, and *h* represents the height of the fluid in metres. The density of water is 1×10^3 kg m^{-3}, under standard conditions, and the acceleration due to gravity is 9.8 m/s, giving a pressure under 1 m^3 of water of 9800 *Pa*.

When the equation is being used to calculate the pressure beneath floodwater in an open air system, atmospheric pressure also needs to be taken into account.

Equation 2-8. The calculation accounting for the effect of atmospheric pressure

$$P_{total} = P_{atmosphere} + P_{fluid}$$

Atmospheric pressure at sea level is 1.01×10^5 Pa. Thus the total pressure under a water column of 1 m x 1 m x 1 m is 110.8 *kPa*.

Medvedev and Cybulko (1995) found that the maximum permissible ground pressure on soils before soil damage was caused was highly dependent on both bulk density and water content, but many of these values (see Table 2-1) are below that of the pressure below 1 m^3 of water.

Table 2-1. The maximum permissible ground pressure on soils of different values of bulk density and water content (Medvedev and Cybulko, 1995; Hamza and Anderson, 2005)

Water Content (kg kg^{-1})	Bulk Density (Mg m^{-3})	Permissible Ground Pressure (kPa)
0.28 – 0.30	1.1	22
	1.2	45
	1.3	63
0.24 – 0.25	1.1	45
	1.2	52
	1.3	68
0.18 – 0.20	1.1	56
	1.2	64
	1.3	88
0.12 – 0.14	1.1	130
	1.2	179
	1.3	226

The pressure exerted beneath 1 m^3 of floodwater is comparable to the recommended tyre inflation pressure beneath agricultural vehicles, which can be as high as 150 kPa (Arvidsson and Keller, 2007), although the likelihood of agricultural soil being flooded to a depth of 1 m is uncertain. In the Somerset Levels flooding of 2014, some flooding was reported as a depth of 3 m (BBC News, 2014). This was an extreme event, but a report published through the Institute of Hydrology in 1996 reported that over 5,000 km^2 of non-built-up areas in England and Wales were at risk of flooding to a depth of 0.9 m, with a further 2657 km^2 at risk of flooding to a depth between 1m and 1.9 m (Morris and Flavin, 1996). Although mechanical compaction is also linked to factors such as the wheel load, high tyre pressure has been shown to be linked to increased compaction (Soane et al., 1982). As the pressure beneath standing water of a depth greater than 1 m is comparable to this tyre pressure, it may be the case that floodwater leads to compaction of soil, particularly when floodwater is deep or flooding continues for a long period of time. However, more work is needed to understand the interaction between floodwater leading to breakdown of soil structural components, such as aggregates, and potential compaction of soil.

2.5.5 Soil organic matter

Regular flooding leads to increases in the soil organic matter content of soils. In areas that regularly flood, sediment deposition by floodwater leads to increases in soil nitrogen and phosphorous (Venterink et al., 2009), in some cases depositing 2.6 g of phosphorous m^{-2} yr^{-1}, and 12.8g of nitrogen m^{-2} yr^{-1} (Johnston et al., 1984). In addition to this deposition, saturation of the soil through flooding leads to increases in soil organic matter content. Saturated soil, as discussed in Section 2.5.1, rapidly reaches anoxic conditions. Under anoxic conditions, rates of decomposition of organic matter are slowed, leading to a build-up of available organic matter (Reddy and Patrick Jr, 1975). Although the weight of floodwater on soil may lead to soil compaction and increased bulk densities after periods of flooding, as discussed in Section 2.5.4, increased organic matter content leads to decreases in soil bulk density (Bronick and Lal, 2005), with a single addition of 15 Mg ha^{-1} of organic matter leading to a reduction of 6.49% in soil bulk density (Barzegar et al., 2002). With regular flooding, the positive impact of soil organic matter accumulation may outweigh any negative impacts of the weight of floodwater on soil bulk density discussed in Section 2.5.4. However, this has not been examined in the literature.

2.6 Earthworm ecology

Earthworm species can be split into three main ecotypes, depending on where they live in the soil and their behaviour within the habitat: anecic, endogeic and epigeic (Bouché, 1977). Epigeic earthworms live within and feed on leaf litter at the soil surface, endogeic earthworms feed on and live within the soil, and anecic earthworms live in deep burrows, emerging to feed on leaf litter at the soil surface (Table 2-2)..

Table 2-2. A summary of earthworm ecotypes, their characteristics, and species belonging to each ecotype (Sims and Gerard, 1999)

Ecotype	Characteristics	UK Species
Epigeic	Live within, and feed on, litter, compost, decomposing tree trunks, etc. at soil surface Typically highly pigmented – red or rosy coloured. Typically small in size.	*Lumbricus castaneus,* *Lumbricus rubellus* *Dendrobaena octaedra*
Endogeic	Live in horizontal burrows in the soil. Ingest food sources with soil (geophagy) – typical food sources include dead roots and microflora. Create casts within the soil. Small to medium in size.	*Allolobophora chlorotica* *Apporectodea caliginosa* *Apporectodea rosea*
Anecic	Live in permanent vertical burrows within the soil. Emerge at night to forage for leaves and plant matter on the soil surface. Create casts on the soil surface.	*Lumbricus terrestris* *Apporectodea longa*

Earthworm populations of all ecotypes are typically lower in arable soils than in pasture soils (Boag et al., 1997; Curry et al., 2002). This is due to a number of factors, such as the low organic matter content of agricultural soils (Reeleder et al., 2006), the use of pesticides (Ball et al., 1986; Pelosi et al., 2013), and crush damage caused to earthworms by agricultural machinery (Tomlin and Miller, 1988). At the ecotype level, endogeic species are some of the most abundant in the UK, with two species, *A. chlorotica* and *A. caliginosa*, forming 53% of sampled earthworm abundance in the UK (Natural England, 2014). In agricultural soils, these endogeic species are also the most abundant (Pelosi et al., 2009), with reduced abundance of anecic and epigeics earthworms (Chan, 2001). This is likely due to two key factors: the avoidance of crush damage by small bodied endogeic earthworms concealed within the soil (Wyss and Glasstetter, 1992), while larger bodied anecic and surface dwelling earthworms are more vulnerable, and the lack of litter cover to act as a suitable food source for epigeics and anecic earthworms in typical cropping systems (House et al., 1985; Pelosi et al., 2009). While these ecotypes are known to respond differently to agricultural soil uses, they are also likely to respond differently to flooding events.

Epigeic species such as *L. rubellus* act as pioneer species in floodplains with poor quality soils, colonising areas within five years of a major event (Fournier et al., 2012). This pioneer species behaviour contributes to the persistence of the species in regularly flooded regions; immediately following a flooding event, Zorn et al. (2005) found that biomass and numbers were reduced to almost zero, but numbers and biomass recovered to pre-flood levels within six months. Furthermore, epigeic species in regularly flooded areas reach maturity at a faster rate than in less frequently flooded areas, with adults in these flooded regions being as much as 0.4 g lighter than adults in unflooded regions (Table 2-3) (Klok et al., 2006). This earlier maturation of epigeic species is characteristic of the population recovery of *L. rubellus* described by Zorn et al. (2005), with an increased rate of cocoon production allowing the population to recover faster following a flooding event.

Table 2-3. The mean weights of juveniles and adults of *L. rubellus* at frequently, moderately and seldom inundated floodplain sites in 2002 (Klok et al., 2006).

Inundation Routine	Juveniles (g)	Adults (g)
Frequent	0.161	0.504
Moderate	0.281	0.714
Seldom	0.384	0.972

Endogeic species are overall the most resistant to flooding events. Pot based laboratory experiments have demonstrated that, when active earthworms were placed in inundated soil, 75% of individuals of *A. chlorotica* were recovered in a quiescent state rather than exhibiting any of the escaping behaviour observed in other species of endogeic earthworm. This quiescent state was not observed in the individuals of *A. chlorotica* at lower rates of soil water content (Zorn et al., 2008). Other laboratory experiments have demonstrated that individuals of *A. chlorotica* are able to survive in excess of seven days in waterlogged soil, but while both *A. caliginosa* and *A. chlorotica* are able to persist in waterlogged soil, choice experiments show a preference for unsaturated soil (Roots, 1956). This ability to survive in waterlogged soil is an essential adaptation given that both *A. chlorotica* and *A. caliginosa* occur in a variety of habitats, including the banks of streams and lakes (Sims and Gerard, 1999). This tolerance to waterlogged soil is also observed in floodplain habitats. A study by Zorn et al. (2005) found that both *A. chlorotica* and *A. caliginosa* showed no response to regular flooding between 2000 and 2003; variations in populations of *A. caliginosa* were not linked to flooding events, and affected all age classes equally, although the causes of the variations were not known.

Anecic species such as *L. terrestris* appear to be the least resistant to flooding events, emerging alive at the soil surface immediately following flooding events (Zorn et al., 2005). This is very similar to the escaping behaviour observed by Roots (1956), where *L terrestris* was the only species of earthworm observed to attempt to escape vivaria of submerged soil. In the Zorn study, where the authors regularly surveyed the earthworm species in a floodplain known to flood regularly, *L. terrestris* was only observed on four occasions, at varied individual biomasses but still representing 50% of the total earthworm biomass within the study. The author indicated that the presence of *L. terrestris* in the study site was unexpected given the flooding regime of the region, and the presence of the species may be attributed to the retreat of the water table to a lower level following the two month flooding period. This in turn would allow the species to recover once the soil was no longer waterlogged. The reason for anecic earthworm evacuation of flooded soil is disputed. While in this thesis the assumption has been that the key driver of earthworm evacuation of flooded soil is the reduced oxygen conditions, it may be the case that waterlogged conditions facilitate the movement of anecic earthworms along the soil surface, allowing migration and avoidance of mating with related individuals (Butt and Nuutinen, 2005). However, whether the soil evacuation is driven by reduced oxygen or by the biological drive to out-breed, flooding still results in reduced soil populations of anecic earthworms.

Recent studies have suggested that earthworms can be divided into four ecotypes, rather than the three listed above from Bouché’s (1977) studies. Based on differences in earthworm physiology, such as the cuticle and epidermis thickness (Briones and Álvarez-Otero, 2018), and differences in feeding behaviours (Hoeffner et al., 2019) and the subsequent distribution of organic matter through the soil (Jégou et al., 1998; Stromberger et al., 2012), anecic earthworms can be subdivided into strict-anecic and epi-anecic ecotypes. Strict anecic earthworms exhibit less exploratory behaviour on the soil surface than epi-anecic earthworms (Hoeffner et al. 2018; Hoeffner et al., 2019), and deposit organic matter at greater depth than epi-anecics, which show a much more even distribution of organic matter along the depth of the burrow (Jégou et al., 1998). While in this thesis strict-and epi-anecic earthworms have been considered under the broad umbrella term of anecics, further work would be needed to understand whether strict- and epi-anecics are likely to respond differently to a flooding event.

2.7 Earthworm physiology and flooding

The abundance of earthworms in regularly flooded areas is generally lower than in irregularly or rarely flooded locations; a meta-analysis by Plum (2005) showed that the abundance of individuals per metre squared in episodically flooded regions was approximately 250 individuals m^{-2}, while the abundance of individuals in areas subject to summer flooding was less than 100 individuals m^{-2}. Earthworm population dynamics are also responsive to flooding regimes, with the population composition of endogeic, anecic, and epigeic earthworms likely to differ between regularly flooded regions and regions not subject to flooding. The question therefore remains about which earthworm ecological and physiological characteristics are driving the population changes, and which characteristics are potential survival mechanisms. Here, these characteristics will be discussed with reference to the most deleterious effects of flooding on soil.

The first assumption to address is that earthworms cannot survive in the anoxic conditions that occur in flooded soil. Earthworms of many species and ecotypes are able to survive in aerated soil under water for very long periods of time; at least 120 days continual submergence when artificially retained in aerated flooded soils (Ausden et al., 2001), with anecic, endogeic and epigeic earthworms all surviving well over 100 days in aerated tap water in the laboratory (Roots, 1956). However, during a flooding event, the oxygen levels in the soil rapidly reduce to anoxic levels within 24 hours of inundation due to root and microbial respiration (Ponnamperuma, 1984); laboratory experiments have found that dissolved oxygen can disappear from flooded soils with no plants growing on them within as little as ten hours when detected using polarographic methods (Scott and Evans, 1955).

There is evidence that earthworms may be able to respire anaerobically. Ralph (1957) first posited this theory after discovering discrepancies between earthworm activity and oxygen consumption, implying that earthworms are able to go into a state of oxygen debt when exposed to anaerobic conditions. Investigations into *L. terrestris* respiration before and after exposure to anaerobic conditions found that lactic acid accumulated in the tissues of the earthworms (Davis and Slater, 1928), with the same mechanisms of anaerobic respiration being observed in *L. rubellus* (Gruner and Zebe, 1978). While anaerobic respiration is certainly a mechanism for surviving in anoxic conditions, an essential mechanism given the anoxic microhabitats that can occur in soils (Sexstone et al., 1985), it is not a process that can continue indefinitely, as it consumes earthworm glycogen reserves at a faster rate than aerobic respiration (Davis and Slater, 1928). A number of questions arise from these findings: Are some species of earthworm able to remain at anaerobic conditions for longer? At what level of dissolved oxygen concentration do earthworms begin to respire anaerobically? And how long can earthworm species maintain anaerobic respiration?

Some species of earthworm are more resistant to low oxygen levels than others. This tolerance seems to be linked to the diurnal rhythms of oxygen consumption: species that have strong diurnal rhythms of oxygen consumption seem to be less resilient to low oxygen concentrations than species that do not display such rhythms (Chuang and Chen, 2008). Chaung and Chen studied two species of earthworm that are not native to the UK; *Amynthas gracilis*, an anecic species, and *Pontoscolezax corethrurus*, an endogeic species. After raising the earthworms in moist soil subject to an alternating 12 hour light and dark period, the authors placed the earthworms into water chambers and measured the rates of oxygen consumption at three distinct time periods over 24 hours. The authors found that for *A. gracilis*, oxygen consumption over the two hour period between 11:00-13:00 was lower than for the periods of 19:00 – 21:00 and 04:00 – 06:00, while *P. corethrurus* showed no change in the rate of oxygen consumption over the same monitoring intervals. The authors drew upon this higher rate of consumption during the night time period to infer that *A. gracilis*, the anecic species, displayed a diurnal rhythm of oxygen consumption, while *P. corethrurus*, the endogeic species, did not. This oxygen consumption may be linked to the foraging strategies of earthworm ecotypes. Anecic earthworms forage on the soil surface for food at night, while endogeic species constantly consume soil as they move through it. These differences in foraging strategies may explain the difference in oxygen consumption, and in turn may result in anecic species being more vulnerable to oxygen deprivation during flooding than endogeic species. However, there are no definitive studies in the literature comparing the oxygen consumption of anecic and endogeic species of UK earthworms.

As discussed in Section 2.5, there is the potential that flooding events lead to compaction within the soil. There is much evidence that earthworm populations are dramatically reduced in both abundances and biomass with regular compaction associated with agricultural activity. One study found that the annual compaction of wet agricultural soil with 10Mg axle load over a period of five years led to a reduction in the proportion of soil fauna represented by earthworms from 65% to 0% (Radford et al., 2001). The reduction of the earthworm population within this study can be attributed both to the initial crush damage from the vehicle and the subsequent evacuation of compacted soil by surviving individuals. However, there is some question as to whether different ecotypes are more intolerant of compacted soil conditions than others (Cluzeau et al., 1992), with behavioural changes being driven by compacted soil. There is evidence that anecic earthworm burrowing activity is influenced by soil compaction (Kretzschmar, 1991; Joschko et al., 1989), with burrows increasing in number, but reducing in continuity, in compacted soil with a bulk density of 1.4 g cm^{-3} (Jégou et al., 2002; Langmaack et al., 1999). Conversely, endogeic burrows increased in continuity with compaction in soils ranging between a bulk densty of 1.48 and 1.61 g cm^{-3} (Langmaack et al., 1999). Both of these behavioural changes are likely due to the increased bulk density of the soil and the way that the different ecotypes process the soil in order to feed. Endogeic species move laterally through the soil, which they ingest as a food source, while anecic species live in vertical burrows and are detritivorous. However, while unfavourable conditions cause rapid and dramatic reductions in earthworm populations, recovery from single mechanical compaction events can be equally rapid. One study found that within three months of a compaction event using laden farm machinery, earthworm abundances were equal to non-compacted soil (Capowiez et al., 2012).

2.8 Sewage sludge usage

In the UK, treated sewage sludge, also known as biosolids, is a by-product of the waste water treatment process. A summary diagram of sewage treatment can be found in Fig. 2-1.

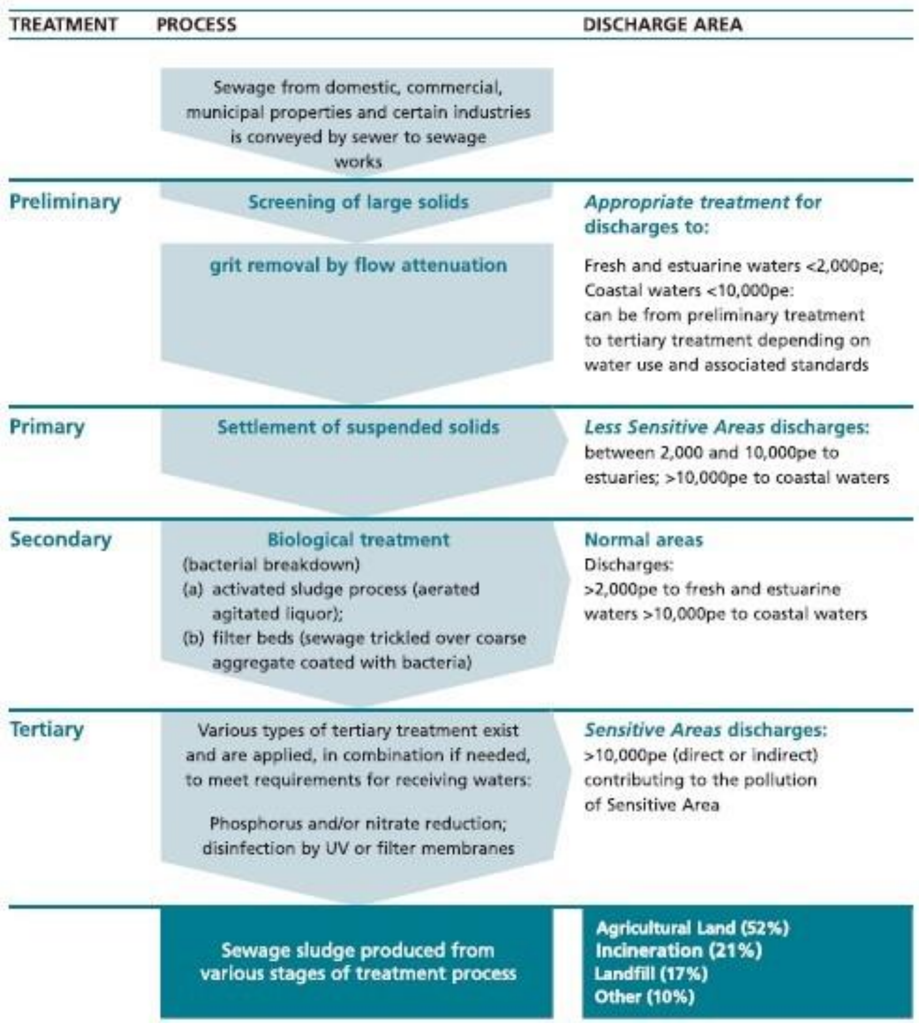

Figure 2-1. The sewage treatment process (DEFRA, 2002)

In 2010, 79% of treated sewage sludge was disposed of through application to approximately 165,000 hectares of farmland (DEFRA, 2012; Water UK, 2010), with approximately 60% of sewage sludge disposed of through land application in the United States and in Canada (Cogger et al., 2006). UK Government legislation provides the regulations and restrictions that apply to the farmland application of sewage sludge. However, while these regulations restrict the application of sludge under certain conditions, including prohibiting its application if the quality of the soil and of surface and groundwater will be impaired (DEFRA, 2013), there are no regulations regarding the application of sewage sludge in areas known to flood regularly.

There are largely three different grades of sewage sludge that are referred to for agricultural purposes: untreated sludge, conventionally treated sludge, and enhanced treated sludge. The application of untreated sewage sludge was banned from application to agricultural soil used for food in 1999, followed by the additional banning of its use for non-food crops on agricultural land in 2005 (ADAS, 2001a). Treated sewage sludge is available in two different grades: conventionally and enhanced treated sludge. Conventionally treated sludge has had 99% of pathogens removed (ADAS, 2001b). This removal could be through a number of chemical and biological methods, but approximately two thirds of the sludge in the UK is treated by anaerobic digestion (DEFRA, 2011). Enhanced treatment sludge has been treated to eliminate 99.99% of all pathogens present in the original sludge (ADAS, 2001b). Examples of treatments for production of enhanced sewage sludge can be found in Table 2-4. The enhanced sewage sludge is then available as different media for application to the soil; the sludge can be applied as a liquid, with approximately 4% dry solids, a dewatered cake, with approximately 25% dry solids, or a granule, with approximately 95% dry solids, with the dewatering process generally achieved through centrifuging the end product (ADAS, 2001b).

Table 2-4. Examples of effective sludge treatment processes (DEFRA, 2002; European Commission, 2010)

Process	Summary
Sludge pasteurisation	Minimum of 30 minutes at 70°C or 4 hours at 55°C, followed by mesophilic anaerobic digestion.
Mesophilic anaerobic digestion	At least 12 days primary digestion at 35°C ± 3°C, or at least 30 days primary digestion at 25°C ± 3°C, followed by a secondary stage with a retention period of at least 14 days.
Thermophilic aerobic digestion	At least 7 days digestion, with sludge treated at a minimum of 55°C for at least 4 hours.
Composting (windrows or aerated piles)	Compost maintained at 40°C for at least 5 days, with 4 hours in that period at a minimum of 55°C. Followed by a maturation process to ensure compost reaction process sufficiently completed.
Lime stabilisation of liquid sludge	Addition of lime to raise pH to greater than 12.0 for a minimum of 2 hours.
Liquid storage	Storage of untreated liquid sludge for at least three months.
Dewatering and storage	Treatment of untreated sludge with lime, followed by dewatering and storage for at least 3 months.

Each of these treatment methods produces enhanced sewage sludge, but the methods vary significantly. This can result in variability of the nutrient content of the sewage sludges applied to the soil. The type of enhanced sewage sludge available locally is largely dependent on the facilities available locally (ADAS, 2001a), with the most common form of enhanced sludge treatment in the UK being anaerobic digestion (European Commission, 2010).

2.9 The impact of sewage sludge on soil

Sewage sludge has two key benefits as a soil amendment for agricultural soils. Its application helps to improve the structure and water holding capacity of poor quality soils (Hamza and

Anderson, 2005), and to increase quantities of limiting nutrients such as nitrogen and phosphorus (Sterritt and Lester, 1980).

2.9.1 Soil physical properties

The addition of sewage sludge to soils reduces soil bulk density, increases soil porosity and increases the stability of aggregates in a variety of soil types. Soil porosity increases in the small to medium pore size range (30 – 50 µm and 50 – 500 µm) with the addition of sewage sludge (Pagliai et al., 1983), which can lead to an increase in water infiltration rates. The increase in aggregate stability is more pronounced in soils with a higher clay content, perhaps due to an already higher tendency to aggregate (García-Orenes et al., 2005), or due to an already low organic matter content within these soils. The increased percentage of stable aggregates initially improves the water holding capacity of the soil, although Epstein (1975) found that while the stable aggregate percentage remained higher in sewage sludge amended soil (28 – 35% for amended soils, compared to 17% of the control soils), the saturated hydraulic conductivity of the soil returned to that of the original soil after 50 to 80 days (Epstein, 1975). As relatively recalcitrant organic matter (Larney and Angers, 2012), the effect of sewage sludge application on saturated hydraulic conductivity may be expected to last longer than Epstein reported, although other studies have found that the benefits persist for at least four years (Wallace et al., 2009; Lindsay and Logan, 1996). However it is important to consider that studies reporting on the longevity of sewage sludges in soil may be dependent on the application methods. Some studies are based on a single, large application of sludge for carbon capture or fertility purposes, which is a very different application regime to that of the uses of sewage sludge as a soil amendment.

These contradictory results regarding the longevity of sewage sludge in soil can potentially be explained by the wide level of heterogeneity between sludges, depending on where they are produced. Sludge composition is affected, not only on the wastewater composition in the area (Sommers, 1977), but also on the processing methods used by the production plant. Different forms of sewage sludge (liquid, dewatered or dry solids) have different nitrogen availability (Smith et al., 1998). Additionally, there is evidence that when sewage sludges are thermally dried at higher temperature, the organic matter is more recalcitrant, and so less available for breakdown by soil organisms (Case et al., 2016). This would result in the sewage sludges remaining in the soil for a longer period of time, and so impacting upon soil structure for longer. However, the environmental conditions of the site of application will also have an impact on the rates of sewage sludge decomposition, with soil water content increasing rates of organic matter breakdown

(Khaleel et al., 1981), and better aerated sites decomposing almost 50% of the waste carbon within 90 days (Mathers and Stewart, 1970).

2.9.2 Soil pH

There is a great amount of variability in the literature as to the effect of the addition of sewage sludge on soil pH. While some papers have found that the addition of sewage sludge reduced the pH of the soil from 6.2 unamended to 5.2 after three years of sludge application (Neilsen et al., 1998), others have found that the soil pH increased towards neutrality, whether the soil began at low pH values of 4.86 or already close to neutral values of 6.30 or 7.09 (Tsadilas et al., 1995). Variability in the content of the sludges, along with variations in the soil themselves, may lead to highly varied effects of the application of sewage sludge on soil pH. As sludges are produced in a variety of processing plants, their composition will be highly dependent on the wastewater produced in the local area. While N, P and K levels may be relatively homogenous, the concentrations of trace elements are more variable (Sommers, 1977). This variation in base material and trace elements may well transfer into the variation observed across various experiments. Variation in sludge pH has been observed across countries, with sewage sludge pHs ranging from 6.8 in Thailand (Parkpain et al., 1998) to 8.6 in Spain (Martinez et al., 2002, in Singh and Agrawal, 2008), and within countries, with sludges in Kolkata, India, ranging in pH from 5.96 to 7.14 (Saha et al., 2018).

2.9.3 Soil fauna

The addition of sewage sludge also impacts soil microbial populations. Bastida et al. (2008) found that, seventeen years after single applications of medium (a soil organic matter content increase of 1%), high (an increase of 1.5%) or very high (2%) quantities of sewage sludges, the presence of the phospho-lipid fatty acid (PLFA) profiles of both soil bacteria and soil fungi was significantly higher than unamended soils, with bacterial and fungal PLFA concentrations in amended soils double those observed in control soils (Bastida et al., 2008). In addition to increasing the microbial populations of soils, the application of sewage sludge also increases the populations of earthworms (Hamilton and Dindal, 1989), although this seems to be highly dependent on the earthworm ecotypes present (Coors et al., 2016), with *L. terrestris* being observed to grow at a faster rate in soil amended with sewage sludge (Hartenstein and Amico, 1983). While most of the existing research regarding interactions between sewage sludge application and earthworms focuses on the uptake of metals and other environmental pollutants, there is very little research into sewage sludge as a food source for earthworms in soil. Despite the reported changes in earthworm populations and growth rates, with the anecic *L. terrestris* seeming to respond positively to the presence of sludge, while Hamilton and Dindal (1989) found that populations of the epigeic *E. fetida* reduced in its presence, many laboratory based experiments have found that many species of earthworm avoid sewage sludge when other food sources such as leaf litter are available (Le Bayon and Binet, 2006; Doube et al., 1997; Artuso et al., 2011). Other studies have found that earthworms willshow a preference for soil that has been amended with sewage sludge as a source of organic matter compared to an OECD artificial soil composed of *Sphagnum* peat mixed with kaolinite clay and sand quartz (Moreira et al., 2008), or over a range of unamended control soils (Bouldin et al., 2016).

2.10 Earthworms, flooding, and sewage sludge: The knowledge gaps

This thesis is part of the research being carried out by the BIOSAS research group. The BIOSAS group is investigating the BIOchemical-physical-biological function of Sludge in Agricultural Soils, and this thesis is investigating the interactions of sludge amended soils, flooding, and earthworm populations.

From the literature, we know that earthworm populations are highly responsive to flooding, with earthworm population structures being highly influenced by the flooding regime at the ecotype level. We also know that there are several advantages to the application of sewage sludge to farmland soil. However, most existing research focuses on the metal content of sludges and the effect that may have on earthworm populations.

There are three key questions that therefore arise for this thesis:

1. Does regular flooding impact earthworm populations in arable and pasture soils differently, given the already low earthworm populations in arable soils?

2. Does increasing organic matter content, in the form of sewage sludge application, lead to faster rates of oxygen depletion when soils floods?

3. Are there species specific responses of earthworms to flooding? And, if so, how might these change with the application of sewage sludge to soil?

2.11 References

ADAS, 2001a. The Safe Sludge Matrix. [Leaflet]

ADAS, 2001b. Guidelines for the Application of Sewage Sludge to Industrial Crops. [Leaflet]

Akhtar, J., Gorham, J., Qureshi, R.H. 1993. Combined Effect of Salinity and Hypoxia in Wheat (*Triticum aestivum* L.) and Wheat-*Thinopyrum* Amphiploids. *Plant and Soil* 166(1): 47 – 54

Artuso, N., Kennedy, T.F., Connery, J., Grant, J., Schmidt, O. 2011. Assessment of Biosolids in Earthworm Choice Tests with Different Species and Soils. *Global Nest Journal*, 13: 255-265.

Arvidsson, J., Keller, T. 2007. Soil Stress as Affected by Wheel Load and Tyre Inflation Pressure. *Soil & Tillage Research*, 96: 284-291.

Ausden, M., Sutherland, W. J., James, R. 2001. The Effects of Flooding Lowland Wet Grassland on Soil Macroinvertebrate Prey of Breeding Wading Birds. *Journal of Applied Ecology*, 38: 320-338.

Ball, D., Tapp, L., Gordon, F.C., Potter, D.A. 1986. Effects of Pesticides on Earthworm Populations in Kentucky Bluegrass, 1985. *Insecticide and Acaricide Tests* 11(1): 395 – 396.

Barzegar, A.R., Yousefi, A., Daryashenas, A. 2002. The Effect of Addition of Different Amounts and Types of Organic Matters on Soil Physical Properties and Yield of Wheat. *Plant and Soil* 247(2): 295 – 301

Bastida, F., Kandeler, E., Hernández, T., García, C. 2008. Long-Term Effect of Municipal Solid Waste Amendment on Microbial Abundance and Humus-Associated Enzyme Activities under Semiarid Conditions. *Microbial Ecology*, 55: 651-661.

Le Bayon, R.C., Binet, F. 2006. Earthworms Change the Distribution and Availability of Phosphorous in Organic Substrates. *Soil Biology & Biochemistry*, 38: 235-246.

BBC News, 2014. What are the Somerset Levels? BBC News, 7th February 2014. Available at https://www.bbc.co.uk/news/uk-england-somerset-26080597 [accessed online 17/09/2019]

Blöschl, G, Hall, J., Viglione, A., Perdigão, R.A., Parajka, J., Merz, B., Lun, D., Arheimer, B., Arnoica, G.T., Bilibashi, A., Boháč, M. 2019. Changing Climate both Increases and Decreases European River Floods. *Nature* 573: 108 - 111

Boag, B., Palmer, L.F., Neilson, R., Legg, R., Chambers, S.J. 1997. Distribution, Prevalence and Intensity of Earthworm Populations in Arable Land and Grassland in Scotland. *Annals of Applied Biology* 130(1): 153 – 165.

Bouldin, J.L., Klasky, J.W.P., Green, V.S. 2016. Earthworm Preference Bioassays to Evaluate Land Management Practices. *Bulletin of Environmental Contamination and Toxicology*, 96: 767-772.

Brinkman, R. 1970. Ferrolysis, A Hydromorphic Soil Forming Process. *Geoderma*, 3: 199-&.

Briones, M.J.I., Álvarez-Otero, R. 2018. Body Wall Thickness as a Potential Functional Trait for Assigning Earthworm Species to Ecological Categories. *Pedobiolgia* 67: 26 – 34

Bronick, C.J., Lal, R. 2005. Soil Structure and Management: A Review. *Geoderma* 124(1-2): 3 – 22

Butt, K.R., Nuutinen, V. 2005. The Dawn of the Dew Worm. *Biologist* 52(4): 218 – 223

Capowiez, Y., Samartino, S., Cadoux, S., Bouchant, P., Richard, G., Boizard, H. 2012. Role of Earthworms in Regenerating Soil Structure after Compaction in Reduced Tillage Systems. *Soil Biology & Biochemistry*, 55: 93-103.

Case, S. D. C., Gomez-Munoz, B., Magid, J., Jensen, L. S. 2016. Increasing Thermal Drying Temperature of Biosolids Reduced Nitrogen Mineralisation and Soil N_2O Emissions. *Environmental Science and Pollution Research*, 23: 14383-14392.

Cave, R.R., Ledoux, L., Turner, K., Jickells, T., Andrews, J.E., Davies, H., 2003. The Humber Catchment and its Coastal Area: From UK to European Perspectives. *Science of the Total Environment*, 314: 31-52.

Chan, K.Y. 2001. An Overview of Some Tillage Impacts on Earthworm Population Abundance and Diversity – Implications for Functioning in Soils. *Soil and Tillage Research* 57(4): 179 – 191.

Chen, W., Hou, Z., Wu, L., Liang, Y., Wei, C. 2010. Evaluating Salinity Distribution in Soil Irrigated with Saline Water in Arid Regions of Northwest China. *Agricultural Water Management* 97(12): 2001-2008

Chuang, S. C. & Chen, J. H. 2008. Role of Diurnal Rhythm of Oxygen Consumption in Emergence from Soil at Night after Heavy Rain by Earthworms. *Invertebrate Biology*, 127: 80-86.

Cluzeau, D., Binet, F., Vertes, F., Simon, J. C., Riviere, J. M., Trehen, P. 1992. Effects of Intensive Cattle Trampling on Soil Plant Earthworms System in 2 Grassland Types. *Soil Biology & Biochemistry*, 24: 1661-1665.

Cogger, C.G., Forge, T.A., Neilsen, G.H. 2006. Biosolids Recycling: Nitrogen Management and Soil. *Canadian Journal of Soil Science* 86: 613 – 620.

Coors, A., Edwards, M., Lorenz, P., Rombke, J., Schmelz, R. M., Topp, E., Waszak, K., Wilkes, G., Lapen, D. R. 2016. Biosolids Applied to Agricultural Land: Influence on Structural and Functional Endpoints of Soil Fauna on a Short- and Long-Term Scale. *Science of the Total Environment*, 562: 312-326.

Curry, J.P., Byrne, D., Schmidt, O. 2002. Intensive Cultivation can Drastically Reduce Earthworm Populations in Arable Land. *European Journal of Soil Biology* 38(2): 127 – 130.

Dawson, R.J., Dickson, M.E., Nicholls, R.J., Hall, J.W., Walkden, J.A., Stansby, P.K., Mokrech, M., Richards, J., Zhou, J., Milligan, J., Jordan, A., Pearson, S., Rees, J., Bates, P.D., Koukoulas, S., Watkinson, A.R., 2009. Integrated Analysis of Risks of Coastal Flooding and Cliff Erosion Under Scenarios of Long Term Change. *Climatic Change*, 95: 249-288.

Davis, J.H., Slater, W.K., 1928. The anaerobic metabolism of the earthworm (*Lumbricus terrestris*). *Biochemical Journal*, 22: 338.

DEFRA, 2002. Sewage Treatment in the UK. UK Implementation of the EC Urban Waste Water Treatment Directive. [Leaflet]

DEFRA, 2011. Anaerobic Digestion Strategy and Action Plan. [Leaflet]

DEFRA, 2012. Waste Water Treatment in the United Kingdom – 2012. Implementation of the European Union Urban Waste Water Treatment Directive – 91/271/EEC. [Leaflet]

DEFRA, 2013. Managing Sewage Sludge, Slurry and Silage [Online]. Available At https://www.gov.uk/guidance/managing-sewage-sludge-slurry-and-silage. [Accessed 16th January 2017].

Doube, B.M., Schmidt, O., Killham, K., Correll, R. 1997. Influence of Mineral Soil on the Palatability of Organic Matter for Lumbricid Earthworms: A Simple Food Preference Study. *Soil Biology & Biochemistry*, 29: 569-575.

Dunfield, P., Knowles, R., Dumont, R., Moore, T. R. 1993. Methane Production and Consumption in Temperate and Sub-Arctic Peat Soils - Response to Temperature and pH. *Soil Biology & Biochemistry*, 25: 321-326.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., Mearns, L. O. 2000. Climate extremes: Observations, Modeling, and Impacts. *Science*, 289: 2068-2074.

Edmunds, W.M., Shand, P., Hart, P., Ward, R.S., 2003. The Natural (Baseline) Quality of Groundwater: a UK Pilot Study. *Science of the Total Environment*, 310: 25-35.

Environment Agency, 2007. Underground, Under Threat. The State of Groundwater in England and Wales. [Leaflet].

Epstein, E. 1975. Effect of Sewage Sludge on Some Soil Physical-Properties. *Journal of Environmental Quality*, 4: 139-142.

European Commission, 2010. Environmental, Economic and Social Impacts of the Use of Sewage Sludge on Land. Final Report. Part III: Project Interim Reports.

Fournier, B., Samaritani, E., Shrestha, J., Mitchell, E. A. D., Le Bayon, R. C. 2012. Patterns of earthworm communities and species traits in relation to the perturbation gradient of a restored floodplain. *Applied Soil Ecology*, 59: 87-95.

García-Orenes, F., Guerrero, C., Mataix-Solera, J., Navarro-Pedreno, J., Gomez, I., Mataix-Beneyto, J. 2005. Factors controlling the aggregate stability and bulk density in two different degraded soils amended with biosolids. *Soil & Tillage Research*, 82: 65-76.

Greenway, H., Armstrong, W., Colmer, T.D. 2006. Conditions Leading to High CO2 (>5Kpa) in Waterlogged-Flooded Soils and Possible Effects on Root Growth and Metabolism. *Annals of Botany*, 98: 9-32.

Gruner, B., Zebe, E. 1978. Studies on Anaerobic Metabolism of Earthworms. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 60: 441-445.

Hall, J.W., Sayers, P.B., Walkden, M.J.A., Panzeri, M. 2006. Impacts of Climate Change on Coastal Flood Risk in England and Wales: 2030 – 2100. *Philosophical Transactions: Mathematical, Physical, and Engineering Sciences* 363(1841): 1027 – 1049

Hamilton, W. E., Dindal, D. L. 1989. Impact of Land-Spread Sewage-Sludge and Earthworm Introduction on Established Earthworms and Soil Structure. *Biology and Fertility of Soils*, 8: 160-165.

Hamza, M. A., Anderson, W. K. 2005. Soil compaction in cropping systems - A review of the nature, causes and possible solutions. *Soil & Tillage Research*, 82: 121-145.

Haque, S.A. 2006. Salinity Problems and Crop Production in Coastal Regions of Bangladesh. *Pakistan Journal of Botany*, 38(5): 1359-1365

Hartenstein, R., Amico, L., 1983. Production and carrying-capacity for the earthworm *Lumbricus terrestris* in culture. *Soil Biology and Biochemistry*, 15: 51-54.

Hoeffner, K., Monard, C., Santonja, M., Cluzeau, D. 2018. Feeding Behaviour of Epi-Anecic Earthworm Species and their Impacts on Soil Microbial Communities. *Soil Biology and Biochemistry* 125: 1 – 9

Hoeffner, K., Santonja, M., Cluzeau, D., Monard, C. 2019. Epi-Anecic Rather than Strict-Anecic Earthworms Enhance Soil Enzymatic Activities. *Soil Biology and Biochemistry* 132: 93 – 100

Hoggart, S.P.G., Hanley, M.E., Parker, D.J., Simmons, D.J., Bilton, D.T., Filipova-Marinova, M., Franklin, E.L., Kotsev, I., Penning-Rowsell, E.C., Rundle, S.D., Trifonova, E., Vergiev, S., White, A.C., Thompson, R.C. 2014. The Consequences of Doing Nothing: The Effects of Seawater Flooding on Coastal Zones. *Coastal Engineering* 87: 169 – 182

Holman, I.P., Hollis, J.M., Bramley, M.E., Thompson, T.R.E., 2003. The contribution of soil structural degredation to catchment flooding: a preliminary investigation of the 2000 floods in England and Wales. *Hydrology and Earth System Sciences Discussions,* 7: 755-766.

House, G.J, Parmelee, R.W. 1985. Comparison of Soil Arthropods and Earthworms from Conventional and No-Tillage Agroecosystems. *Soil and Tillage Research* 5(4): 351 – 360

Hughes, A., Vounaki, T., Peach, D.W., Ireson, A.M., Jackson, C.R., Butler, A.P., Bloomfield, J.P., Finch, J., Wheater, H.S. 2011. Flood Risk for Groundwater: Examples from a Chalk Catchment in Southern England. *Journal of Flood Risk Management* 4(3): 143 – 155

Jégou, D., Brunotte, J., Rogasik, H., Capowiez, Y., Diestel, H., Schrader, S., Cluzeau, D. 2002. Impact of soil compaction on earthworm burrow systems using X-ray computed tomography: preliminary study. *European Journal of Soil Biology*, 38: 329-336.

Jégou, D., Cluzeau, D., Balesdent, J, Tréhen, P. 1998. Effects of Four Ecological Categories of Earthworms on Carbon Transfer in Soil. *Applied Soil Ecology* 9(1-3): 249 – 255

Johnston, C.A., Bubenzer, G.D., Lee, G.B., Madison, F.W., McHenry, J.R. 1984. Nutrient Trapping by Sediment Deposition and Seasonally Flooded Lakeside Wetland 1. *Journal of Environmental Quality* 13(2): 283 – 290

Joschko, M., Diestel, H., Larink, O. 1989. Assessment of Earthworm Burrowing Efficiency in Compacted Soil with a Combination of Morphological and Soil Physical Measurements. *Biology and Fertility of Soils,* 8: 191-196.

Khaleel, R., Reddy, K, R., Overcash, M.R., 1981. Changes in soil physical-properties due to Organic Waste Applications – A Review. *Journal of Environmental Quality*, 10: 133-141.

Klok, C., Zorn, M., Koolhaas, J. E., Eijsackers, H. J. P. & Van Gestel, C. A. M. 2006. Does Reproductive Plasticity in *Lumbricus Rubellus* Improve the Recovery of Populations in Frequently Inundated River Floodplains? *Soil Biology & Biochemistry*, 38: 611-618.

Kögel-Knabner, I., Amelung, W., Cao, Z. H., Fiedler, S., Frenzel, P., Jahn, R., Kalbitz, K., Kolbl, A. & Schloter, M. 2010. Biogeochemistry of Paddy Soils. *Geoderma*, 157: 1-14.

Kretzschmar, A. 1991. Burrowing Ability of the Earthworm *Aporrectodea longa* Limited by Soil Compaction and Water Potential. *Biology and Fertility of Soils*, 11: 48-51.

Langmaack, M., Schrader, S., Rapp-Bernhardt, U., Kotzke, K. 1999. Quantitative Analysis of Earthworm Burrow Systems with Respect to Biological Soil-Structure Regeneration after Soil Compaction. *Biology and Fertility of Soils*, 28: 219-229.

Larney, F. J., Angers, D. A. 2012. The Role of Organic Amendments in Soil Reclamation: A Review. *Canadian Journal of Soil Science,* 92: 19-38.

Lindsay, B.J., Logan, T.J., 1998. Field Response of Soil Physical Properties to Sewage Sludge. *Journal of Environmental Quality*, 27: 534-542.

Maraun, D., Osborn, T. J., Gillett, N. P. 2008. United Kingdom Daily Precipitation Intensity: Improved Early Data, Error Estimates and an Update from 2000 to 2006. *International Journal of Climatology*, 28: 833-842.

Mathers, A.T., Stewart, B.A. 1970. Nitrogen Transformations and Plant Growth as Affected by Applying Large Amounts of Cattle Feedlot Wastes to Soil. In Cornell *Univ Conf Agr Waste Manage Relat Agr Soil Water*.

Mcmichael, A. J., Woodruff, R. E., Hales, S. 2006. Climate Change and Human Health: Present and Future Risks. *Lancet*, 367: 859-869.

Medvedev, V. V., Cybulko, W. G. 1995. Soil Criteria for Assessing the Maximum Permissible Ground Pressure of Agricultural Vehicles on Chernozem Soils. *Soil & Tillage Research*, 36: 153-164.

Milly, P. C. D., Wetherald, R. T., Dunne, K. A., Delworth, T. L. 2002. Increasing Risk of Great Floods in a Changing Climate. *Nature*: 415, 514-517.

Moreira, R., Sousa, J.P., Canhoto, C., 2008. Biological Testing of a Digested Sewage Sludge and Derived Composts. *Bioresource Technology*, 99: 8382-8389.

Morris, D.G., Flavin, R.W. 1996. Report No. 130. Flood Risk Map for England and Wales. Institute of Hydrology

Natural England, 2014. Earthworms in England: Distribution, Abundance and Habitats (NECR145)

Neilsen, G. H., Hogue, E. J., Neilsen, D., Zebarth, B. J. 1998. Evaluation of Organic Wastes as Soil Amendments for Cultivation of Carrot and Chard on Irrigated Sandy Soils. *Canadian Journal of Soil Science*, 78: 217-225.

Ohu, J. O., Folorunso, O. A. 1989. The Effect of Machinery Traffic on the Physical-Properties of a Sandy Loam Soil and on the Yield of Sorghum in Northeastern Nigeria. *Soil & Tillage Research*, 13: 399-405.

Owojori, O.J., Reinecke, A.J., Rozanov, A.B. 2008. Effects of Salinity of Partitioning, Uptake and Toxicity of Zinc in the Earthworm *Eisenia fetida*. *Soil Biology and Biochemistry* 40(9): 2385-2393.

Owojori, O.J., Reinecke, A.J., Voua-Otomo, P., Reinecke, S.A. 2009. Comparative Study of the Effects of Salinity on Life-Cycle Parameters of Four Soil-Dwelling Species (*Folsomia candida, Enchytraeus doerjesi, Eisenia fetida* and *Aporrectodea Caliginosa*). *Pedobiologia*, 52(6): 351-360

Page, H.J., Williams, W., 1926. The Effect of Flooding With Sea-Water on the Fertility of the Soil. *Journal of Agricultural Science*, 16: 551-573.

Pagliai, M., Bisdom, E.B.A., Ledin, S., 1983. Changes in Surface-Structure (Crusting) After Application of Sewage-Sludge and Pig Slurry to Cultivated Agricultural Soils in Northern Italy. *Geoderma:* 30, 35-53.

Parkpain, P., Sirisukhodom, S., Carbonell-Barrachina, A.A. 1998. Heavy Metals and Nutrients Chemistry in Sewage Sludge Amended Thai Soils. *Journal Of Environmental Science And Health, Part A* 33(4): 573-597.

Parsons, D.A., Apmann, R.P., Decker, G.H. 1963. The Determination of Sediment Yields from Flood Water Sampling. *International Association Scientific Hydrology Publication* 65: 7 – 15

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulcke, F. Pesticides and Earthworms. A Review. 2013. *Agronomy for Sustainable Development* 34(1): 199 – 229.

Pelosi, C., Bertrand, M., Roger-Estrade, J. 2009. Earthworm Community in Conventional, Organic, and Direct Seeding with Living Mulch Cropping Systems. *Agronomy for Sustainable Development* 29(2): 287 – 295

Plum, N. 2005. Terrestrial Invertebrates in Flooded Grassland: A Literature Review. *Wetlands*, 25: 721-737.

Ponnamperuma, F.N., 1984. Effects of Flooding on Soils. In: Flooding and Plant Growth. London; Academic Press, Inc.

Prudhomme, C., Jakob, D., Svensson, C. 2003. Uncertainty and Climate Change Impact on the Flood Regime of Small UK Catchments. *Journal of Hydrology*, 277, 1-23.

Radford, B. J., Wilson-Rummenie, A. C., Simpson, G. B., Bell, K. L., Ferguson, M. A. 2001. Compacted Soil Affects Soil Macrofauna Populations in a Semi-Arid Environment in Central Queensland. *Soil Biology & Biochemistry*, 33: 1869-1872.

Ralph, C.L., 1957. Persistent Rhythms of Activity and O_2-Consumition in the Earthworm. *Physiological and Biochemical Zoology*: 30, 41-55.

Raw, F. 1959. Estimating Earthworm Populations by Using Formalin. *Nature:* 184, 1661-1662.

Reddy, K.R., Patrick Jr, W.H. 1975. Effect of Alternate Aerobic and Anaerobic Conditions on Redox Potential, Organic Matter Decomposition and Nitrogen Loss in a Flooded Soil. *Soil Biology and Biochemistry* 7(2); 87-94

Reeleder, R.D., Miller, J.J., Ball-Coelho, B.R., Roy, R.C. 2006. Impacts of Tillage, Cover Crop, and Nitrogen on Populations of Earthworms, Microarthropods, and Soil Fungi in a Cultivated Fragile Soil. *Applied Soil Ecology* 33(3): 243 – 257.

Roots, B. I. 1956. The Water Relations of Earthworms .2. Resistance to Desiccation and Immersion, and Behaviour When Submerged and When Allowed a Choice of Environment. *Journal of Experimental Biology*, 33, 29-44.

Rowell, D. L. 1994. Soil science: Methods and applications. Longman Group, Essex, UK

Saha, S., Saha, B.N., Hazra, G.C., Pati, S., Pal, B., Kundu, D., Ghosh Bag, A., Chatterjee, N., Batabyal, K. 2018. Assessing The Suitability of Sewage-Sludge Produced in Kolkata, India, For Their Agricultural Use. *Proceedings of the Indian National Science Academy* 84(3): 781-792.

Sahrawat, K. L. 2005. Fertility and Organic Matter in Submerged Rice Soils. *Current Science*, 88: 735-739.

Schlesinger, W.H., 2013. Biogeochemistry: An Analysis of Global Change. *Oxford: Academic Press.*

Scott, A.D., Evans, D.D., 1955. Dissolved Oxygen in Saturated Soil. *Soil Science Society of America*, 19, 7-12.

Sexstone, A. J., Revsbech, N. P., Parkin, T. B., Tiedje, J. M. 1985. Direct Measurement of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society of America Journal*, 49: 645-651.

Seybold, C. A., Mersie, W., Huang, J. Y., Mcnamee, C. 2002. Soil Redox, pH, Temperature, and Water-Table Patterns of a Freshwater Tidal Wetland. *Wetlands*, 22: 149-158.

Sims, R.W., Gerard, B.M. 1991. Earthworms. FSC Publications, London

Singh, R.P., Agrawal, M. 2008. Potential Benefits and Risk of Land Application of Sewage Sludge. *Waste Management* 28(2): 347-358

Smith, S. R., Woods, V., Evans, T. D. 1998. Nitrate Dynamics in Biosolids-Treated Soils. I. Influence of Biosolids Type and Soil Type. *Bioresource Technology*, 66: 139-149.

Soane, B. D. 1990. The Role of Organic-Matter in Soil Compactibility - A Review of Some Practical Aspects. *Soil & Tillage Research*, 16: 179-201.

Soane, B. D., Dickson, J. W., Campbell, D. J. 1982. Compaction by Agricultural Vehicles - A Review .3. Incidence and Control of Compaction in Crop Production. *Soil & Tillage Research*, 2: 3-36.

Sommers, L. E. 1977. Chemical Composition of Sewage Sludges and Analysis of their Potential Use as Fertilizers. *Journal of Environmental Quality*, 6: 225-232.

Stanford, G., Dzienia, S., Vanderpol, R. A. 1975. Effect of Temperature on Denitrification Rate in Soils. *Soil Science Society of America Journal*, 39: 867-870.

Sterritt, R. M., Lester, J. N. 1980. The Value of Sewage-Sludge to Agriculture and Effects of The Agricultural Use of Sludges Contaminated With Toxic Elements - A Review. *Science of the Total Environment*, 16: 55-90.

Stromberger, M. E., Keith, A. M., Schmidt, O. 2012. Distinct Microbial and Faunal Communities and Translocated Carbon in *Lumbricus terrestris* Drilospheres. *Soil Biology and Biochemistry* 46: 155 – 162

Taboada, M. A., Lavado, R. S. 1988. Grazing Effects of the Bulk-Density in a Natraquoll of the Flooding Pampa of Argentina. *Journal of Range Management*, 41: 500-503.

Tanji, K. K., Gao, S., Scardaci, S. C., Chow, A. T. 2003. Characterizing Redox Status of Paddy Soils with Incorporated Rice Straw. *Geoderma*, 114: 333-353.

Tomlin, A.D., Miller, J.J. 1988. Impact of Ring-Billed Gull (*Larus delawarensis* Ord.) Foraging on Earthworm Populations of Southwestern Ontario Agricultural Soils. *Agriculture, Ecosystems & Environment* 20(3): 165 – 173.

Tsadilas, C. D., Matsi, T., Barbayiannis, N., Dimoyiannis, D. 1995. Influence of Sewage-Sludge Application on Soil Properties and on the Distribution and Availability of Heavy-Metal Fractions. *Communications in Soil Science and Plant Analysis*, 26: 2603-2619.

Tsilingridis, G., Papakostas, K., 2014. Investigating the Relationship between Air and Ground Temperature Variations in Shallow Depths in Northern Greece. *Energy*, 73: 1007-1016.

Unger, I. M., Motavalli, P. P., Muzika, R. M. 2009. Changes in Soil Chemical Properties with Flooding: a Field Laboratory Approach. *Agriculture Ecosystems & Environment*, 131: 105-110.

Van Ranst, E., De Coninck, F. 2002. Evaluation of Ferrolysis in Soil Formation. *European Journal of Soil Science*, 53: 513-519.

Venterink, H.O., Vermaat, J.E., Pronk, M., Wiegman, F., van der Lee, G.E.M., van den Hoorn, M.W., Higler, L.W.G., Verhoeven, J.T.A. 2009. Importance of Sediment Deposition and

Denitrification for Nutrient Retention in Floodplain Wetlands. *Applied Vegetation Science* 9(2): 163 – 174

Wallace, B. M., Krzic, M., Forge, T. A., Broersma, K., Newman, R. F. 2009. Biosolids Increase Soil Aggregation and Protection of Soil Carbon Five Years after Application on a Crested Wheatgrass Pasture. *Journal of Environmental Quality*, 38: 291-298.

Water UK (2010). Recycling Of Biosolids to Agricultural Land. [Briefing Pack]

Wyss, E., Glasstetter, M. 1992. Tillage Treatments and Earthworm Distribution in a Swiss Experimental Corn Field. *Soil Biology and Biochemistry*. 24(12): 1635 – 1639

Zorn, M. I., Van Gestel, C. A. M., Eijsackers, H. 2005. Species-Specific Earthworm Population Responses in Relation to Flooding Dynamics in a Dutch Floodplain Soil. *Pedobiologia*, 49: 189-198.

Zorn, M. I., Van Gestel, C. A. M., Morrien, E., Wagenaar, M., Eijsackers, H. 2008. Flooding Responses of Three Earthworm Species, *Allolobophora chlorotica*, *Aporrectodea caliginosa* and *Lumbricus rubellus*, in a Laboratory-Controlled Environment. *Soil Biology & Biochemistry*, 40: 587-593.

3. Seasonal differences in earthworm populations in pasture and arable fields with different localised flooding regimes

3.1. Abstract

Earthworms are a key component of soil's natural capital that give rise to a range of ecosystem services. As relatively slow moving, soil dwelling organisms they are vulnerable to flooding. As our climate changes, extreme weather events such as heavy rainfall that can give rise to flooding are becoming more common. A pasture and arable field, each known to regularly flood, were selected and divided into different flooding regimes based on communications with land users and the topography of the fields. The sites were visited every three months over a period of eighteen months for the pasture field and twelve months for the arable field. At each visit earthworms were sampled by hand-sorting six pits per field zone and identified live in the laboratory using a field guide. Total earthworm abundance, biomass, and Shannon diversity of the populations were determined, and characteristics of the earthworm population such as the percentage of different earthworm ecotypes and the difference in mass of individuals of particular species across the sites determined. Environmental factors of soil temperature, bulk density, moisture content, pH, and carbon and nitrogen percentages were also determined. Datasets were compared within the pasture and arable site, and across the two sites.

Earthworm abundance, biomass, and diversity were significantly lower in the arable field than in the pasture field. Across both field sites, increased intensity of flooding led to decreases in earthworm populations, but the response was greater in the arable field crop sites than in the arable field margin or pasture sites. The earthworm population within the regularly-flooded sites and in the crop sites was almost entirely endogeic earthworm species, while the other regions of the field had a higher proportion of anecic and epigeic earthworms. Environmental factors differed significantly across both the regions of the field and the time of year. The regularly-flooded sites showed higher soil moisture, lower bulk density, and higher carbon and nitrogen percentages than the rarely flooded regions. Soil pH decreased with increased flooding in the pasture site, but increased with flooding in the arable site.

The study suggests that some earthworm species, particularly endogeic earthworm species, are better able to survive both flooding and the soil conditions caused by regular flooding than others, but that not all earthworm species that were able to survive the flooding events flourished, with

some individuals of *A. caliginosa* showing a reduced body mass in regularly flooded areas. Increased intensity and occurrence of flooding in the UK may lead to shifts in the earthworm populations of pasture soils towards lower biodiversity, which may have knock-on impacts on soil function and ecosystem food webs.

3.2. The scope of the chapter

This chapter aims to summarise the different earthworm populations observed between seasons in both a pasture field, located near the river Loddon in Reading, England, and an arable field, located near the river Swale in Holme-On-Swale, England. Each of these fields had sites within them that were subject to differing levels of flooding. In the pasture field, there were three regions: regularly flooded, fast-draining; regularly flooded, slow-draining; and rarely flooded. The arable field was more complex, and could be divided into: rarely flooded crop and margin; occasionally flooded crop and margin; regularly flooded crop and margin; and field and river sides of the river bank.

As the methods listed generated large amounts of data, this chapter has been split into multiple component parts.

Chapter 3: This section consists of the abstract, scope of the chapter, introduction, methods, and hypotheses that apply to both the pasture and the arable site

Chapter 3A: This section consists of the results, discussion, and conclusion for the pasture field.

Chapter 3B: This section consists of the results, discussion, and conclusion for the arable field.

Chapter 3C: This section discusses the similarities and differences observed between the pasture and the arable field, and considers the key factors driving the earthworm population in both.

3.3. Introduction

The UK climate is changing. Flooding events associated with increased rainfall have been increasing in both occurrence and intensity (Prudhomme et al., 2003) and the mean annual floodwater discharge in the UK increasing by approximately 12% between 1960 and 2010. While these events can cause catastrophic damage to urban regions they are also affecting arable and pasture regions of the UK, leading not only to losses of crops and livestock, but also to long term damage of agricultural land. For example, flooding in the Somerset levels in late 2013 and early

2014 affected over 13,000 ha of agricultural land, reducing winter cereal crop yields by 20%, leaving 50% of winter crops unviable, and reducing grassland availability for grazing by an estimated 30% (ADAS, 2014). With the threat of flooding increasing on agricultural land, one question that arises is what will be the impact of these flooding events on soil fauna – particularly earthworms?

Earthworms are an important organism in the soil ecosystem. They are a key food source for many animal species: nocturnal foragers such as badgers (Skinner and Skinner, 1988) and foxes (Macdonald, 1980), birds (Ausden et al., 2001; Wilson et al., 1999), and soil dwelling fauna such as moles (Funmilayo, 1979) consume earthworms as a greater or lesser part of their diet. Earthworms have also been referred to as 'ecosystem engineers' (Jones et al., 1994): organisms which, through their behaviours, cause changes to abiotic or biotic materials, altering the availability of resources to other organisms (Lawton, 1994). Earthworms fulfil this role in the soil environment by their behaviours; for example, their tunnelling increases soil porosity and air spaces (Stork and Eggleton, 1992), consumption of the soil and organic matter contribute to the nutrient turnover of the soil, and casting of digested material increases the aggregate stability of the soil (Zhang and Schrader, 1992). Given that earthworms play such an important role in the soil, it is important to consider whether changes in flooding regimes with changing climatic conditions will impact earthworm populations, and the further implications this may have on crop yields or grassland production.

It has long been an accepted observation that earthworms emerge from the soil after heavy rainfall. However, the precise reason for earthworm emergence remains debated. The early theory that the earthworms were already in poor condition and emerged in order to die (Darwin, 1881) seems unlikely, but other theories have been suggested. Perhaps the most commonly accepted theory is that the earthworms are 'escaping drowning'. As all earthworm species use the skin as the organ of oxygen transfer, a more accurate description here may be that the earthworms are escaping suffocation. When soil floods, the available oxygen in the soil is rapidly depleted, falling to anoxic levels within a few hours (Ponnamperuma, 1984). With no available oxygen, the earthworms may flee flooded soil in order to avoid suffocation. As a short-term survival strategy, fleeing an anoxic environment may be very successful for earthworms, provided they can move rapidly enough to entirely escape a flooded region. However, over multiple repeated flooding events, this tactic may result in reduced earthworm populations, with earthworms on the soil surface vulnerable to predation (Tomlin and Miller, 1988) and potentially unable to feed. Within the soil itself, regular inundation may cause physical and chemical changes within the soil that create an environment that is either unsuitable for earthworms, such as the reduced oxygen conditions, or which favours one particular ecotype or behavioural subtype over another, such as changes in soil pH, as discussed in Section 2.6. Population fluctuations in regularly flooded regions, therefore, may depend on a number of factors: how likely earthworms are to survive on the surface and repopulate the flooded regions; how viable earthworm cocoons and juveniles remain during and after a flooding event, and whether earthworm species belonging to different ecotypes respond in the same way.

Within arable soils, the role of earthworms becomes even more important; the changes that earthworms cause in soil through their behaviour as ecosystem engineers are vital for crop growth (Bertrand et al., 2015). They alter the physical environment of the soil, increasing soil water infiltration rates (Ernst et al., 2009) and increasing soil aggregate stability (Maeder et al., 2002). Earthworms also alter the nutritional content of soil. They free nutrients essential for crop growth such as nitrogen from soil, either through excretion of consumed soil matter (Barley and Jennings, 1959) or through release from tissue on earthworm death (Syers and Springett, 1984), while their casts contain high quantities of macro- and micronutrients necessary for plant growth and metabolism (Tomati and Galli, 1995). Through their burrowing, casting, and excretion of nutrients, earthworms create a soil environment more conducive for plant growth (Scheu et al., 1999; Tomati et al., 1988), in turn increasing crop yield by 25% when soil nitrogen is otherwise limited (van Groenigen et al., 2014). However, in arable soils earthworm populations are also greatly reduced in comparison to pasture soils (Curry et al., 2002; Boag et al., 1997) due to a number of factors including crush or cutting damage from agricultural machinery (Boström, 1995; Tomlin and Miller, 1988), the use of pesticides (Pelosi et al., 2013; Ball et al., 1986) and the generally low organic matter contents found in arable soils being unsuitable for large earthworm populations (Reeleder et al., 2006).

This chapter aims to address the differences in earthworm populations in sites of a pasture field and an arable field that are subject to different flooding regimes. The chapter will examine the differences first independently, in Chapters 3A and 3B, and then broadly across the whole dataset, in Chapter 3C. The chapter will address these aims through measures of earthworm abundance, biomass, and diversity, alongside measures of environmental variables that may also change with different flooding regimes.

Five broad hypotheses were considered for this chapter:

1. Soil environmental factors will vary with both the time and the flooding regime. Factors such as soil temperature and soil moisture content will vary seasonably, while factors such as soil carbon and nitrogen content will likely be affected by the flooding regime. As discussed in Section 2.5.2, regular flooding may cause soil pH to be less acidic, may lead to reductions in soil bulk density, and may lead to the accumulation of organic matter in soils.

2. Populations of earthworms found in different regions of the field would be greater in the autumn than in the summer, and would be lower in the regularly-flooded regions than in the rarely flooded regions of the field (Section 2.7; Plum 2005).

3. Earthworm populations in regularly-flooded regions are dominated by endogeic earthworm species, as these are generally the most resistant to flooded conditions (Section 2.6; Roots, 1956; Zorn et al, 2008).

4. The abundance and biomass of individual adult earthworms of different species differs according to the flooding regime, with the abundance of species of adult anecic and epigeic earthworms and the biomass of epigeic adults lower in the regularly-flooded areas than in the rarely flooded (Section 2.6; Klok et al, 2006) and differs over the sampling timepoints, with fewer endogeic earthworms in the warmer months due to aestivation.

5. Earthworm population variables are correlated with soil environmental factors. Earthworm populations may be positively associated with factors such as soil carbon and nitrogen percentage, and negatively correlated with soil moisture.

3.4. Methods

3.4.1. Field sites

3.4.1.1. Pasture

The pasture field is located at grid reference SU 75153 68746 near Reading, England. The field runs adjacent to the river Loddon, which regularly escapes its banks during the winter period (T Sizmur, Department of Geography and Environmental Science, University of Reading, 2016, personal communication, 4th October). The site also regularly experiences groundwater flooding, with both fluvial and groundwater flooding events often occurring concurrently and sometimes difficult to separate (T Sizmur, 2019, personal communication, 30th August). Three regions of the field were chosen that were known to undergo different flooding regimes due to the topography of the field (Fig. 3-1). Site 1 regularly floods, but drains rapidly. Site 2 regularly floods, but does not drain rapidly, and is often under standing water for some time following flooding events. Site 3 very rarely floods.

Figure 3-1. A. Google earth image (screen captured 08/01/2020) of the three sampling sites within the pasture field bordering the river Loddon. Site 1 regularly floods but drains rapidly. Site 2 also regularly floods but drains slowly, and is often under standing water. Site 3 very rarely floods. B. Ordnance survey map of the same field at the 1:25 00 scale, with the approximate locations of the sampling sites marked with red pins.

The pasture site was visited every three months over a period of eighteen months, from November 2016 to February 2018. On each visit, six samples were randomly taken in each flooding regime region, giving a total of eighteen samples per visit.

3.4.1.2. Arable

The arable field is located at grid reference SE 36200 81600 near Holme-On-Swale in Yorkshire, England. The field runs adjacent to the river Swale, and is regularly inundated during the winter (M Marley 2017, Holme House Farm, Holme-On-Swale, personal communication). Eight regions of the field were chosen that are subject, not only to different flooding regimes due to the topography of the field, but also to different treatments of crops or field margin (Fig. 3-2).

Sites 1, 3, and 5 are regions of the cropped soil that never flood, occasionally flood, and regularly flooded respectively. Sites 2, 4, and 6 are subject to the same flooding regimes, but within the field margin. Sites 7 and 8 are the field and river side of the riverbank that is separated from the field by a fence, and not farmed or accessed by machinery. The bank lies between the field and the river Swale. A phone interview with a representative of the Environment Agency suggested that the bank has likely been there for a period greater than 100 years, and was a dumping location for silt from river dredging to keep the river channel clear (O Saunders, Environment Agency, personal communication, 01/08/2018).

Figure 3-2. A. Google earth image (screen captured 08/01/2020) of each of the 8 sites in the arable field bordering the river Swale Sites 1 and 2 rarely flood. Sites 3 and 4 occasionally flood. Sites 5 and 6 regularly flood. Sites 7 and 8 are the field and river sides of the bank, respectively. B. Ordnance survey map of the same field at the 1:25 00 scale, with the approximate locations of the sampling sites marked with red pins.

The arable field was visited approximately every three months, from April 2017 to January 2018. Due to poor weather conditions, some of the sampling during the January 2018 period was delayed. The decision to only sample for the year once sampling had been completed in each season, a spring, summer, autumn and winter sampling, was due to the generally low earthworm abundances across all sites. On each visit, six samples were randomly taken in each flooding regime region, giving a total of forty-eight samples per visit.

3.4.2. Earthworm sampling

Samples were taken by digging a pit measuring approximately 20 cm x 20 cm x 20 cm. The soil was extracted using a sharp levering motion with a spade in order to prevent earthworm escape, and the soil mass was rapidly removed from the soil into a high sided tray. The extracted soil was hand sorted for live earthworms, while any earthworms living deeper within the soil were extracted using one litre of 0.13 ml L^{-1} concentration allyl isothiocyanate in deionised water (Zaborski, 2003; Pelosi et al., 2009), which was poured into the pit and left for 30 minutes to drain into the soil. Emerging earthworms were rinsed with deionised water, and stored separately from earthworms collected from the pit. Earthworms were collected live and transported back to the laboratory in damp soil. The soil temperature at 5 cm and 10 cm depths for each pit was recorded by inserting a soil temperature probe into the intact soil adjacent to the pit. A soil sample was collected by hammering a bulk density ring of volume 63.62 cm^3 (height 4 cm, diameter 5.5 cm) into the side of the freshly dug pit, approximately 10 cm below the soil surface, taking the measurement between approximately 8 cm and 12 cm soil depth, before the addition of the allyl isothiocyanate solution. The sample was brought back to the lab for analysis of soil moisture content, bulk density, soil pH, and soil carbon and nitrogen content.

In the laboratory, live earthworms were identified using the OPAL “Key to Common British Earthworms” (Jones and Lowe, 2016) and weighed. As only adult earthworms can be identified using that key, juvenile earthworms were weighed and categorised by their colour and size. Juvenile earthworm categories were: greater than 1 gram, as any juveniles greater than a gram in weight were likely to belong to one of two species of earthworm; *Lumbricus terrestris* and *Aporrectodea longa*, as both *L. terrestris* and *A. longa* achieve mass > 1 g within 12 weeks of hatching (Lowe and Butt, 2002); unpigmented and less than 1g; pigmented and less than 1 g; and green and less than 1 g. The majority of juveniles were beneath 1 g in weight. . Green juvenile earthworms were likely to only belong to endogeic earthworms, as they were likely juveniles of *Allolobophora chlorotica*. Earthworms below 1 g in biomass and unpigmented in colour were considered likely to be endogeics. Earthworms below 1 g in biomass and pigmented, such as a dark brown or red colour, were considered likely to be epigeics. Earthworm fragments or dead earthworms were recorded as such, along with their weight and any colour that could be determined.

3.4.3. Soil analysis

Soil samples were dried at 105°C for 24 hours. The weights of the soils were recorded before and after drying. The difference in the weights was used to calculate the gravimetric moisture content of the soil samples, and the weight of the dried soil sample was used in conjunction with the known volume of the sample ring used in the field in order to calculate the bulk density in grams per centimetre cubed (g cm^{-3}) of oven-dried soil. There were no large stones found in the bulk density sampling at either site. The dried soil samples were stored for future analysis.

Soil pH was determined by adding 40 ml of deionised water to 10 g of the dried soil sample in 50ml Fisher centrifuge tubes. These tubes were shaken for two hours and left to stand for one hour in order to allow any particulate matter to settle. Soil pH readings were taken using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers.

Soil carbon and nitrogen content was determined by grinding a subsample of the dried soil in a ball mill. Approximately 100 mg (c. 95 to 105 mg) of the dried, finely ground soil sample was then analysed in a Vario Macro C/N analyser, which gave the percentages of carbon and nitrogen in the sample and the C:N ratio. The C/N analyser was calibrated using samples of glutamic acid in the same weight range as the dried soil. A certified organic analytical standard of Peaty soil from Elemental Microanalysis Ltd (B2176 – batch 133519), gave recoveries of 97% (± 2.21) and 100% (± 2.94) percent of 15.95% C and 1.29% N respectively.

Multiplying the soil carbon percentage by 1.72 gives an estimate of the soil organic matter content, based on the principal that 58% of the soil organic matter is composed of carbon. However, as this conversion factor is not suited to all soil types (Pribyl, 2010), the obtained soil carbon values were not converted into soil organic matter and instead the soil percent carbon and nitrogen values obtained in this analysis were used.

3.4.4. Data analysis

Data were analysed using R version 3.4.2.The environmental factors used in further analysis were: the mean of the soil temperature at 5 cm and 10 cm (°C), soil pH, soil moisture content (%), soil bulk density (g cm^{-3}), soil carbon percentage, and soil nitrogen percentage. The separate datasets of soil temperature at 5 cm and 10 cm were combined into one mean value.

The earthworm population data were analysed in three different ways. The total abundance of earthworms which had been extracted from the pit through both hand sorting and chemical extraction was calculated for each pit. Partial earthworms were not included in this calculation. The abundance was then divided by 0.04 to convert the values from the 20 cm x 20cm x 20 cm area to an area of 1 m x 1 m to a depth of 20 cm. Secondly, the total biomass of earthworms(g m^{-2}) was calculated by summing the biomass of each individual and partial earthworm body fragments and treating the values in the same manner as the total earthworm abundance; dividing by 0.04. Thirdly, the Shannon diversity index (Equation 3-1) was calculated for each pit. When calculating the diversity index, juveniles were classified as separate species based on the categories listed above. Partial or dead individuals, while included in the total biomass data, were not included in the diversity data. The Shannon index was chosen as the diversity index for this study as it is widely accepted to be more robust with smaller populations potentially dominated by only a few species than indices such as the Simpson Diversity Index (Morris et al., 2014), an advantage when some pits contained very low numbers (i.e. one individual).

$$H = \sum (Pi) \times \ln(Pi)$$

Equation 3-1. The formula for determining the Shannon Diversity Index value for each species, where *H* = Shannon Diversity Index, and *Pi* = the proportion of the total sample represented by species *i*.

Datasets were tested for normality and heteroscedascity, and non-normal datasets were transformed. For the both the pasture and arable sites, no transformations were performed on the on the environmental datasets. Within the pasture dataset, the total biomass of earthworms was square root transformed, while the Shannon diversity index values were raised to the power of two. Within the arable dataset, the total biomass of earthworms was square root transformed, while the Shannon diversity index values were log10 +1 transformed. For the other data analyses performed, statistical tests were used which did not require normality, or were non-parametric equivalents to parametric tests.

Repeated measures ANOVAs were not used in the statistical testing of the hypotheses as the same site was being measured, but not the same pit. As the pits were at least 1 m apart, they were considered sufficiently independent to not require a repeated measures ANOVA.

3.5. Data analyses and statistics

The data were then analysed using various statistical methods to address the five broad hypotheses listed in Section 3.5. The methods for each hypothesis are listed below.

1.. The variability of environmental factors over sampling times and flooding regimes was tested by comparing each environmental variable to both the time period and the flooding regime using a two way ANOVA.

2. The variability of earthworm populations over sampling times and flooding regimes was tested by comparing each earthworm population variable to both the time period and the site of the field using a generalised linear model (GLM) with a Poisson link for earthworm abundance and a two way ANOVA for earthworm biomass and diversity. As the abundance data was count data, a two way ANOVA was not suitable for use with that data type, so a generalised linear model was used.

3. The differences in earthworm population structure at the ecotype level was tested by first calculating the overall proportions of adults and juveniles of each earthworm ecotype within each pit, at each timepoint and site, and assessing the differences between the proportions of the ecotypes using a GLM with a Quasipoisson link.

4.. The difference in abundances and biomass of adult earthworms of different species between flooding regimes was tested using two way ANOVAs for earthworm biomass, and GLMs with a Poisson link for earthworm abundance.

5. The association between earthworm population variables and soil environmental factors was tested using Spearman's Rank Correlations of each population variable against each environmental factor. Spearman's Rank was used as the statistical test due to the non-normal nature of the data. Stepwise model selection was then used to determine which soil environmental factors were the most influential on the measures of earthworm population.

3.6. References

ADAS, 2014. Impact of 2014 Winter Floods on Agriculture in England [Leaflet]

Ausden, M., Sutherland, W.J., James, R. 2001. The Effects of Flooding Lowland Wet Grassland on Soil Macroinvertebrate Prey of Breeding Wading Birds. *Journal of Applied Ecology* 38: 320-338

Ball, D., Tapp, L., Gordon, F.C., Potter, D.A. 1986. Effects of Pesticides on Earthworm Populations in Kentucky Bluegrass, 1985. *Insecticide and Acaricide Tests* 11(1): 395 – 396.

Barley, K.P., Jennings, A.C. 1959. Earthworms and Soil Fertility. III. The Influence of Earthworms on the Availability of Nitrogen. *Australian Journal of Agricultural Research* 10(3); 364 – 370.

Bertrand, M., Barot, S., Blouin, M., Whalen, J., de Oliveira, T., Roger-Estrade, J. 2015. Earthworm Services for Cropping Systems. A Review. *Agronomy for Sustainable Development* 35(2): 553 – 567

Blöschl, G, Hall, J., Viglione, A., Perdigão, R.A., Parajka, J., Merz, B., Lun, D., Arheimer, B., Arnoica, G.T., Bilibashi, A., Boháč, M. 2019. Changing Climate both Increases and Decreases European River Floods. *Nature* 573: 108 - 111

Boag, B., Palmer, L.F., Neilson, R., Legg, R., Chambers, S.J. 1997. Distribution, Prevalence and Intensity of Earthworm Populations in Arable Land and Grassland in Scotland. *Annals of Applied Biology* 130(1): 153 – 165.

Boström, U. 1995. Earthworm Populations (Lumbricidae) in Ploughed and Undisturbed Leys. *Soil and Tillage Research* 35(3): 125 – 133

Bouché, M. B., 1977. Strategies Lombriciennes. *Ecological Bulletins* 25: 122-132.

Curry, J.P., Byrne, D., Schmidt, O. 2002. Intensive Cultivation can Drastically Reduce Earthworm Populations in Arable Land. *European Journal of Soil Biology* 38(2): 127 – 130.

Darwin, C. 1881. The Formation of Vegetable Mould, Through the Action of Worms: With Observations on their Habits Illustrated Edition. Milton Keynes: Dodo Press

Ernst, G., Felten, D., Vohland M., Emmerling, C. 2009. Impact of Ecologically Different Earthworm Species on Soil Water Characteristics. *European Journal of Soil Biology* 45(3): 207 – 213.

Funmilayo, O. 1979. Food Consumption, Preferences and Storage in the Mole. *Acta theriologica* 24: 379-389

Jones, C.G., Lawton, J.H., Shachak, M. 1994. Organisms as Ecosystem Engineers. *Oikos* 69(3): 373-386

Jones, D.T., Lowe, C.N. 2016. Key to Common British Earthworms. The OPAL Soil and Earthworm Survey. Imperial College London.

Klok, C., Zorn, M., Koolhass, J.E., Eijsackers, H.J.P., van Gestel, C.A.M. 2006. Does Reproductive Plasticity in *Lumbricus rubellus* Improve the Recovery of Populations in Frequently Inundated River Floodplains? *Soil Biology and Biochemistry* 38(3): 611 – 618

Lawton, J.H., 1994. What Do Species Do in Ecosystems? *Oikos* 71(3): 367-374

Lowe, C.N., Butt, K.R. 2002. Growth of Hatching Earthworm in the Presence of Adults: Interactions in Laboratory Culture. *Biology and Fertility of Soils* 35(3): 204 – 209

Macdonald, D.W., 1980. The Red Fox, *Vulpes vulpes*, as a Predator upon Earthworms*, Lumbricus terrestris*. *Zeitshrift für Tierpsychologie* 52(2): 171-200

Maeder, P., Fliessbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U. 2002. Soil Fertility and Biodiversity in Organic Farming. *Science* 296(5573): 1694 – 1697.

Morris, E.K., Caruso, T., Buscot, F., Fischer, M., Hancock, C., Maier, T.S., Meiners, T., Müller, C., Obermaier, E., Prati, D., Schoer, S.A., Sonnemann, I., Wäschke, T., Wurst, S., Rillig, M.C. 2014. Choosing and Using Diversity Indices: Insights for Ecological Applications from the German Biodiversity Exploratories. *Ecology and Evolution* 4(18): 3514 – 3524

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulcke, F. Pesticides and Earthworms. A Review. 2013. *Agronomy for Sustainable Development* 34(1): 199 – 229.

Pelosi, C., Bertrand, M., Capowiez, Y., Boizard, H., Roger-Estrade, J. 2009. Earthworm Collection from Agricultural Fields: Comparisons of Selected Expellants in Presence/Absence of Hand-Sorting. *European Journal of Soil Biology* 45(2): 176 – 183

Plum, N.M. 2005. Terrestrial Invertebrates in Flooded Grassland: A Literature Review. *Wetlands* 25(3): 721-737

Ponnamperuma, F. N. 1984. Effects of Flooding on Soils. In: Kozlowski, T.T, ed. Flooding and Plant Growth. Orlando: Academic Press, 9 - 45

Pribyl, D.W. 2010. A Critical Review of the Conventional SOC to SOM Conversion Factor. *Geoderma* 156(3-4): 75 – 83

Prudhomme, C., Jakob, D., Svensson, C. 2003. Uncertainty and Climate Change Impact on the Flood Regime of Small UK Catchments. *Journal of Hydrology* 277: 1-23

Reeleder, R.D., Miller, J.J., Ball-Coelho, B.R., Roy, R.C. 2006. Impacts of Tillage, Cover Crop, and Nitrogen on Populations of Earthworms, Microarthropods, and Soil Fungi in a Cultivated Fragile Soil. *Applied Soil Ecology* 33(3): 243 – 257.

Roots, B. I. 1956. The Water Relations of Earthworms II. Resistance to Desiccation and Immersion, and Behaviour when Submerged and when Allowed a Choice of Environments. *Journal of Experimental Biology* 33: 29-44.

Scheu, S., Theenhaus, A, Jones, H. 1999. Links between the Detritivore and the Herbivore System: Effects of Earthworms and Collembola on Plant Growth and Aphid Development. *Oecologia* 119(4): 541 – 551.

Skinner, C.A., Skinner, P.J. 1988. Food of Badgers (*Meles meles*) in an Arable Area of Essex. *Journal of Zoology: London* 215: 360-362

Stork, N.E., Eggleton, P. 1992. Invertebrates as Determinants and Indicators of Soil Quality. *American Journal of Alternative Agriculture* 7: 38 – 47

Syers, J.K., Springett, J.A. 1984. Earthworms and Soil Fertility. *Plant and Soil* 76(1-3): 93 – 104.

Tomati, U., Galli, E. 1995. Earthworms, Soil Fertility, and Plant Productivity. *Acta Zoologica Fennica*. 196: 11 – 14.

Tomati, U., Grappelli, A., Galli, E. 1988. The Hormone-Like Effect of Earthworm Casts on Plant Growth. *Biology and Fertility of Soils* 5(4): 288 – 294.

Tomlin, A.D., Miller, J.J. 1988. Impact of Ring-Billed Gull (*Larus delawarensis* Ord.) Foraging on Earthworm Populations of Southwestern Ontario Agricultural Soils. *Agriculture, Ecosystems & Environment* 20(3): 165 – 173.

van Groenigen, J.W., Lubbers, I.M, Vos, H.M.J., Brown, G.G., De Deyn, G. B., van Groenigen, K.J. 2014. Earthworms Increase Plant Production: a Meta-Analysis. *Scientific Reports* 4: 6365

Wilson, J.D., Morris, A.J., Arroyo, B.E., Clark, S.C., Bradbury, R.B. 1999. A Review of the Abundance and Diversity of Invertebrate and Plant Foods of Granivorous Birds in Northern Europe in Relation to Agricultural Change. *Agriculture, Ecosystems & Environment* 75: 13 – 30

Zaborski, E.R. 2003. Allyl Isothiocyanate: An Alternative Chemical Expellant for Sampling Earthworms. *Applied Soil Ecology* 22(1): 87 – 95

Zhang, H., Schrader, S. 1992. Earthworm Effects of Selected Physical and Chemical Properties of Soil Aggregates. *Biology and Fertility of Soils* 15: 229 – 234.

Zorn, M.I., Van Gestel, C.A.M., Morrien, E., Wagenaar, M., Eijsackers, H. 2008. Flooding Responses of Three Earthworm Species, *Allolobophora chlorotica, Aporrectodea caliginosa*, and *Lumbricus rubellus*, in a Laboratory-Controlled Environment. *Soil Biology and Biochemistry* 40(3): 587 – 59

3A. Seasonal differences in earthworm populations in a pasture field with different localised flooding regimes

3A.1 Results

3A.1.1 Environmental factors across site and time

Spearman's Rank correlations were performed to determine how soil environmental factors across all sampling timepoints and sites related to each other (Table 3A-1). Significant positive correlations were observed between soil moisture and: bulk density, percent carbon and percent nitrogen, and between percent carbon and percent nitrogen. Negative correlations were observed between soil temperature and soil moisture, and bulk density and both percent carbon and percent nitrogen.

Table 3A-1. Spearman's Rank rho (ρ) values of correlations between soil environmental factors. Shaded cells indicate a statistically significant correlation (*P* < 0.01; n = 108).

	Bulk Density	**Soil Moisture**	**pH**	**Percent Carbon**	**Percent Nitrogen**
Temperature	ρ = 0.004	ρ = - 0.45	ρ = - 0.11	ρ = - 0.07	ρ = - 0.04
Bulk Density	-	ρ = - 0.64	ρ = 0.11	ρ = - 0.82	ρ = - 0.83
Soil Moisture	-	-	ρ = - 0.11	ρ = 0.78	ρ = 0.78
pH	-	-	-	ρ = - 0.13	ρ = - 0.16
Percent carbon	-	-	-	-	ρ = 0.98

Two way ANOVAs were performed to determine the effects of the time of year and the flooding regime on a number of environmental factors: the mean of the temperature at 5 and 10 cm, bulk density, soil moisture percentage, pH, percent carbon, and percent nitrogen. A significant effect of site, time, and an interaction term was observed for all environmental factors except for bulk density, which had no significant effect of time, and pH, which had no significant interaction term. *P* values are summarised in Table 3A-2.

Table 3A-2 - The outcomes of two way ANOVAs performed to determine the differences between a number of soil environmental factors across different sampling sites and time within the Loddon pasture site (n = 108).

Soil environmental factor	Variable	*P* value (DF, F)
Mean temperature (°C)	Timepoint	< 0.0001 (5, 5262.78)
	Site	< 0.0001 (2, 40.49)
	Interaction term	< 0.0001 (10, 12.394)
Bulk density (g cm^{-3})	Timepoint	0.9401 (5, 0.247)
	Site	< 0.0001 (2, 153.443)
	Interaction term	0.0312 (10, 2.113)
Soil moisture percentage	Timepoint	< 0.0001 (5, 76.539)
	Site	< 0.0001 (2, 401.012)
	Interaction term	< 0.0001 (10, 5.655)
pH	Timepoint	0.0079 (5, 3.359)
	Site	0.0132 (2, 4.544)
	Interaction term	0.1468 (10, 1.515)
Percent carbon	Timepoint	0.0010 (5, 4.519)
	Site	< 0.0001 (2, 333.021)
	Interaction term	0.0141 (10, 2.402)
Percent nitrogen	Timepoint	0.0034 (5, 3.827)
	Site	< 0.0001 (2, 520.772)
	Interaction term	0.0076 (10, 2.624)

Following the two way ANOVAs, Tukey post-hoc tests were performed to determine where the significant differences lay within the environmental factors.

Soil temperature (Figure 3A-1) showed a significant effect of both site and sampling timepoint. Post hoc testing showed that in the November 2016 and May 2017 timepoints there was no significant difference in soil temperature across the three sites, while all other sampling time points showed differences across the three flooding regimes. Soil bulk density (Figure 3A-2) showed no significant variation with time, but did show a significant effect of sampling site. Post hoc testing showed that the soil bulk density in the regularly flooded, slow-draining site was significantly lower than in the rarely flooded site. Soil moisture percentage (Figure 3A-3) showed a significant effect of both sampling site and sampling timepoint. As with soil temperature, there were significant differences between the sites dependent on the time of year, with soil temperatures significantly lower in May 2017. Soil pH (Figure 3A-4) did not show a significant interaction term between the sampling site and the sampling timepoint. The soil pH was significantly higher in the rarely flooded site than in the regularly flooded sites. Soil pH was significantly lower in the November 2016 timepoint than in the August 2017 timepoint. Percent carbon (Figure 3A-5) and percent nitrogen (Figure 3A-6) were significantly lower in the rarely flooded site than in the fast-draining site, which was significantly lower than the slow-draining site.

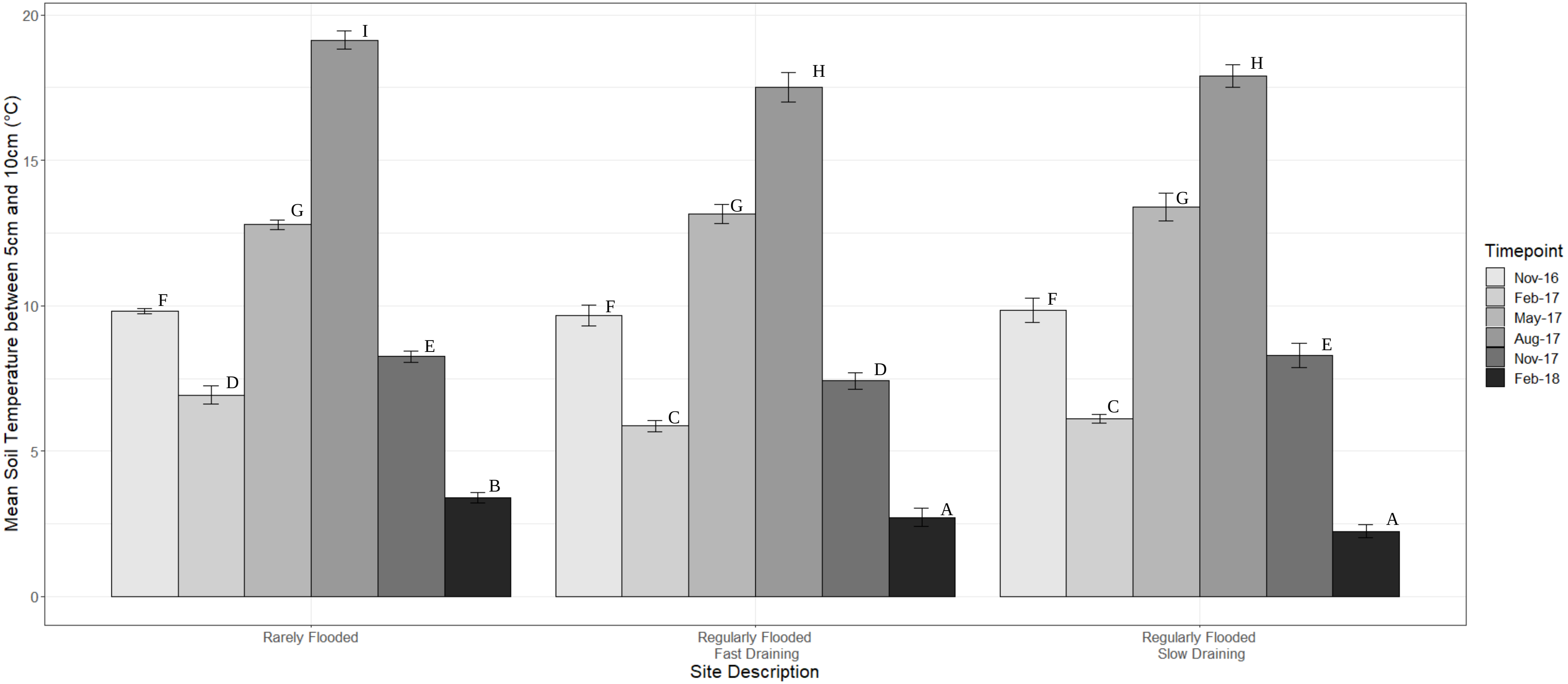

Figure 3A-1. The mean soil temperature between 5 cm and 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). Bars marked with the same letter are not significantly different (*P* < 0.05).

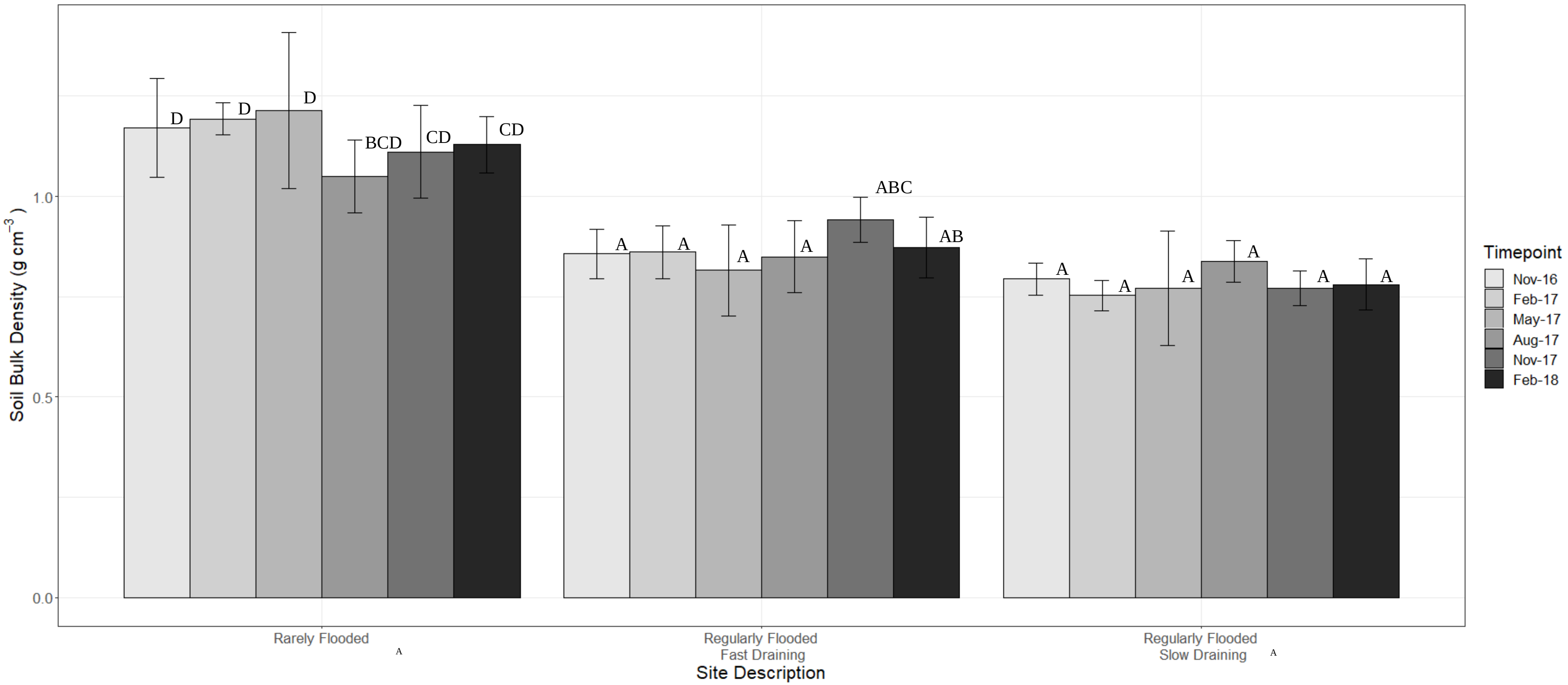

Figure 3A-2. The mean soil bulk density (g cm^{-3}) at a depth of approximately 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). Bars marked with the same letter are not significantly different (P < 0.05).

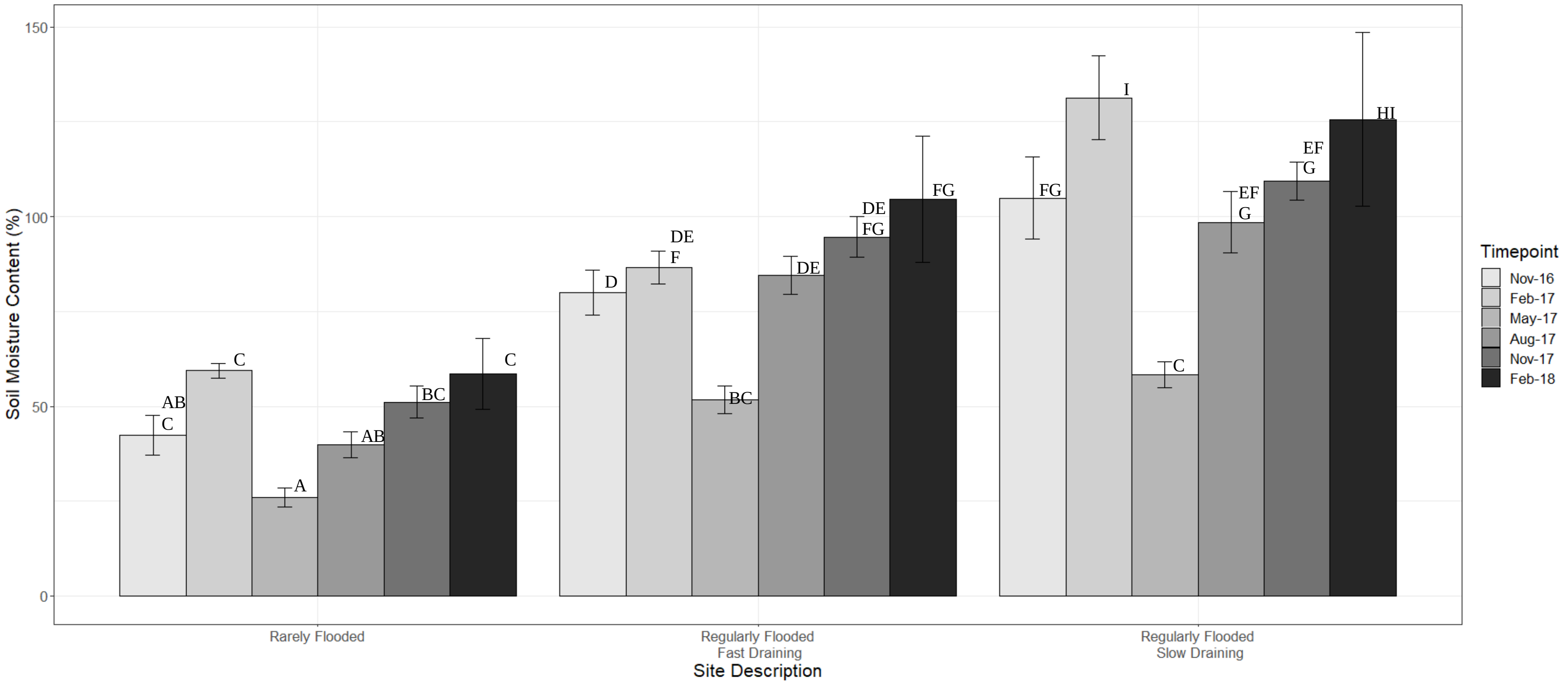

Figure 3A-3. The mean soil moisture content (%) at a depth of approximately 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). Bars marked with the same letter are not significantly different ($P < 0.05$).

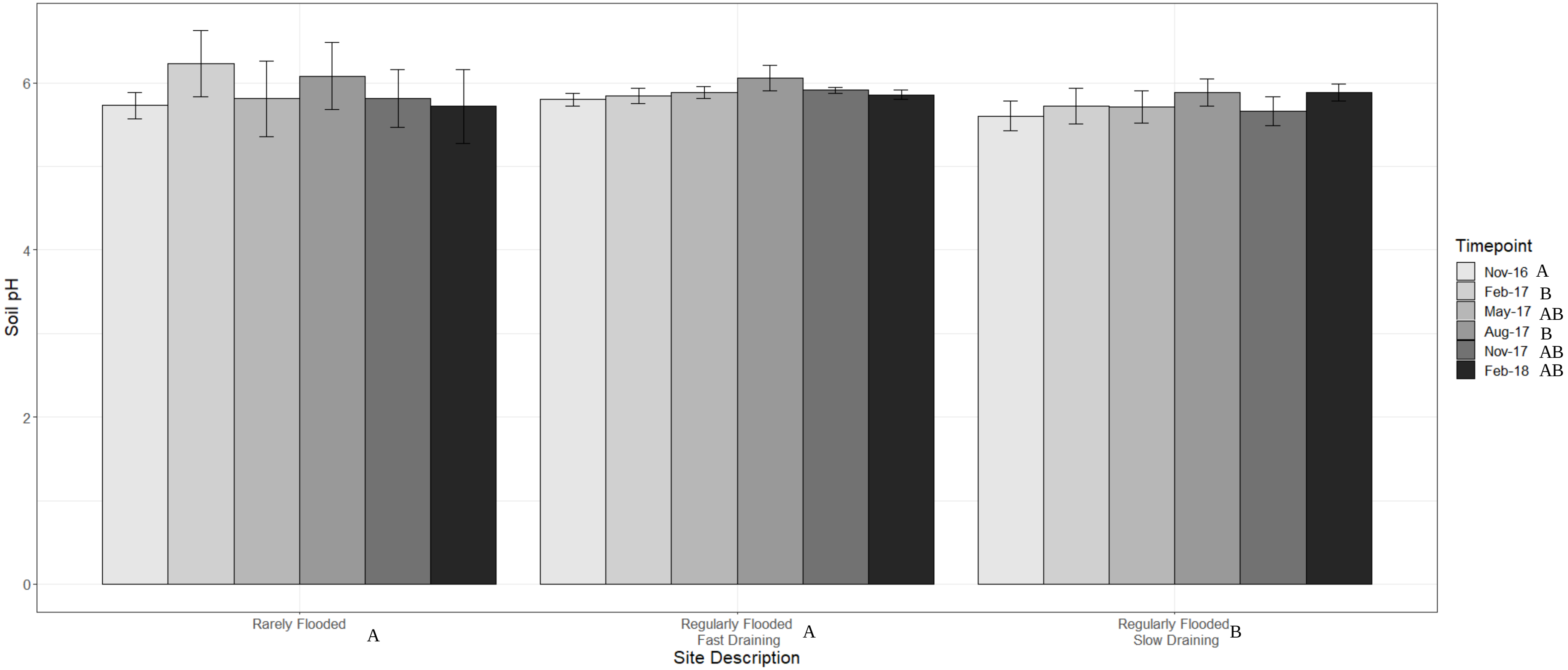

Figure 3A-4. The mean soil pH at a depth of approximately 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). X axis site descriptions marked with the same letter show no significant difference between sites across all timepoints (*P* < 0.05). Legend labels marked with the same letter are not significantly different across all sites (*P* < 0.05).

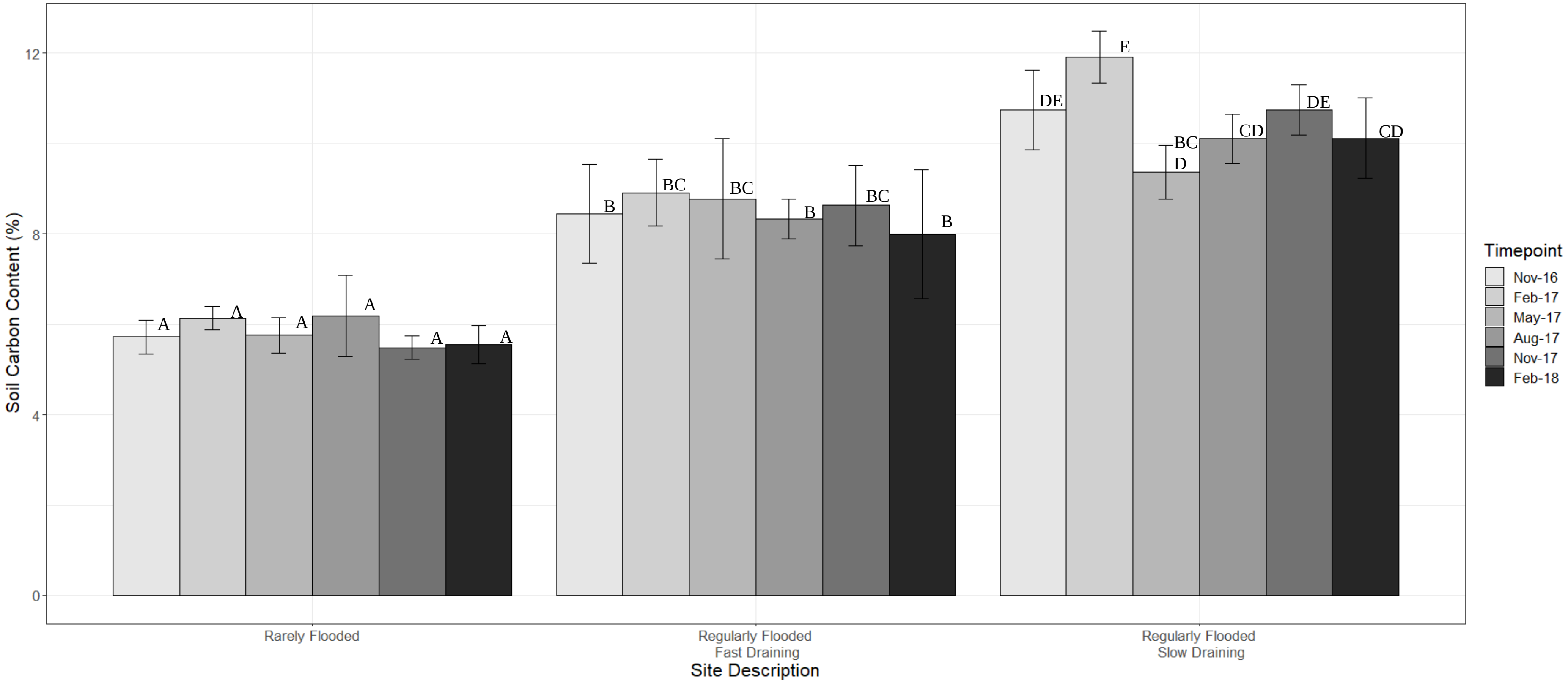

Figure 3A-5. The mean soil carbon content (%) at a depth of approximately 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). Bars marked with the same letter are not significantly different (*P* < 0.05).

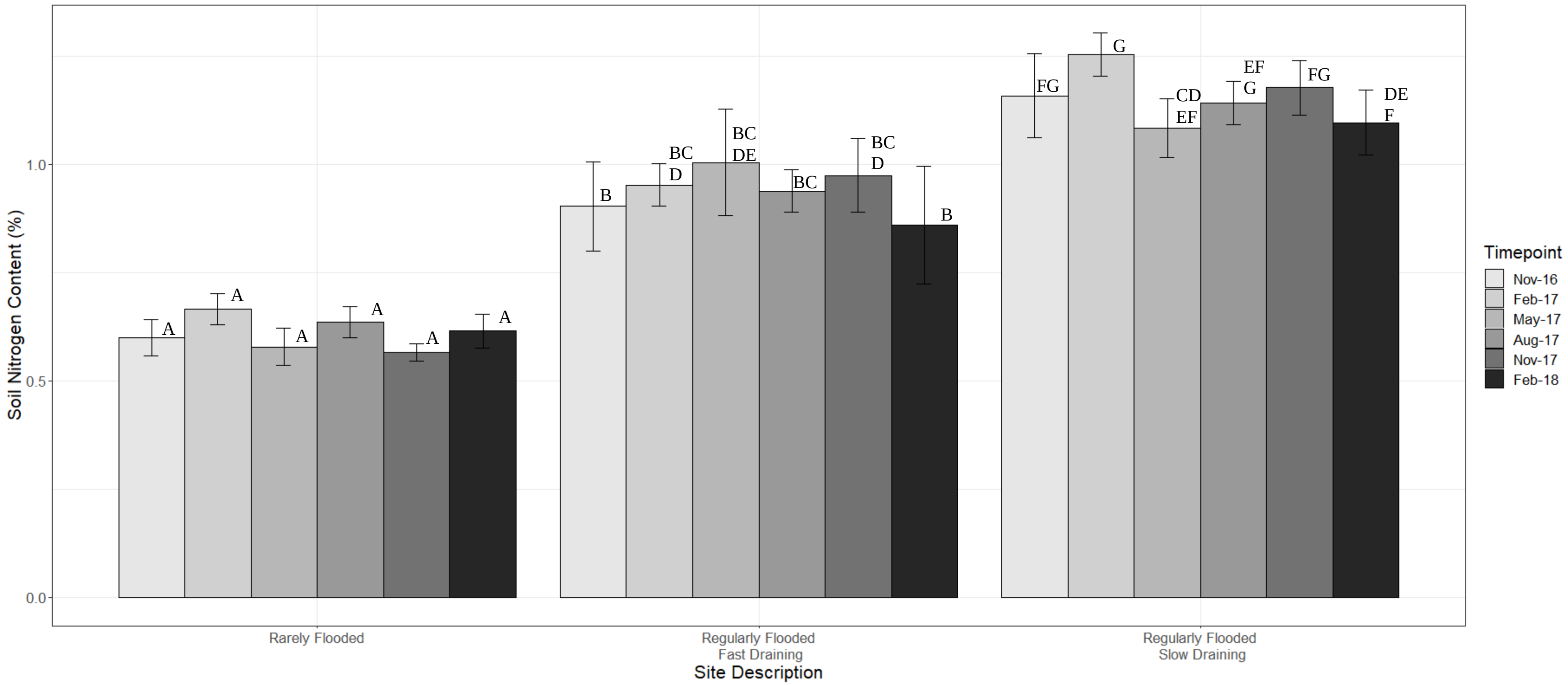

Figure 3A-6. The mean soil nitrogen content (%) at a depth of approximately 10 cm for the different sites over the six sampling timepoints, with error bars showing standard deviation (n = 108). Bars marked with the same letter are not significantly different (*P* < 0.05).

3A.1.2 Population metrics across site and time

A GLM, for earthworm abundance, and two way ANOVAs, for total earthworm biomass and Shannon diversity index values, were performed to determine the effects of the time of year and the flooding regime on the three measures of earthworm population. A significant effect of site, time, and an interaction term was observed for all three population metrics at the level of $P < 0.01$. *P* values are summarised in Table 3A-3.

Table 3A-3. The outcomes of the GLM and two way ANOVAs performed to determine the differences between a number of population metrics across differing flooding regimes and time within the Loddon pasture site (n=108).

Earthworm population metric	**Variable**	***P* value (DF, F/X^2)**
Total abundance (ind m^{-2})	Timepoint	< 0.0001 (5, 47.40)
(untransformed)	Site	< 0.0001 (2, 86.92
	Interaction term	2.76×10^{-10} (10, 65.88)
Total biomass (g m^{-2})	Timepoint	< 0.0001 (5, 12.23)
(square root transformed)	Site	< 0.0001 (2, 26.12)
	Interaction term	0.005 (10, 2.81)
Shannon diversity index	Timepoint	0.0004 (5,5.01)
(raised to the power of 2 transformed)	Site	< 0.0001 (2, 52.27)
	Interaction term	< 0.0001^5 (10, 4.51)

Following the statistical testing, a Tukey post-hoc test was performed to determine where the significant differences lay within the population metrics.

Earthworm abundance (Figure 3A-7), biomass (Figure 3A-8), and diversity (Figure 3A-9) were significantly affected by both site and timepoint. However, there was a significant interaction term between site and sampling timepoint for all earthworm population metrics. The earthworm abundance, biomass, and diversity were most similar across all sampling sites in May 2017, with a general trend of higher earthworm populations in the rarely flooded sites compared to the two flooded sites.

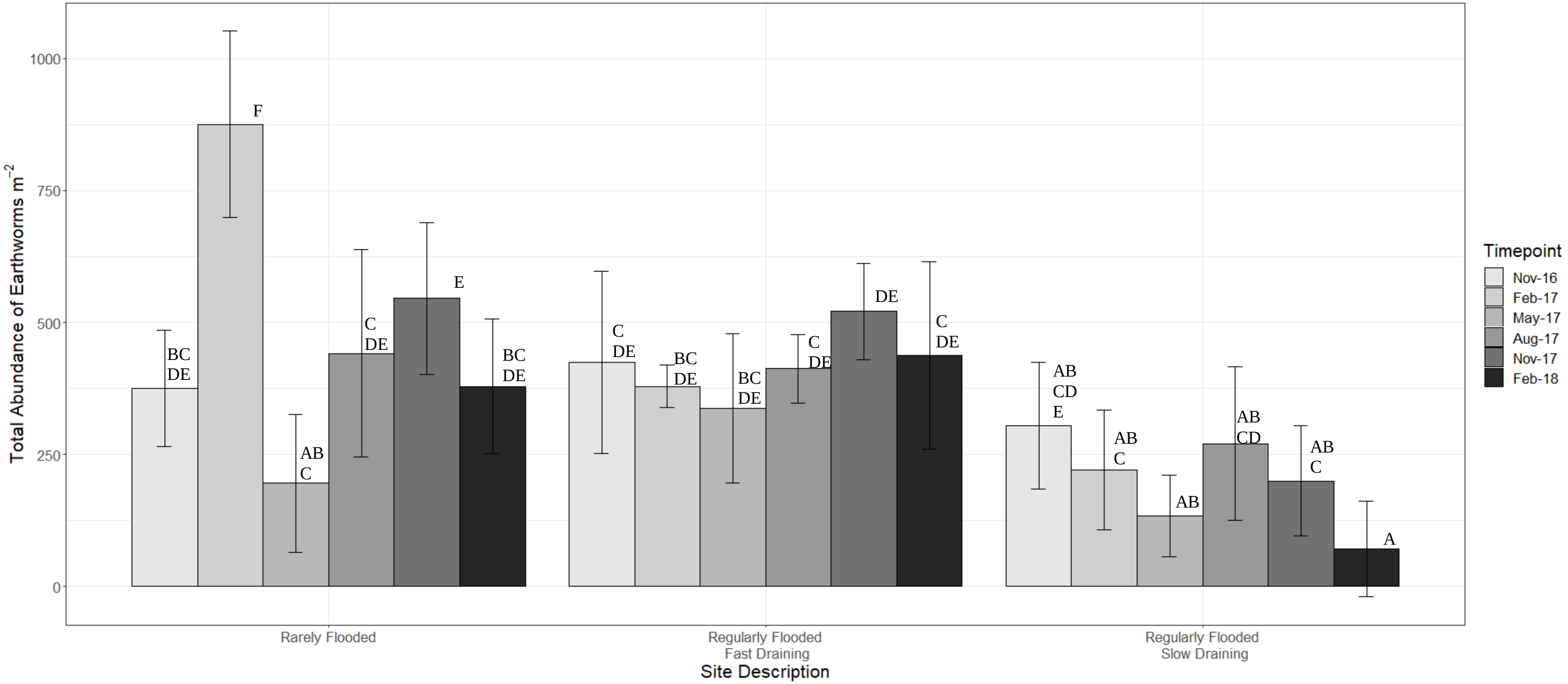

Figure 3A-7. The mean total abundance of earthworms m^{-2} for the different sites over the six sampling timepoints, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

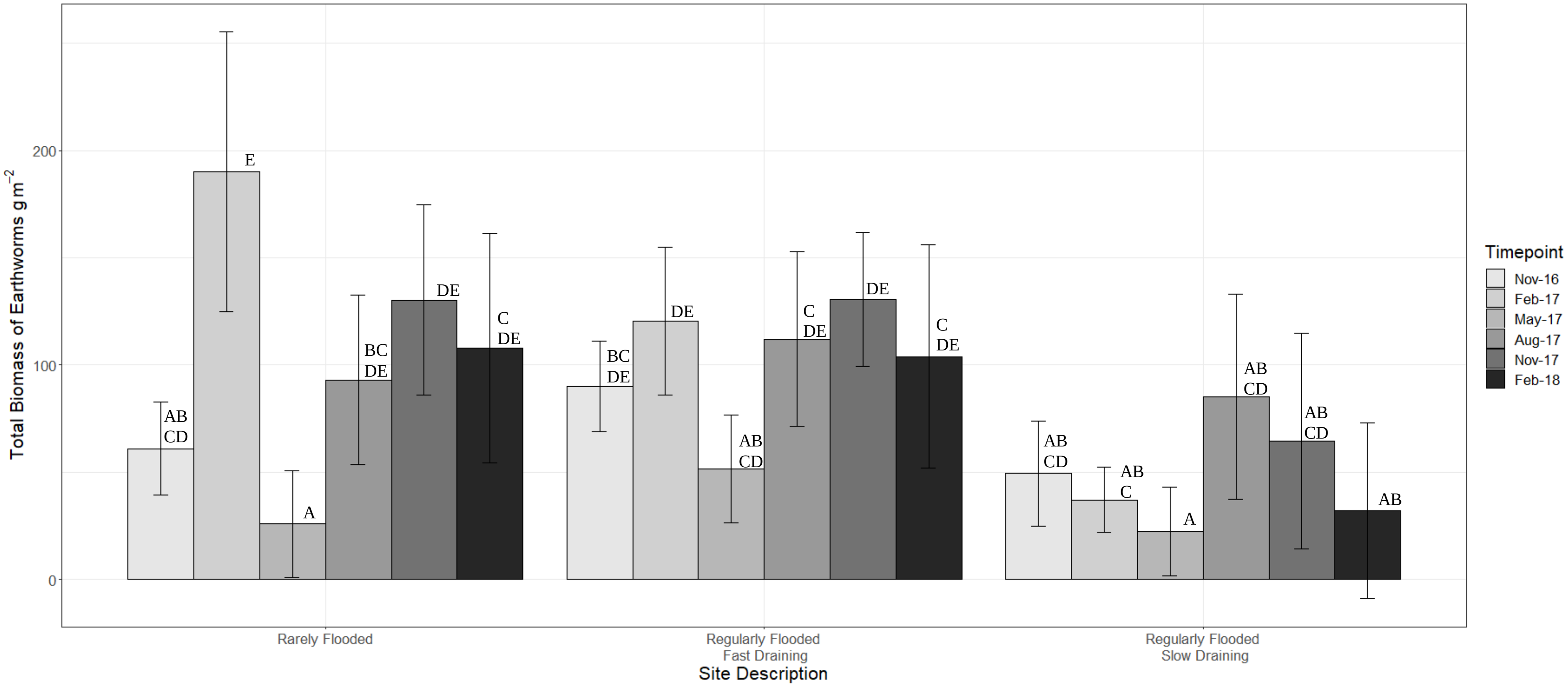

Figure 3A-8. The mean total biomass of earthworms per m^2 for the different sites over the six sampling timepoints, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

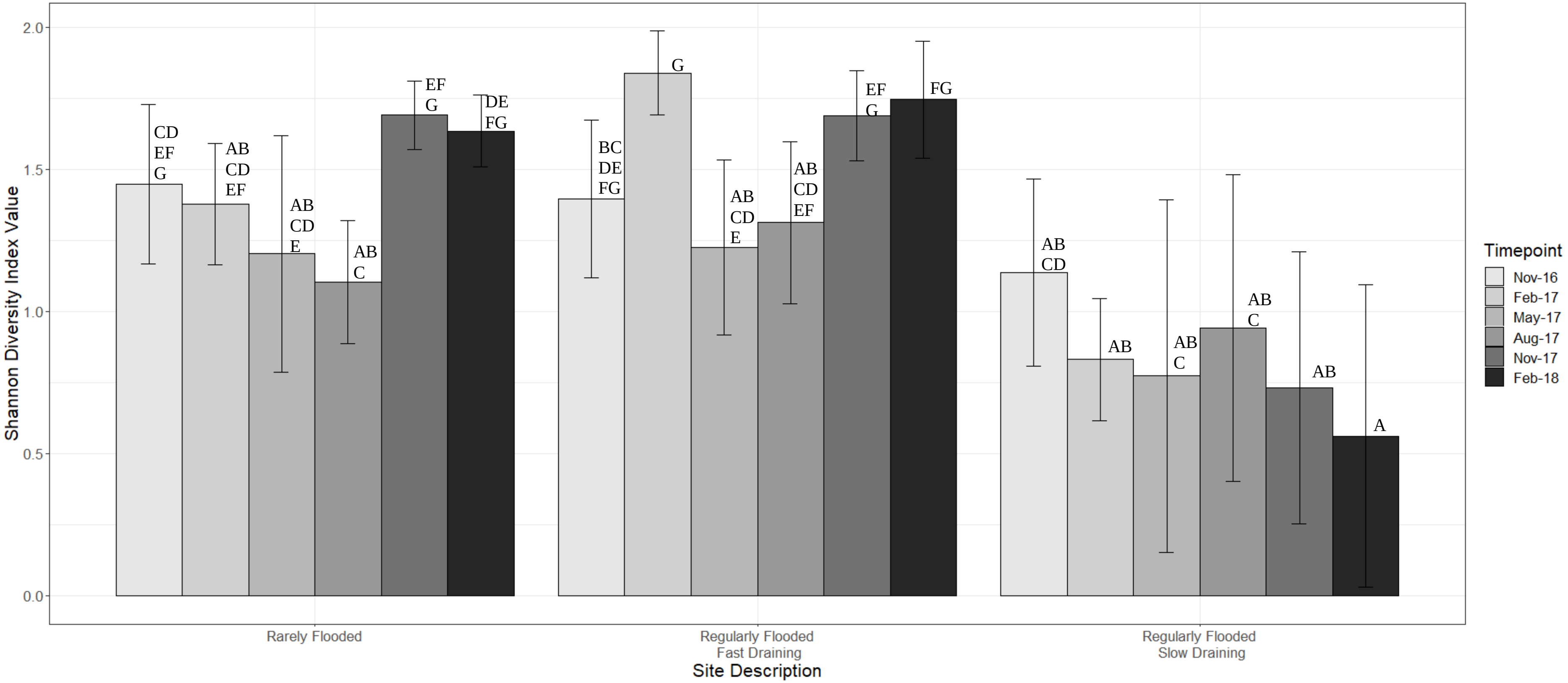

Figure 3A-9. The mean Shannon Diversity Index values of earthworms per pit for the different sites over the six sampling timepoints with error bars showing standard deviation. Bars marked with the same letter are not significantly different (*P* < 0.05).

3A.1.3 Population structure at the ecotype level across sites

Figure 3A-10 shows the relative proportions of the different earthworm ecotypes by the number of adult or juvenile individuals of the anecic, endogeic or epigeic ecotypes for each site at the different sampling time points. GLMs with a Quasipoisson link indicated that all categories of adult and juvenile anecic, endogeic, and epigeic earthworms differed between both the site and timepoints ($P < 0.05$). There was a significant interaction between the site and time of sampling for juvenile anecics, juvenile endogeics, and juvenile epigeics ($P < 0.01$). The P values for the significance of the timepoint, site, and interaction term are summarised in Table 3A-4.

Table 3A-4. The outcomes of GLMs performed to determine how the proportions of the population composed of adult or juvenile individuals of the anecic, endogeic, or epigeic ecotypes differed across the sampling timepoints and sampling sites.

Ecotype and age of individual (n)	**Variable**	***P* value (DF, LR Chi sq))**
Adult anecics (n = 14)	Timepoint	0.0003 (5, 23.033)
	Site	0.0098 (2, 9.250)
	Interaction term	0.1715 (10, 14.032)
Juvenile anecics (n = 34)	Timepoint	0.0144 (5, 14.189)
	Site	< 0.0001 (2, 41.021)
	Interaction term	0.0031 (10, 26.516)
Adult endogeics (n = 358)	Timepoint	< 0.0001 (5, 76.942)
	Site	< 0.0001 (2, 67.969)
	Interaction term	0.1980 (10, 13.481)
Juvenile endogeics (n = 936)	Timepoint	< 0.0001 (5, 38.112)
	Site	0.0016 (2, 12.905)
	Interaction term	0.0112 (10, 22.860)
Adult epigeics (n = 33)	Timepoint	0.0010 (5, 20.529)
	Site	0.0269 (2, 7.232)
	Interaction term	0.0732 (10, 17.052)
Juvenile epigeics (n = 191)	Timepoint	< 0.0001 (5, 28.683)
	Site	< 0.0001 (2, 56.070)
	Interaction term	0.0008 (10, 30.231)

For adult anecic and adult epigeic earthworms, Tukey post hoc testing showed no significant difference at the $P < 0.05$ level for either the difference sites or timepoints, with the same effect observed for juvenile anecic and epigeic earthworms when examining the interaction between both sites and timepoints.

Tukey post hoc testing indicated that adult endogeic earthworms were a significantly greater proportion of the community in the regularly flooded, slow-draining site (*P* = 0.05), and a significantly greater proportion of the population in November 2017 than in all other timepoints except February 2018 (*P* = 0.05), which did not significantly differ from November 2016 and February 2018, but was significantly greater than May and August 2017 (*P* = 0.04).

Tukey post hoc testing on the proportion of the community represented by juvenile endogeics across both sites and timepoints indicated that the proportions were broadly lower in May 2017 and August 2017, and higher in the slow draining site of the field. More in depth representation of the post hoc testing is shown on Figure 3A-10.

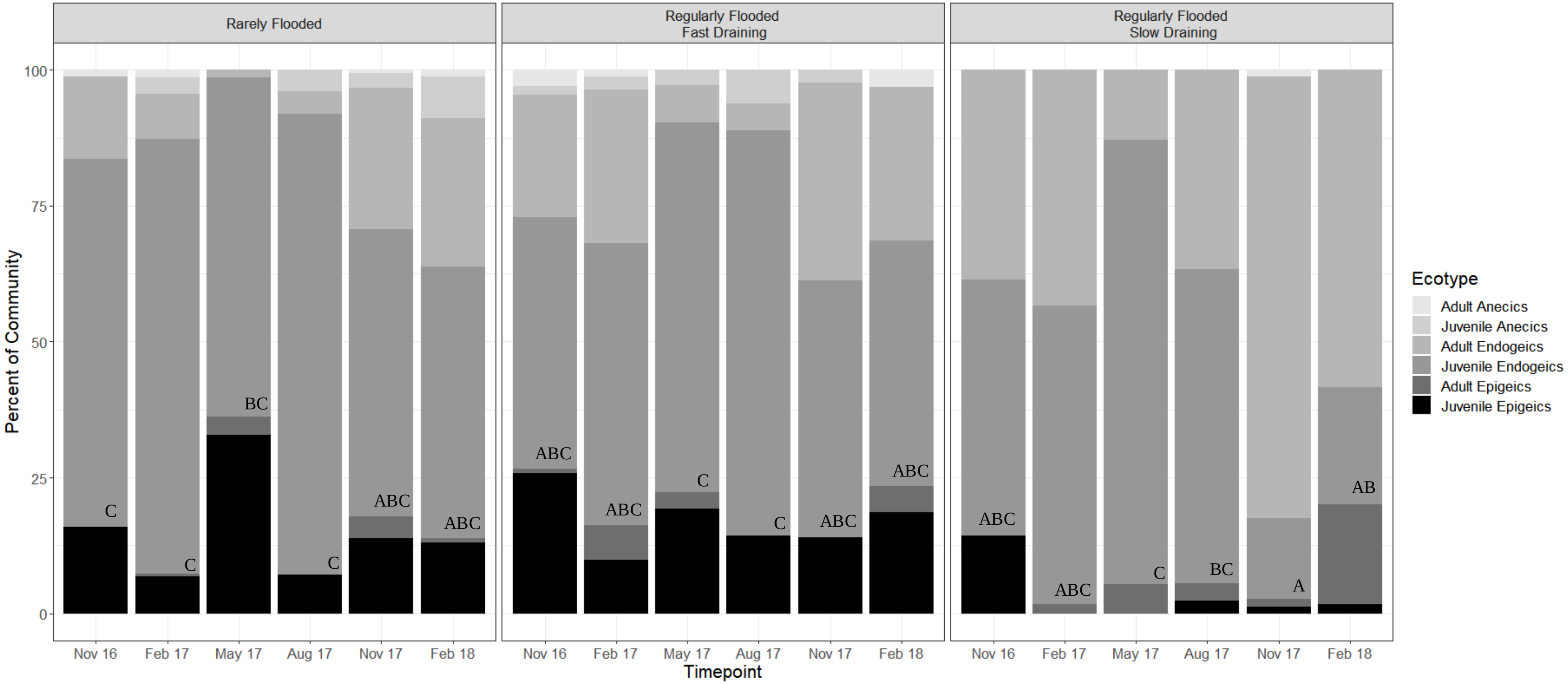

Figure 3A-10. The proportions of the community represented by adults and juveniles of the three earthworm ecotypes between the three sites in the Loddon floodplain. Juvenile endogeic bars marked with the same letter are not significantly different (*P* < 0.05).

3A.1.4 Differences in abundance and mass of mature individuals of species across sites

Mature adults individuals of six earthworm species were present across multiple sites and timepoints; *A caliginosa*, *A. chlorotica*, *A. rosea*, *L. castaneus*, *L. rubellus* and *L. terrestris*. Tables Table 3A-5 and Table 3A-6 summarise the differences in earthworm abundances and biomass respectively across all sites and timepoints. As overall abundances were low for several species, statistical testing was only performed for individuals of *A. chlorotica* (n = 264) and *A. caliginosa* (n = 74), with all other species present at n < 25.

To determine if the abundance of individuals of adult earthworms of each of the six species differed across the sites, GLMs with a Poisson link were performed between the timepoint and the site. The only significant effect observed was the effect of the time of year on *A. chlorotica* ($P < 0.01$), with abundances significantly lower in August 2017 ($P < 0.05$) and higher in November 2017 ($P < 0.05$).

Table 3A-5. The mean and range of the abundances of individuals m^{-2} of the six species present in the Loddon field site. Shaded row headings for individual species indicate a significant difference assessed by generalised linear model with a Poisson link function ($P < 0.01$), while row heading cells marked with the same letter are not significantly different ($P < 0.05$). N/A indicates no range, with only one earthworm found in a set of six pits.

A. caliginosa			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	50 (25 – 75)	65 (25 – 175)	30 (25 – 50)
Feb. 17	37.5 (25 – 50)	-	25 (N/A)
May. 17	32.5 (25 – 50)	-	-
Aug. 17	32.5 (25 – 50)	25 (N/A)	25 (N/A)
Nov. 17	57.5 (25 – 100)	25 (N/A)	25 (N/A)
Feb. 18	50 (25 – 75)	-	50 (25 – 100)

A. chlorotica			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16 A	80 (50 – 125)	80 (25 – 150)	40 (25 – 50)
Feb. 17 A	80 (25 – 125)	87.5 (50 – 160)	70 (50 – 100)
May. 17 A	125 (N/A)	30 (25 – 50)	25 (N/A)
Aug. 17 C	-	88 (25 – 225)	-
Nov. 17 B	125 (75 – 200)	162.5 (50 – 300)	130 (50 – 225)
Feb. 18 A	87.5 (25 – 150)	62.5 (25 – 125)	67.5 (25 – 125)

A. rosea			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	32.5 (25 – 50)	-	25 (N/A)
Feb. 17	25 (N/A)	-	25 (N/A)
May. 17	-	-	-
Aug. 17	25 (N.A)	-	25 (N/A)
Nov. 17	25 (N/A)	-	25 (N/A)
Feb. 18	75 (N/A)	-	25 (N.A)

L. rubellus			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	25 (N/A)	-	-
Feb. 17	25 (N/A)	25 (N/A)	25 (N/A)
May. 17	25 (N/A)	37.5 (25 – 50)	25 (N/A)
Aug. 17	-	37.5 (25 – 50)	-
Nov. 17	-	25 (N/A)	32.5 (25 – 50)
Feb. 18	37.5 (25 – 50)	25 (N/A)	25 (N/A)

L. castaneus			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	-	-	-
Feb. 17	25 (N/A)	-	-
May. 17	25 (N/A)	-	50 (N/A)
Aug. 17	-	-	-
Nov. 17	-	-	-
Feb. 18	25 (N/A)	25 (N/A)	-

L. terrestris			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	37.5 (25 – 50)	-	25 (N.A)
Feb. 17	-	-	25 (N/A)
May. 17	-	-	-
Aug. 17	-	-	-
Nov. 17	-	-	-
Feb. 18	-	-	-

To determine if the mass of adult individuals of given species differed across the sites, two way ANOVAs were performed between the timepoints and the site. To achieve normality, results for *A. chlorotica* were transformed with a reciprocal transformation.

The biomass of adult individuals of both *A. chlorotica* and *A. caliginosa* was significantly different between sites ($P < 0.01$) and timepoints ($P < 0.01$), and also showed an interaction term between the two terms ($P < 0.01$).

Post hoc testing showed general trends of the biomass of adult individuals differing in the May 2017 and August 2017 sampling from the February 2017 and 2018 and November 2016 and 2017 samplings. For *A. chlorotica*, the biomass was lower in the May 2017 samples than in the other time periods. Despite having significant interaction terms, *A. chlorotica* and *A. caliginosa,* showed different patterns of earthworm biomass. Individuals of *A. chlorotica* were of a higher biomass in the slow-draining site than in the fast-draining and rarely flooded sites. In contrast, individuals of *A. caliginosa* were a higher biomass in the fast-draining site. Individuals in August generally had a higher biomass than individuals in the February 2017 and February 2018 samples, but this was more noticeable in the slow-draining site.

Table 3A-6. The mean and range of the biomass (g) of individuals of the six species present in statistically valid quantities in the Loddon field site. Shaded row and column headings for individual species indicate a significant difference assessed by two way ANOVAs between sites and sampling times (lighter shading indicates a significance of P < 0.05; darker shading a significance of P < 0.01). For any given species values, cells with the same letter are not significantly different (P < 0.05). N/A indicates no range, with only one earthworm found in a set of six pits.

A. caliginosa			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	0.41 (0.23 – 0.54) **AB**	0.25 (0.07 – 0.58) **A**	0.37 (0.28 – 0.45) **AB**
Feb. 17	0.31 (0.05 – 0.65) **AB**	-	0.69 (N/A) **AB**
May. 17	0.39 (0.32 – 0.47) **AB**	-	-
Aug. 17	0.58 (0.47 – 0.75) **B**	0.78 (N/A) **B**	0.45 (0.31 – 0.70) **AB**
Nov. 17	0.44 (0.28 – 0.56) **B**	0.65 (N/A) **AB**	0.41 (0.31 – 0.51) **AB**
Feb. 18	0.47 (0.40 – 0.75) **B**	-	0.54 (0.44 – 0.75) **B**

A. chlorotica			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	0.23 (0.15 – 0.32) **CD**	0.28 (0.14 – 0.45) **ABCD**	0.21 (0.17 – 0.28) **CDE**
Feb. 17	0.23 (0.13 – 0.32) **CD**	0.22 (0.15 – 0.50) **DE**	0.20 (0.13 – 0.24) **DE**
May. 17	0.18 (N/A) **ABCDE**	0.16 (0.12 – 0.20) **E**	0.16 (N/A) **ABCDE**
Aug. 17	-	0.35 (0.19 – 0.51) **A**	-
Nov. 17	0.23 (0.46 – 0.3) **CD**	0.27 (0.14 – 0.48) **BC**	0.23 (0.15 – 0.31) **CD**
Feb. 18	0.21 (0.15 – 0.27) **DE**	0.35 (0.25 – 0.49) **AB**	0.22 (0.16 – 0.58) **CD**

A. rosea			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	0.22 (0.19 – 0.25)	-	0.95 (N/A)
Feb. 17	0.25 (N.A)	-	0.16 (N/A)
May. 17	-	-	-
Aug. 17	0.17 (N.A)	-	0.28 (N/A)
Nov. 17	0.23 (0.22 – 0.25)	-	0.20 (0.14 – 0.24)
Feb. 18	0.26 (0.21 – 0.34)	-	0.26 (0.23 – 0.28)

L. castaneus			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	-	-	-
Feb. 17	0.24 (N/A)	-	-
May. 17	0.67 (N/A)	-	0.10 (0.09 – 0.11)
Aug. 17	-	-	-
Nov. 17	-	-	-
Feb. 18	0.15 (0.13 – 0.6)	0.22 (N/A)	-

L. rubellus			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	0.46 (N/A)	-	-
Feb. 17	0.47 (0.26 – 0.69)	0.43 (N/A)	0.12 (N/A)
May. 17	0.29 (N/A)	0.56 (0.39 – 0.79)	0.56 (N.A)
Aug. 17	-	0.92 (0.76 – 1.16)	-
Nov. 17	-	0.63 (N/A)	0.39 (0.30 – 0.48)
Feb. 18	0.36 (0.13 – 0.54)	0.49 (N/A)	0.43 (N/A)

L. terrestris			
	Fast draining	**Slow draining**	**Rarely flooded**
Nov. 16	1.04 (0.27 – 2.37)	-	0.61 (N/A)
Feb. 17	-	-	4.09 (3.50 – 4.68)
May. 17	-	-	-
Aug. 17	-	-	-
Nov. 17	-	-	-
Feb. 18	-	-	-

3A.1.5 Correlations between soil environmental factors and earthworm population metrics

To determine if there was a linear relationship between the soil environmental factors and the earthworm population metrics of abundance, biomass, and Shannon Diversity, Spearman's Rank correlations were performed (Table 3A-7).

Table 3A-7. Spearman's Rank rho (ρ) values of correlations between soil environmental factors and earthworm population metrics (n=108). Lighter shaded cells indicate a statistically significant correlation of *P* < 0.01. Darker shaded cells indicate a statistically significant correlation of *P* < 0.05.

	Abundance m^{-2}	Total earthworm biomass ($g\ m^{-2}$)	Shannon Diversity Index
Temperature	ρ = - 0.04	ρ = - 0.13	ρ = -0.28
Bulk density	ρ = 0.42	ρ = 0.33	ρ = 0.37
Soil moisture content	ρ = - 0.23	ρ = - 0.06	ρ = -0.21
pH	ρ = 0.25	ρ = 0.24	ρ = 0.06
Percent carbon	ρ = - 0.38	ρ = -0.3	ρ = - 0.41
Percent nitrogen	ρ = - 0.40	ρ = - 0.21	ρ = - 0.42

Earthworm abundance, biomass and diversity are all negatively associated with the soil carbon and nitrogen percentages, with abundance and diversity also negatively associated with the soil moisture content. Earthworm diversity was negatively associated with the mean soil temperature. Positive associations were observed for abundance and biomass with pH and bulk density. Earthworm diversity was positively associated with bulk density.

To determine which environmental factors were the most influential on earthworm populations, GLMs were produced comparing all environmental factors to the individual earthworm population metrics. Using stepwise model selection of GLMs (Zhang, 2016), the environmental factors which explained the most variance within the model, and their statistical significance on the population variable, could be selected for each earthworm population variable.

Stepwise model selection of a GLM using a Poisson link indicated that the key environmental factors predicting total earthworm abundance were soil moisture, bulk density, and percent carbon ($P < 0.01$).The key statistically significant environmental factors predicting the total biomass of earthworms were soil moisture and percent carbon ($P < 0.01$). While it was important in explaining some of the variance in the model, pH was not significant, although the P value was very close to significance ($P = 0.07$). The key environmental factors predicting the Shannon diversity index of earthworms were the mean temperature of the soil between 5cm and 10cm and soil percent carbon ($P < 0.01$).

3A.2 Discussion

3A.2.1 Soil environmental factors

3A.2.1.1 Soil temperature and soil moisture

Soil temperatures were typically lower in the November 2016 and 2017 and February 2017 and 2018 samplings than in the May 2017 and August 2017 samplings, and were typically lowest in the regularly-flooded, fast-draining site and highest in the rarely flooded site. While the association with the time of year is not unexpected, the association with site is possibly due to the sampling order performed on the day of sampling. On all sampling visits, the sampling was completed in one day, starting in the regularly-flooded, fast-draining site, then the slow-draining, and finally the rarely flooded sites. Typically increased soil moisture content helps to limit both the gain and loss of heat, with drier soils gaining and losing stored heat more rapidly than wetter soils (Al-Kayssi et al., 1990). As the third site sampled, the rarely flooded site had both the highest soil temperatures and the lowest soil moisture contents, it may be that the warming effect of sunlight was more noticeable than in the wetter, thermally buffered sites.

Soil moisture and soil temperature were negatively correlated, with lower soil temperatures and higher soil moisture content observed during the winter months of November and February and higher soil temperatures and lower soil moisture content in the sampling months of May and August. The soil moisture reflects rainfall patterns reported by other organisations during the eighteen month sampling period (Table 3A-7); the exceptionally low soil moisture observed in May 2017 is likely due to the fact that April 2017 was at the time the driest April on record. Met Office (UK) figures state that the UK had just 47% of average rainfall for April that year (Met Office, 2017), with an average rainfall of 0.2 mm per day (Table 3A-8).

Table 3A-8. The average daily rainfall (mm) and total month's rainfall (mm) reported at Wokingham Weather Monitoring Station (SU 80012 70401) (Wokinghamweather.co.uk, 2019). This is the closest weather monitoring station to the field site at a distance of 3.2 miles

Month	Average rainfall per day (mm)	Total month's rainfall (mm)	Mean daily maximum temperature (°C)	Mean daily minimum temperature (°C)
September 2016	3.21	96.3	21.1	12.0
October 2016	0.97	30	15.2	6.9
November 2016	2.83	84.9	9.7	2.5
December 2016	0.45	13.8	9.4	2.2
January 2017	1.79	55.5	7.5	-0.4
February 2017	1.2	33.6	9.7	3.9
March 2017	0.71	21.9	14.1	6.1
April 2017	0.2	6	15.7	4.3
May 2017	2.061	63.9	19.4	9.2
June 2017	1.17	35.1	23.0	12.7
July 2017	3.831	118.8	23.1	13.9
August 2017	2.24	69.3	21.8	12.5
September 2017	2.11	63.3	18.4	9.9
October 2017	0.55	17.1	16.3	9.4
November 2017	1.22	36.6	10.9	3.5
December 2017	2.42	75	8.5	2.5
January 2018	2.07	64.2	9.4	3.0
February 2018	0.80	22.5	7.0	-0.7

The soil moisture contents observed throughout this study were frequently very high in the frequently flooded sites. In February 2017 and 2018 in the slow draining site, soil moisture content was 130% of the dry soil mass in the slow draining site, significantly higher than the soil moisture content of the rarely flooded site. This saturation of the soil in the February samplings does not coincide with periods of exceptionally high rainfall, with total rain for the month higher in November 2016, May 2017, August 2017 and November 2017 than in February 2018. However, in both February 2017 and 2018 rainfall in the preceding month (55.5 mm and 64.2 mm in January 2017 and 2018 respectively) was high, suggesting that the slow-draining site remains saturated for some time following periods of heavy rainfall.

3A.2.1.2 Soil bulk density

Soil bulk density was higher in the rarely flooded site than in the regularly flooded sites, while soil percent carbon and nitrogen were lower, with a strong negative correlation between soil bulk density and percent carbon and nitrogen. The rarely flooded site had the highest temperature and lowest soil moisture content. The differences in bulk density are likely due to the effect of the increased organic matter accumulation in the regularly flooded sites. With regular flooding, the soils are often under anaerobic conditions, which leads to a build-up of organic matter due to reduced rates of decomposition (Reddy and Patrick Jr, 1975). The accumulation of organic matter, which increases percent carbon and nitrogen in the soil, leads to decreased soil bulk density (Bronick and Lal, 2005) and increased soil water holding capacity (Carter, 2002; Rawls et al., 2003). This is consistent with the results observed in this study, with significantly lower bulk density and higher soil moisture content and percent carbon and nitrogen observed across all months in the regularly flooded, slow-draining site.

Some earthworm species act to reduce soil bulk density (Blouin et al., 2013), either through their burrowing activity creating large macropores (Blanchart et al., 1999; Francis and Fraser, 1998; van Schaik et al., 2013) or through the increases in soil aggregation they cause (Brown et al., 2000; Lee et al., 1991), which in turn leads to protection of soil carbon within aggregates (Beare et al., 1994; Franzluebbers and Arshad, 1996). Earthworms are also observed to play a key role in the incorporation of organic matter into soil (Clements et al., 1991). However, the strong associations between earthworms and soil organic matter content may also be due to the nature of the soils. Undisturbed soils accumulate greater levels of soil organic matter than disturbed soils such as agricultural soils (Woods, 1989; Lal, 2004). These undisturbed conditions allow diverse populations of earthworms, as in agricultural soils earthworm populations are greatly reduced (Boag et al., 1997; Curry et al., 2002), in part due to crush or cutting damage to individuals caused by agricultural machinery (Tomlin and Miller, 1988), leading to an association of higher earthworm populations in soils with higher organic matter content. In this study, earthworm abundances and biomass were lower in the regularly flooded, slow draining soils than in the fast-draining or rarely flooded soils, where bulk density and organic matter content of soil were increased. However, despite low abundance and biodiversity, there may still be an effect of earthworm burrowing on the soil bulk density. Earthworms can broadly be grouped into two functional groups of ‘compactors’ and ‘decompactors’ (Blanchart et al., 1999). This grouping is not based on ecotype, with studies finding both compacting and decompacting earthworms belonging to the endogeic ecotype (Rossi, 2002). While the majority of experiments have focused on tropical earthworm species, experiments investigating European earthworm species have found that *A. caliginosa* was associated with increases in soil compaction (Ester and van Rozen, 2002), while the pink morph of *A. chlorotica* was associated with decreases in compaction (Amossé et al., 2015). *A. rosea* was included in both experiments, and associated with increases and decreases in compaction respectively. Although abundances of individuals of *A. chlorotica* and *A. caliginosa* did not significantly differ between the sites, there were more individuals of *A. chlorotica* in total than individuals of *A. caliginosa* across the field. With low overall abundances, biomass and diversity of earthworms in the slow-draining site, the combination of the increased organic matter accumulation with flooding and the dominating presence of the decompacting earthworm species led to lower soil bulk density in this site than the rarely flooded site.

3A.2.1.3 Soil pH

The soil pH was lower in the November and February sampling periods than in the summer months. This may be because soil pH variations are associated with extreme climatic conditions such as abnormally hot or wet conditions, likely due to the moisture content of the soil in these periods (Conyers et al., 1997). During this study, shifts in the pH occur as the pH increases significantly from May to August before returning to pre-summer levels in November and February. This coincides with the generally dry conditions in the UK reported by the Met Office in April 2017. Generally, between the sites there is a trend for the pH to be slightly more acidic within the regularly-flooded, slow-draining sites than the fast-draining or rarely flooded sites. There is some evidence that soil moisture content is influential on soil pH, with experiments that have compared soil incubated under moist, ‘autumnal’ conditions with drier, ‘summer’ conditions finding that soil pH tends to be less acidic under drier conditions (Conyers et al., 1995). Conyers et al. attributed this to the influence of soil moisture on soil microbial activity, with increased moisture leading to greater rates of oxidation of nitrogen within soil, which leads in turn to decreases in the soil pH. A later study by Zhang and Wienhold found that the pH reducing oxidation of inorganic nitrogen occurs under anaerobic conditions, with very little change in pH occurring under aerobic conditions (Zhang and Wienhold, 2002). Although in this study only total nitrogen content was measured, rather than the organic and inorganic fractions, it may be the case that the higher nitrogen content of the regularly flooded sites, combined with the frequently high soil moisture contents leading to anaerobic conditions in soil, led to more acidic conditions observed in the regularly flooded sites. The accumulation of organic matter in soil can act to buffer soils against dramatic pH change by increasing the cation exchange capacity of soils (Ritchie and Dolling, 1985). While the accumulation of organic matter may lead to more acidic conditions during flooding events in the regularly flooded site, it does also protect the soils against the high levels of variability in soil pH observed in the rarely flooded site.

Under laboratory conditions, earthworms are able to tolerate soil pH ranges of 4.5 to 7 in culture (Lowe and Butt, 2005). With a measured pH range of between 5.0 and 6.6, none of the values observed at the Loddon sites shifted the soil pH range beyond tolerable levels for earthworms. The earthworm populations in the most acidic, slow-draining site, were dominated by *A. chlorotica* with a lower presence of *A. caliginosa;* if the populations were driven by soil pH, it is unlikely that these species, considered acid-intolerant, would be present in such a high proportion of the population (Satchell, 1955, in Butt and Briones, 2017). It is likely that the seasonal fluctuations observed in the earthworm populations are not driven by changes in the soil pH. While seasonal fluctuations in earthworm populations can be driven by other factors, such as earthworm life cycles (Evans and Guild, 1948a), the stepwise model selection between the measures of earthworm population and the environmental variables found a significant link between earthworm abundance and soil pH, explaining some of the variance within the populations without causing a significant effect. The model selection also indicated that soil pH was nearly significantly associated with total earthworm biomass. It may be the case that pH and earthworm biomass are strongly linked to other environmental factors, leading to the presence of pH in the selected model. As discussed above, soil pH is lowest in the high percent carbon, high soil moisture content conditions of the regularly flooded sites. The regularly flooded sites have the lowest total earthworm biomass. This suggests that the soil pH and total earthworm biomass are linked, but unlikely to be directly influencing each other.

3A.2.1.4 Soil carbon and nitrogen content

Finally, both soil carbon and soil nitrogen percentages were significantly higher in the regularly-flooded, slow-draining site than within the regularly-flooded, fast-draining site, which in turn is significantly higher than in the rarely flooded site. The strong positive correlation observed between the soil percent carbon and percent nitrogen suggests that the carbon and nitrogen present in the soil are held in the same soil component, mostly likely the organic matter fraction of the soil (Six et al., 2001). This is likely due to the fact that soil conditions in the regularly-flooded, slow-draining site are optimal for slow rates of organic matter breakdown, of which carbon and nitrogen are components. However, higher inputs of organic matter to these sites could also occur due to deposition of organic matter from flood waters, which may explain the temporal differences in the percent carbon and nitrogen observed. Soil carbon and nitrogen percentages were higher in February 2017 than in May 2017, which may be due to winter flooding occurring before the February 2017 sampling, with some organic matter lost by degradation before the May 2017 sampling. The lower soil moisture content in May 2017, and the low total rainfall in April 2017

(Table 3A-7), suggests that drying of the soil lead to an increase in the breakdown of soil carbon. This pattern was particularly noticeable in the slow-draining site, with both the soil moisture content and percent carbon of the soil significantly lower in May 2017 than all other sampling timepoints in the slow-draining site. Wetting and drying cycles of soil can lead to increased consumption of soil organic matter (Reddy and Patrick Jr, 1975), and would explain the observed pattern of soil carbon and nitrogen percentages.

3A.2.2 **Earthworm Populations**

Consistently across earthworm abundance, biomass, and Shannon Diversity, the measures of earthworm populations are lower in the regularly-flooded, slow-draining site than in the fast-draining or the rarely flooded site. This pattern of earthworm populations being lower in a region subject to regular, long term flooding stress has been observed in previous studies (Plum, 2005; Plum and Filser, 2005; Zorn et al., 2005).

3A.2.2.1 Association with soil environmental factors

When considering the effects of the environmental factors, there were statistically significant correlations with all factors except temperature, in the case of abundance, temperature and soil moisture content, in the case of biomass, and pH, in the case of the Shannon diversity index values (Table 3A-5). When using stepwise model selection to find the environmental factors with the most influence on earthworm populations, there was some overlap. All three measures of earthworm population were highly influenced by the soil carbon percentage, and both earthworm abundance and biomass were strongly influenced by soil moisture content, while the Shannon diversity was strongly influenced by temperature. However, it is important to note that the association with the soil carbon percentage is negative; despite soil carbon being an important food source for endogeic earthworms (Lavelle, 1988; Curry and Schmidt, 2007), populations are reduced in the highest carbon regions, and higher in the lower carbon regions. This negative association was also observed for soil moisture, with earthworm abundances and biomass higher in lower moisture conditions, and the diversity of earthworms higher at lower soil temperatures. As soil moisture content was positively correlated with the soil carbon percentage, the negative association with both for earthworm abundance and biomass is unsurprising. Overall, these results, and the studies performed by Plum (2005), Plum and Filser (2005), and Zorn et al. (2005), suggest that while flooding leads to some soil conditions that may be beneficial to earthworms, such as increased carbon and nitrogen content, it also has strongly reducing effects on the earthworm populations, as individuals either flee flooded soil or drown when soil oxygen reaches anoxic conditions.

3A.2.2.2 Seasonal variation in earthworm populations

The measures of earthworm abundance also show a significant difference based on the time of sampling. Across all sites, earthworm abundance and biomass were lowest in May 2017, and earthworm biodiversity was lowest in May 2017 and August 2017 in the fast-draining and rarely flooded sites. While this may be attributed to environmental conditions associated with the summer sampling months, soil temperature and moisture content were significantly different for the same time period. It may be the case that the conditions of lower temperature and drier soil in May were equally as unsuitable for earthworm populations as the higher temperature but slightly wetter soil in August, as soil temperature and moisture are highly influential on earthworm lifecycles (Edwards and Lofty, 1977) and behaviour such as casting (Gerard, 1967) and reproduction (Evans and Guild, 1948b). The similarity in populations may also indicate slow population recovery after stressful conditions in May 2017. The twelve weeks between sampling in May 2017 and August 2017 exceeds the cocoon incubation period of many earthworm species (Evans and Guild, 1948b). The model selection suggests that soil temperature and soil moisture are both factors affecting earthworm population metrics. This is consistent with existing studies of seasonal patterns of earthworm abundance. Earthworm abundances are higher in soils in the winter months (Gerard, 1967), cocoon production peaks during the period of February to July (Evans and Guild, 1948a), and hatching occurs 10 to 20 weeks later (Wilcke, 1952, in Edwards and Lofty, 1977), coinciding with wetter, cooler weather conditions.

3A.2.2.3 Earthworm population composition

The lower diversity observed in the regularly flooded, slow-draining site reflects the increased proportion of the population in this site that are endogeic earthworms, with comparatively low representation of other earthworm ecotypes. At the ecotype level, the endogeic earthworms made up a large proportion of all three field sites. This is not surprising; a survey undertaken by Natural England found that, across a number of habitats, the two most numerous earthworms (jointly making up 53% of identified adult earthworms) were *A. chlorotica* and *A. caliginosa,* both of which are endogeic species (Natural England, 2014). Within the field sites, the proportion of the population represented by adults and juveniles of the endogeic ecotype is significantly greater within the regularly-flooded, slow-draining site than within the fast-draining or the rarely flooded sites. As there were three endogeic species (*A. chlorotica*, *A. caliginosa* and *A. rosea*) observed across the field, compared to three epigeic (*L. castaneus, L. rubellus, D. octaedra)* and two anecic species (*L. terrestris* and *A. longa*), their dominant presence within the regularly-flooded, slow-draining site leads to an overall low biodiversity compared to the fast-draining and rarely flooded sites, and suggests that endogeic earthworms are better able to survive regularly flooded conditions than anecic or epigeic earthworms.

However, even within the dominating endogeic earthworm groups, there was variation at the species level. *A. rosea* was not recorded within the slow-draining site, instead being present only in the fast-draining and the rarely flooded sites. The presences of *A. rosea* within the rarely flooded and fast-draining sites were low, meaning that statistical testing could not be performed, but it may be the case that *A. rosea* may be highly sensitive to the effects of flooding, or it may be outcompeted by other endogeic earthworm species in the area. Previous studies have found that *A. rosea* is not present in floodplain meadows (Ivask et al., 2007), and is a species that arrives and recolonises disturbed soil later than other endogeic species (Eijsackers et al., 2009). Other studies have found that the abundance of *A. rosea* is negatively associated with the presence of other endogeic earthworm species (Gutiérrez-López et al., 2010). However, *A. rosea* seems to feed at a different trophic level to both *A. chlorotica* and *A. caliginosa*, despite belonging to the same ecotype. Unlike *A. chlorotica* and *A. caliginosa, A. rosea* feeds on a broad range of soil fungi (Bonkowski et al., 2000), and shows preference for different sources of nitrogen within soil (Schmidt et al., 1997). This differentiation of food preferences, combined with the presence of *A. rosea* in the fast-draining and the rarely flooded regions, both of which also have a high abundance of competing endogeic earthworms, suggests that it is sensitivity to persistent waterlogging and to the soil conditions that is driving the absence of *A. rosea* in the slow-draining region, rather than direct competition for resources.

A. chlorotica significantly differed in abundance based on the interaction term between the time of sampling and the location, while both *A. chlorotica* and *A. caliginosa* showed a difference in abundance and biomass both based on the interaction between time of sampling and on the location.. Individuals of *A. caliginosa* had, on average, significantly lower biomass in the slow-draining site than in the fast-draining site or the rarely flooded site. Individuals of *A. chlorotica* showed the opposite pattern, and were of significantly higher biomass in the slow-draining site than within the other two sites. This indicates that although the endogeic earthworms are able to survive in the regularly-flooded, slow-draining environment of the field, they do not necessarily thrive, in the case of *A. caliginosa*. *A. chlorotica*, with their higher average biomass in the slow-draining site, may be outcompeting *A. caliginosa* by virtue of being better able to exploit either the higher organic matter or soil moisture content found in this region of the field. *A. chlorotica* has two colour morphs found in UK soils: a pink morph and a green morph. The green morphs are known to dominate regions with a higher soil moisture content (Satchell, 1967), and in this study only green individuals of *A. chlorotica* were found. There are a number of adaptations to high soil moisture conditions observed in the green morph, such as higher cocoon production and fast growth rates than in the pink colour morph (Lowe and Butt, 2007). Studies into earthworm populations have found no effect of flooding on survival and abundance of *A. caliginosa* (Zorn et al., 2005), but laboratory experiments found that in flooded conditions individuals of *A. caliginosa* lost biomass (Zorn et al., 2008). These studies support the field observations obtained, where both *A. caliginosa* and *A. chlorotica* were able to survive flooding with no effect on the abundance of individuals of the respective species, but individuals of *A. caliginosa* are not as well adapted as the green morph of *A. chlorotica* to these conditions, and exhibit a stress response.

3A.2.2.4 Earthworm sampling methodology

It may be the case that some of the reduced populations recorded in the study are due to inefficiencies in the sampling methods. Although the combination of hand sorting and application of dilute allyl isothiocyanate is considered an efficient sampling method to determine earthworm populations (Zaborski, 2003; Čoja et al., 2008, Pelosi et al., 2009), it does favour the extraction of mature anecic earthworms (Bartlett et al., 2006), while inefficiently sampling inactive endogeic earthworms during periods when aestivation is likely to occur (Eisenhauer et al., 2008). Endogeic earthworms such as *A. chlorotica, A. caliginosa* and *A. rosea,* and the anecic earthworm *A. longa*, are all known to retreat into deeper soil layers during periods of high temperature and low soil moisture (Evans and Guild, 1948b; Gerard, 1967; Nordström, 1975; Morgan and Winters, 1990). However, it could be argued that the sampling that occurs at the discrete timepoints needs to reflect the active population within the soil, and sampling of inactive earthworms may inaccurately represent the processes occurring in the soil. At the time of sampling, earthworms were not assessed to determine whether they were in an aestivating state, partly due to the sampling methodology of transporting earthworms back to the laboratory for identification. Future population study could record earthworm behaviours in addition to abundances.

3A.3 **Conclusion**

Regular flooding of regions of a pasture field increased the soil moisture content, decreased soil bulk density and pH, and increased the percent carbon and percent nitrogen of the soil. The regular flooding and the increases it caused in factors such as soil moisture also helped buffer soil temperatures against dramatic increases and decreases. Flooding induces a number of changes in soil physical properties, but pH remained within a range suitable for earthworm activity. Stepwise model selection of GLMs showed that percent carbon has a very strong negative correlation with earthworm populations, and that measures of earthworm population abundance and biomass were influenced by soil moisture, while diversity was influenced by soil temperature. Although it may be expected that higher soil carbon percentage (representing increased soil organic matter contents) leads to higher earthworm populations, the accumulation of soil carbon and nitrogen in these sites is a result of regular flooding. The regular flooding leads to earthworm absence in these sites, either as a result of earthworm expulsion from the soil or death, and results in the strong negative association between earthworm population metrics and soil carbon percent.

Sites within the pasture field that flooded regularly and remained saturated for some time had a reduced earthworm population compared to the sites that drained rapidly or rarely flooded. The most common ecotype within the pasture field was endogeic earthworms, and the most common endogeic earthworm species was *A. chlorotica*. The regularly flooded, fast-draining and the rarely flooded sites had a higher earthworm diversity that the regularly flooded, slow-draining site. The regularly flooded, slow-draining site was dominated by *A. chlorotica*, with some epigeic earthworms but very few anecic earthworms present. This suggests that endogeic earthworm species, particularly *A. chlorotica*, may have adaptations that allow them to either exploit the higher carbon percentage of the slow-draining site or survive the negative flooding effects that lead to anecic or epigeic earthworm either dying or being expelled from the soil.

As the flooding regime has been continuing within the field for some time, it is impossible to say with certainty whether the flooding itself or the unfavourable soil conditions created by flooding lead to the reduced earthworm populations. With the removal of anecic and epigeic earthworms from the system with flooding, the recovery of a number of soil factors may be reduced. Anecic earthworms play important roles in increasing the quantity of small pores in the soil (Pérès et al., 1998; Görres et al., 2001), which in turn increases the infiltration of water into soil (Lipiec et al., 2006). Epigeic earthworms also create burrows in the upper levels of soil, to a depth of 21cm (Francis and Fraser, 1998; Francis et al., 2001), and break down the layer of organic matter and leaf litter on the soil surface (Gundale, 2002; Hale, 2005), leading to changes in soil nutrient contents (Suthar, 2007). The removal of anecic and epigeic earthworms from the pasture soil ecosystem by flooding may therefore result in feedback loops where soils are more likely to remain saturated for longer following a flooding event.

As this chapter has discussed, there are many factors which affect the populations of earthworms in a pasture field. Chapter 3B will examine the effects of flooding, and the environmental changes it causes, on earthworm populations in an arable field.

3A.4 References

Al-Kayssi, A.W., Al-Karaghouli, A.A., Hasson, A.M., Beker, S.A. 1990. Influence of Soil Moisture Content on Soil Temperature and Heat Storage under Greenhouse Conditions. *Journal of Agricultural Engineering Research* 45: 141 – 252

Amossé, J., Turberg, P., Kohler-Milleret, R., Gobat, J-M., Le Bayon, R-C. 2015. Effects of Endogeic Earthworms on the Soil Organic Matter Dynamics and the Soil Structure in Urban and Alluvial Soil Materials. *Geoderma* 243: 50 – 57

Bartlett, M.S., Harris, J.A., James, I.T., Ritz, K. 2006. Inefficiency of Mustard Extraction Technique for Assessing Size and Structure of Earthworm Communities in UK Pasture. *Soil Biology and Biochemistry* 38(8): 2990 – 2992

Beare, M.H., Cabera, M.L., Hendrix, P.F., Coleman, D.C. 1994. Aggregate-Protected and Unprotected Organic Matter Pools in Conventional- and No-Tillage Soils. *Soil Science Society of America Journal* 58(3): 787 – 795

Blanchart, E., Albrecht, A., Alegre, J., Duboisset, A., Gilot, C., Pashanasi, B., Lavelle, P., Brussaard, L. 1999. Effects of Earthworms on Soil Structure and Physical Properties. *Earthworm Management in Tropical Agroecosystems* 5: 149 – 171

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., Brun, J. –J. 2013. A Review of Earthworm Impact of Soil Function and Ecosystem Services. *European Journal of Soil Science* 64(2): 161 – 182

Boag, B., Palmer, L.F., Neilson, R., Legg, R., Chambers, S.J. 1997. Distribution, Prevalence and Intensity of Earthworm Populations in Arable Land and Grassland in Scotland. *Annals of Applied Biology* 130(1): 153 – 165.

Bonkowski, M., Griffiths, B.S., Ritz, K. 2000. Food Preferences of Earthworms for Soil Fungi. *Pedobiologia* 44(6): 666 – 676

Bronick, C.J., Lal, R. 2005. Soil Structure and Management: A Review. *Geoderma* 124(1-2): 3 – 22

Brown, G.G., Barois, I., Lavelle, P. 2000. Regulation of Soil Organic Matter Dynamics and Microbial Activity in the Drilosphere and the Role of Interactions with other Edaphic Functional Domains. *European Journal of Soil Biology* 36(3 – 4): 177 – 198

Butt, K.R., Briones, M.J.I. 2017. Earthworms and Mesofauna from an Isolated, Alkaline Chemical Waste Site in Northwest England. *European Journal of Soil Biology* 78: 43 – 49

Carter, M.R. 2002. Soil Quality for Sustainable Land Management. *Agronomy Journal* 94(1): 38 – 47

Clements, R.O., Murray, P.J., Sturdy, R.G. 1991. The Impact of 20 Years' Absence of Earthworms and Three Levels of N Fertilizer on a Grassland Soil Environment. Agriculture, *Ecosystems & Environment* 36(1-2): 75 – 85

Čoja, T., Zehetner, K., Bruckner, A., Watzinger, A., Meyer, E. 2008. Efficacy and Side Effects of Five Sampling Methods for Soil Earthworms (Annelida, Lumbricidae). *Ecotoxicology and Environmental Safety* 71(2): 552 – 565

Conyers, M.K., Uren, N.C., Helyar, K.R. 1995. Causes of Changes in pH in Acidic Mineral Soils. *Soil Biology and Biochemistry* 27(11): 1383 – 1392

Conyers, M.K., Uren, N.C., Helyar, K.R., Poile, G.J., Cullis, B.R. 1997. Temporal Variation in Soil Acidity. *Australian Journal of Soil Research* 35(5): 1115 – 1130

Curry, J.P., Byrne, D., Schmidt, O. 2002. Intensive Cultivation can Drastically Reduce Earthworm Populations in Arable Land. *European Journal of Soil Biology* 38(2): 127 – 130.

Curry, J.P., Schmidt, O. 2007. The Feeding Ecology of Earthworms – A Review. *Pedobiologia* 50(6): 463 – 477

Edwards, C.A., Lofty, J.R. 1977. Biology of Earthworms (2nd Edition). London: Chapman and Hall.

Eijsackers, H., Bruggeman, J., Harmsen, J., de Kort, Th., Schakel, A. 2009. Colonisation of PAH-Contaminated Dredged Sediment by Earthworms. *Applied Soil Ecology* 43: 216 – 225

Eisenhauer, N., Straube, D., Scheu, S. 2008. Efficiency of Two Widespread Non-Destructive Extraction Methods under Dry Soil Conditions for Different Ecological Earthworm Groups. *European Journal of Soil Biology* 44(1): 141 – 145

Ester, A., van Rozen, K. 2002. Earthworms (*Aporrectodea* spp.; Lumbricidae) Cause Soil Structure Problems in Young Dutch Polders. *European Journal of Soil Biology* 38(2): 181 – 185

Evans, A.C., Guild, W.J. 1948a. Studies on the Relationships between Earthworms and Soil Fertility. *Annals of Applied Biology* 35(4): 471 – 484.

Evans, A.C., Guild, W.J. 1948b. Studies on the relationships between earthworms and soil fertility. IV. On the life cycles of some British Lumbricidae. *Annals of Applied Biology* 35, 473-493

Francis, G.S., Fraser, P.M. 1998. The Effects of Three Earthworm Species of Macroporosity and Hydraulic Conductivity. *Applied Soil Ecology* 10(1-2): 11 – 19

Francis, G.S., Tabley, F.J., Butler, R.C., Fraser, P.M. 2001. The Burrowing Characteristics of Three Common Earthworm Species. *Australian Journal of Soil Research* 39(6): 1453 – 1465

Franzluebbers, A.J., Arshad, M.A. 1996. Water-Stable Aggregation and Organic Matter in Four Soils under Conventional and Zero Tillage. *Canadian Journal of Soil Science* 76(3): 387 – 393

Gerard, B.M. 1967. Factors Affecting Earthworms in Pastures. *Journal of Animal Ecology* 36(1): 235 – 252

Görres, J.H., Savin, M.C., Amador, J.A. 2001. Soil Micropore Structure and Carbon Mineralisation in Burrows and Casts of an Anecic Earthworm (*Lumbricus terrestris*). *Soil Biology and Biochemistry* 33(14): 1881 - 1887

Gutiérrez-López, M., Jesús, J.B., Trigo, D., Fernández, R., Novo, M., Díaz-Cosín, D.J. 2010. Relationships among Spatial Distribution of Soil Microarthropods, Earthworm Species and Soil Properties. *Pedobiologia* 53(6): 381 – 389

Gundale, M.J. 2002. Influence of Exotic Earthworms on the Soil Organic Horizon and the Rare Fern *Botrychium mormo*. *Conversation Biology* 16(6): 1555 – 1561

Hale, C.M., Frelich, L.E., Reich, P.B., Pastor, J. 2005. Effects of European Earthworm Invasion on Soil Characteristics in Northern Hardwood Forests of Minnesota, USA. *Ecosystems* 8(8): 911 – 927

Ivask, M., Truu, J., Kuu, A., Truu, M., Leito, A, 2007. Earthworm Communities of Flooded Grasslands in Matsalu, Estonia. *European Journal of Soil Biology* 43(2): 71 – 76

Lal, R. 2004. Agricultural Activities and the Global Carbon Cycle. *Nutrient Cycling in Agroecosystems* 70(2): 103 – 116

Lavelle, P. 1988. Earthworm Activities and the Soil System. *Biology and Fertility of Soils* 6(3): 237 – 251

Lee, K.E., Foster, R.C. 1991. Soil Fauna and Soil Structure. *Australian Journal of Soil Research* 29(6): 745 – 775

Lipiec, J., Kuś, J., Słowińska-Jurkiewicz, A., Nosalewicz, A. 2006. Soil Porosity and Water Infiltration as Influenced by Tillage Methods. *Soil and Tillage Research* 89(2): 210 – 220

Lowe, C.N., Butt, K.R. 2005. Culture Techniques for Soil Dwelling Earthworms: A Review. *Pedobiologia* 49(5): 401 – 413.

Lowe, C.N., Butt, K.R. 2007. Life-Cycle Traits of the Dimorphic Earthworm Species *Allolobophora chlorotica* (Savigny, 1826) Under Controlled Laboratory Conditions. *Biology and Fertility of Soils* 43(4): 495 – 499

Met Office, 2017. Statistics for April 2017. Available at: https://www.metoffice.gov.uk/news/releases/2017/statistics-for-april-2017 [accessed 15/08/2018].

Morgan, A.J., Winters, C. 1990. Diapause in the Earthworm, *Apporectodea longa*: Morphological and Quantitative X-Ray Microanalysis of Cryosectioned Chloragogenous Tissue. *Scanning Microscopy* 5(1): 219 – 227

Natural England, 2014. Earthworms in England: Distribution, Abundance and Habitats (NECR145)

Nordström, S. 1975. Seasonal Activity of Lumbricids in Southern Sweden. *Oikos* 26(3): 307 – 315

Pelosi, C., Bertrand, M., Capowiez, Y., Boizard, H., Roger-Estrade, J. 2009. Earthworm Collection from Agricultural Fields: Comparisons of Selected Expellants in Presence/Absence of Hand-Sorting. *European Journal of Soil Biology* 45(2): 176 – 183

Pérès, G., Cluzeau, D., Curmi, P., Hallaire, V. 1998. Earthworm Activity and Soil Structure Changes due to Organic Enrichments in Vineyard Systems. *Biology and Fertility of Soils* 27(4): 417 – 424

Plum, N.M. 2005. Terrestrial Invertebrates in Flooded Grassland: A Literature Review. *Wetlands* 25(3): 721-737

Plum, N.M., Filser, J. 2005. Floods and Drought: Response of Earthworms and Potworms (Oligochaeta: Lumbricidae, Enchytraeidae) to Hydrological Extremes in Wet Grassland. *Pedobiologia* 49(5): 443-453

R Core Team 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rawls, W.J., Pachepsky, Y.A., Ritchie, J.C., Sobecki, T.M., Bloodworth, H. 2003. Effect of Soil Organic Carbon on Soil Water Retention. *Geoderma* 116(1-2): 61 – 76

Reddy, K.R., Patrick Jr, W.H. 1975. Effect of Alternate Aerobic and Anaerobic Conditions on Redox Potential, Organic Matter Decomposition and Nitrogen Loss in a Flooded Soil. *Soil Biology and Biochemistry* 7(2); 87-94

Ritchie, G.S.P., Dolling, P.J. 1985. The Role of Organic Matter in Soil Acidification. *Soil Research* 23(4): 569 – 576

Rossi, J.P. 2003. The Spatiotemporal Pattern of a Tropical Earthworm Species Assemblage and its Relationship with Soil Structure: The 7th International Symposium on Earthworm Ecology – Cardiff – Wales 2002. *Pedobiologia* 47(5-6): 497 – 503

Satchell, J.E. 1967. Colour Dimorphism in *Allolobophora chlorotica* Sav. (Lumbricidae). *Journal of Animal Ecology* 36(3): 623 – 630.

Schmidt, O., Scrimgeour, C.M., Handley, L.L. 1997. Natural Abundance of ^{15}N and ^{13}C in Earthworms from a Wheat and Wheat-Clover Field. *Soil Biology and Biochemistry* 29(9-10): 1301 – 1308

Six, J., Guggenberger, G., Paustian, K., Haumaier, L., Elliott, E.T., Zech, W. 2001. Source and Composition of Soil Organic Matter Fractions Between and Within Soil Aggregates. *European Journal of Soil Science* 52(4): 607 – 618

Suthar, S. 2007. Nutrient Changes and Biodynamics of Epigeic Earthworm *Perionyx excavatus* (Perrier) During Recycling of Some Agriculture Wastes. *Bioresource Technology* 98(8): 1608 – 1614

Tomlin, A.D., Miller, J.J. 1988. Impact of Ring-Billed Gull (*Larus delawarensis* Ord.) Foraging on Earthworm Populations of Southwestern Ontario Agricultural Soils. *Agriculture, Ecosystems & Environment* 20(3): 165 – 173.

van Schaik, L., Palm, J., Klaus, J., Zehe, E., Schröder, B. 2013. Linking Spatial Earthworm Distribution to Macropore Numbers and Hydrological Effectiveness. *Ecohydrology*. 7(2): 401 – 408

Wokinghamweather.co.uk [website]. Accessed online 02/09/19. Available from http://www.wokinghamweather.co.uk/weather/historicweatherdata.htm

Woods, L.E. 1989. Active Organic Matter Distribution in the Surface 15cm of Undisturbed and Cultivated Soil. *Biology and Fertility of Soils* 8(3): 271 – 278

Zaborski, E.R. 2003. Allyl Isothiocyanate: An Alternative Chemical Expellant for Sampling Earthworms. *Applied Soil Ecology* 22(1): 87 – 95

Zhang, Z. 2016. Variable Selection with Stepwise and Best Subset Approaches. *Annals of Translational Medicine* 4(7): 136

Zhang, R., Wienhold, B.J. 2002. The Effect of Soil Moisture on Mineral Nitrogen, Soil Electrical Conductivity, and pH. *Nutrient Cycling in Agroecosystems* 63(2 – 3): 251 – 254

Zorn, M.I., Van Gestel, C.A.M., Eijsackers, H. 2005. Species-Specific Earthworm Population Responses in Relation to Flooding Dynamics in a Dutch Floodplain Soil. *Pedobiologia* 49(3): 189 – 198

3B. The long-term fluctuations of earthworm populations in an arable field with differing localised flooding effects

3B.1 Results

3B.1.1 Environmental factors across site and time

Spearman’s Rank correlations were performed to determine how soil environmental factors across all sampling timepoints and sites related to each other across the whole dataset and within the land use categories of crop, margin, and riverbank (Table 3B-1). The site variable combined the flooding regime classification (rarely, occasionally, and regularly flooded) with the location within the arable field (crop, margin, or riverbank). The riverbanks, while regularly flooded, were considered separately to the crop and margin sites due to differences in the soil uses discussed in Section 3B.3.1. Positive correlations were observed between soil moisture and: bulk density, percent carbon, and percent nitrogen, and between percent carbon and percent nitrogen. Negative correlations were observed between soil temperature and soil moisture and between bulk density and both percent carbon and percent nitrogen. Within the crop, temperature correlated positively with percent nitrogen. Bulk density was negatively correlated with soil moisture, pH, percent carbon and percent nitrogen. Soil moisture, percent carbon, percent nitrogen and pH were all positively correlated with each other. Within the margin, soil moisture negatively correlated with temperature, but positively correlated with the percent nitrogen. Bulk density negatively correlated with soil moisture, percent carbon and percent nitrogen. Positive correlations were observed between soil moisture and both pH and percent carbon. pH positively correlated with percent carbon, and percent carbon positively correlated with percent nitrogen. Within the river bank sites, temperature negatively correlated with soil moisture. Bulk density negatively correlated with soil moisture, percent carbon and percent nitrogen. Soil moisture positively correlated with percent carbon and percent nitrogen. Percent carbon positively correlated with percent nitrogen.

Table 3B-1. Spearman’s Rank rho (ρ) values of correlations between soil environmental factors within the whole dataset and when the dataset is divided into land use categories. Shaded cells indicate a statistically significant correlation of *P* < 0.01. Darker shaded cells indicate a statistically significant correlation of *P* < 0.05.

Whole dataset	**Bulk density**	**Soil moisture**	**pH**	**Percent carbon**	**Percent nitrogen**
Temperature	ρ = -0.05	ρ = - 0.23	ρ = - 0.07	ρ = 0.09	ρ = 0.56
Bulk density	-	ρ = - 0.68	ρ = -0.33	ρ = - 0.78	ρ = - 0.59
Soil moisture	-	-	ρ = 0.63	ρ = 0.68	ρ = 0.44
pH	-	-	-	ρ = 0.51	ρ = 0.36
Percent carbon	-	-	-	-	ρ = 0.79

Crop	**Bulk density**	**Soil moisture**	**pH**	**Percent carbon**	**Percent nitrogen**
Temperature	ρ = - 0.13	ρ = - 0.22	ρ = - 0.09	ρ = 0.13	ρ = 0.37
Bulk density	-	ρ = - 0.82	ρ = - 0.53	ρ = - 0.80	ρ = - 0.58
Soil moisture	-	-	ρ = 0.56	ρ = 0.70	ρ = 0.36
pH	-	-	-	ρ = 0.52	ρ = 0.33
Percent carbon	-	-	-	-	ρ = 0.61

Margin	**Bulk density**	**Soil moisture**	**pH**	**Percent carbon**	**Percent nitrogen**
Temperature	ρ = 0.13	ρ = - 0.32	ρ = - 0.06	ρ = - 0.06	ρ = 0.30
Bulk density	-	ρ = - 0.51	ρ = - 0.13	ρ = - 0.64	ρ = - 0.38
Soil moisture	-	-	ρ = 0.63	ρ = 0.55	ρ = 0.14
pH	-	-	-	ρ = 0.40	ρ = 0.19
Percent carbon	-	-	-	-	ρ = 0.72

Riverbank	**Bulk density**	**Soil moisture**	**pH**	**Percent carbon**	**Percent nitrogen**
Temperature	ρ = - 0.07	ρ = - 0.36	ρ = - 0.20	ρ = 0.16	ρ = - 0.01
Bulk density	-	ρ = - 0.49	ρ = 0.15	ρ = - 0.38	ρ = - 0.40
Soil moisture	-	-	ρ = 0.29	ρ = 0.53	ρ = 0.59
pH	-	-	-	ρ = 0.25	ρ = 0.23
Percent carbon	-	-	-	-	ρ = 0.93

Two way ANOVAs were performed to determine the effects of the time of year and the site on a number of soil environmental factors: the mean of the temperature at 5 and 10 cm, bulk density, soil moisture percentage, pH, percent carbon and percent nitrogen. A significant effect of site, time, and an interaction term was observed for all environmental factors except pH, which had no significant interaction term, and percent carbon, which had no effect of time point. *P* values are summarised in Table 3B-2.

Table 3B-2. The outcomes of two way ANOVAs performed to determine the differences between a number of soil environmental factors across differing sampling sites and time within the Swale arable site (n=192).

Soil environmental factor	Variable	*P* value (DF, F)
Mean temperature (°C)	Timepoint	< 0.0001 (3, 8540.02)
	Site	< 0.0001 (7, 28.71)
	Interaction term	< 0.0001 (21, 42.39)
Bulk density (g cm^{-3})	Timepoint	0.0003 (3, 6.593)
	Site	< 0.0001 (7, 20.273)
	Interaction term	0.0006 (21, 2.07),
Soil moisture percentage	Timepoint	< 0.0001 (3, 45.204)
	Site	< 0.0001 (7, 28.383)
	Interaction term	< 0.0001 (21, 4.641)
pH	Timepoint	0.005 (3, 4.43)
	Site	< 0.0001 (7, 75.603)
	Interaction term	0.066 (21, 1.559)
Percent carbon	Timepoint	0.554 (3, 0.698)
	Site	< 0.0001 (7, 57.91)
	Interaction term	0.013 (21, 1.92)
Percent nitrogen	Timepoint	< 0.0001 (3, 13.98)
	Site	< 0.0001 (7, 30.17)
	Interaction term	< 0.0001 (21, 4.93)

Following the two way ANOVAs, Tukey post-hoc tests were performed to determine where the significant differences lay within the interaction between sampling timepoint and site. Soil temperature (Figure 3B-1) was significantly higher in July 2017 than in October 2017, which was significantly higher than April 2017, which was in turn significantly higher than January 2018. There were no overlaps of temperature across the four sampling timepoints. Within each sampling timepoint, July 2017 and April 2017 showed a trend of soil temperatures increasing with proximity to the riverbank sites, with the riverbank sites significantly higher in temperature than the rarely flooded crop. January 2018 showed the opposite pattern, with temperatures decreasing as proximity to the river increased, with the riverbank sites significantly lower in soil temperature than the rarely flooded crop and margin sites. Soil bulk density (Figure 3B-2) was significantly higher in the rarely flooded crop and margin sites than in the frequently flooded, riverbank sites, with the exception of the river side riverbank site in April 2017. Generally there was a trend of reduced bulk density with increased flooding frequency, and in the regularly flooded regions bulk density was generally lower in the October 2017 and January 2018 sampling timepoints than in April and July 2017. Soil moisture percentage (Figure 3B-3) showed a general trend of increasing with increased flooding frequency, and was generally lower in the April and July 2017 samplings than in October 2017 and January 2018. The soil moisture in the rarely flooded crop and margin and occasionally flooded crop sites was significantly lower in April 2017 than in all sampling timepoints in the regularly flooded margin, and field and river side riverbank sites. Soil pH (Figure 3B-4) showed no interaction term between the site and sampling timepoint, but was significantly higher in January 2018 than in April 2017. Soil pH was not significantly different between the regularly flooded margin and the riverbank sites, but was significantly different between all other sites, with a general trend of a significantly lower pH in the margin than in the crop. In the rarely flooded sites, the soils were slightly acidic, with the margin sites more acidic than the crop sites. Both crop and margin sites increased towards neutrality with increased flooding. Percent carbon (Figure 3B-5) was significantly higher in the regularly flooded and riverbank sites than the rarely flooded crop across all sampling timepoints. In the occasionally and regularly flooded crop, soil carbon increased in October 2017 and January 2018, with the soil carbon content in the occasionally flooded crop significantly higher in October 2017 than in April 2017. There was a general trend of carbon content of the soil increasing with increased flooding frequency for both percent carbon and percent nitrogen. Percent nitrogen (Figure 3B-6) was significantly higher in the river side riverbank site than in the rarely flooded crop and margin sites for all timepoints except July 2017. In July 2017 the soil percent nitrogen content was significantly higher in the rarely flooded crop than the other sampling timepoints, and was higher than April 2017, October 2017, and January 2018 in the rarely flooded margin, the occasionally flooded crop, and the occasionally flooded margin.

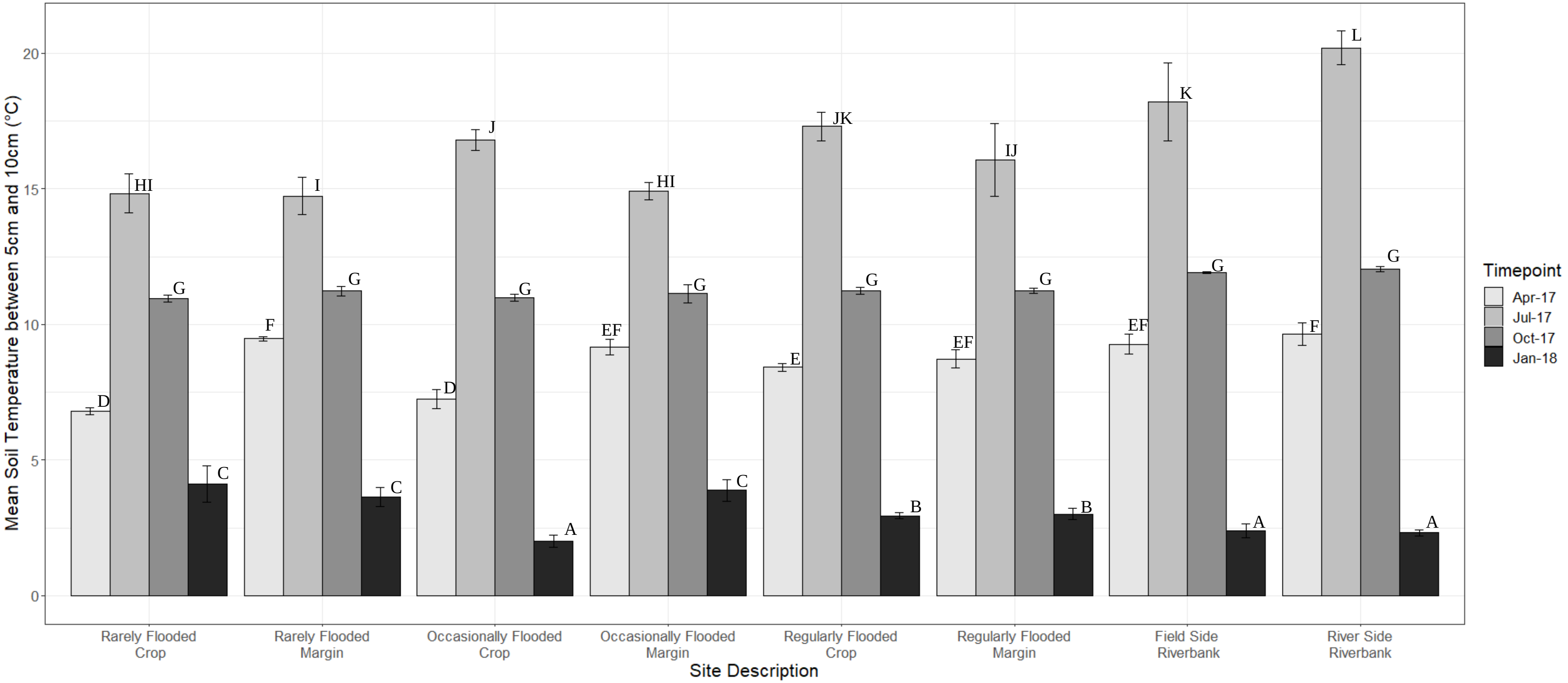

Figure 3B-1. The mean soil temperature between depths of 5 cm and 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192). Bars marked with the same letter are not significantly different (*P* < 0.05).

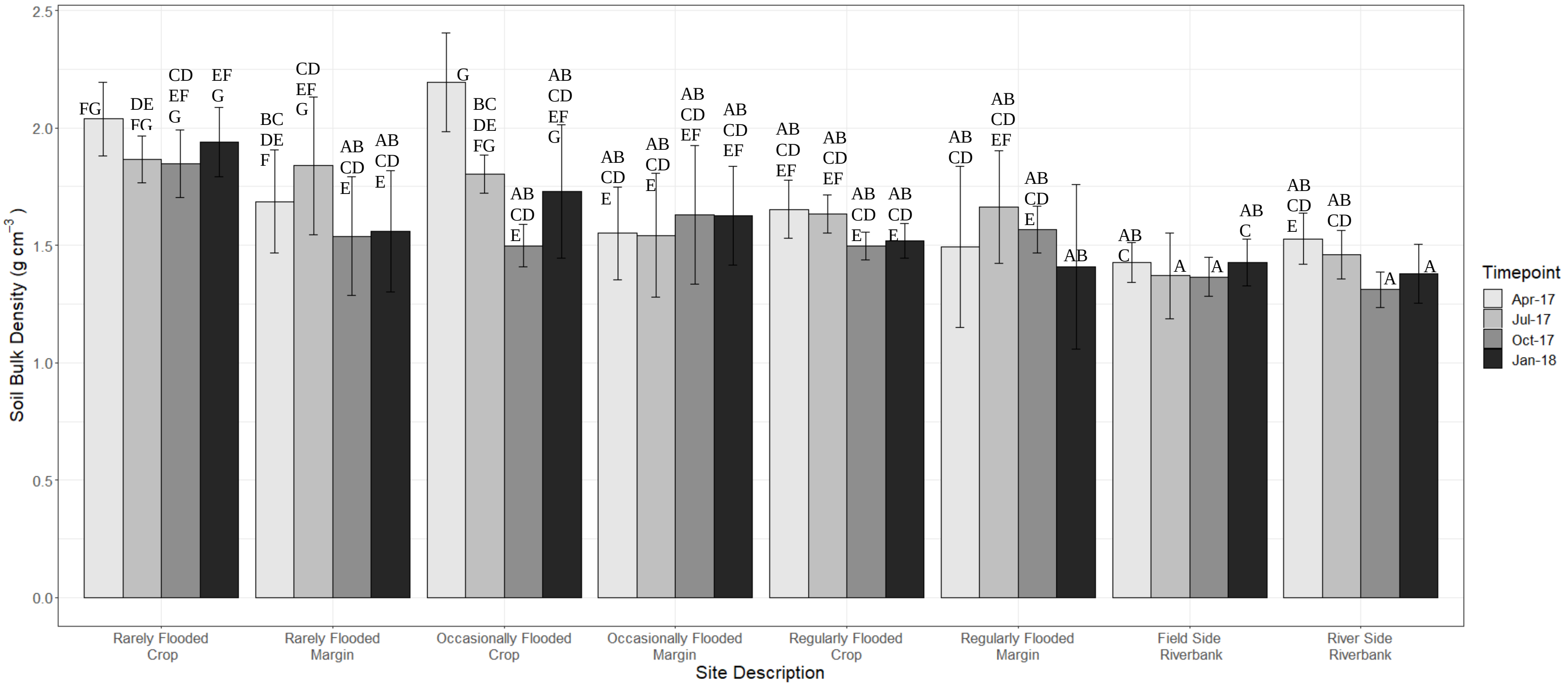

Figure 3B-2. The mean soil bulk density (g cm^{-3}) at a depth of approximately 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192). Bars marked with the same letter are not significantly different (*P* < 0.05).

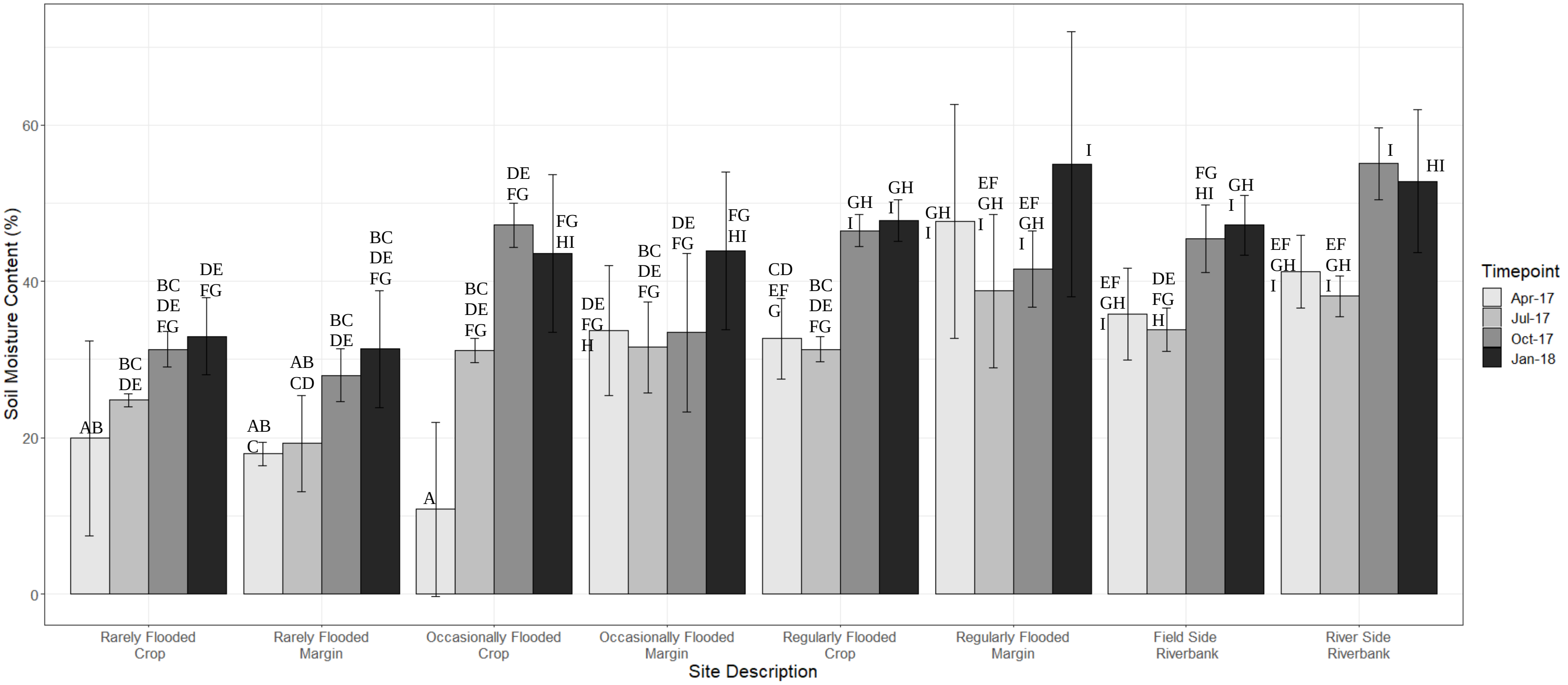

Figure 3B-3. The mean soil moisture content (%) at a depth of approximately 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192). Bars marked with the same letter are not significantly different (*P* < 0.05).

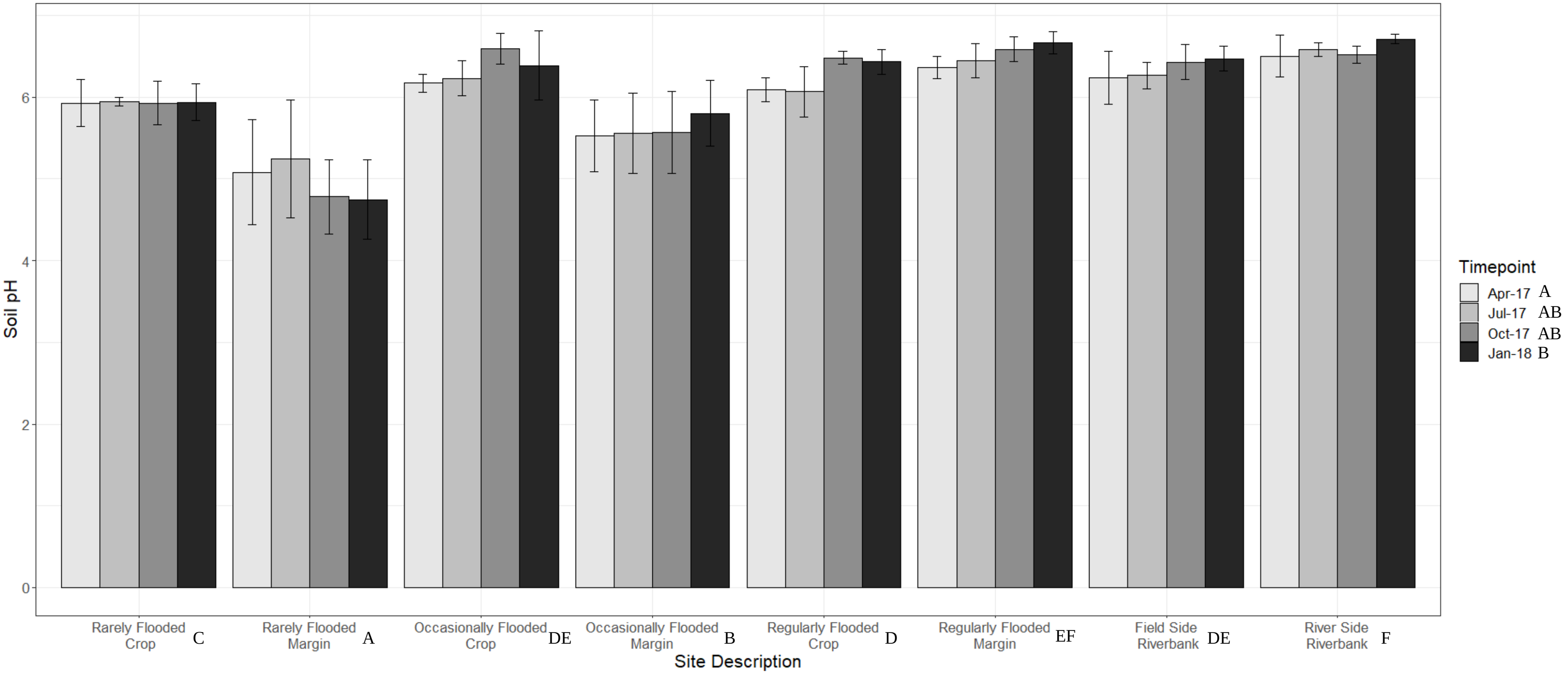

Figure 3B-4. The mean soil pH at a depth of approximately 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192). X axis site descriptions marked with the same letter show no significant difference between sites across all timepoints (*P* < 0.05). Legend labels marked with the same letter are not significantly different across all sites (*P* < 0.05).

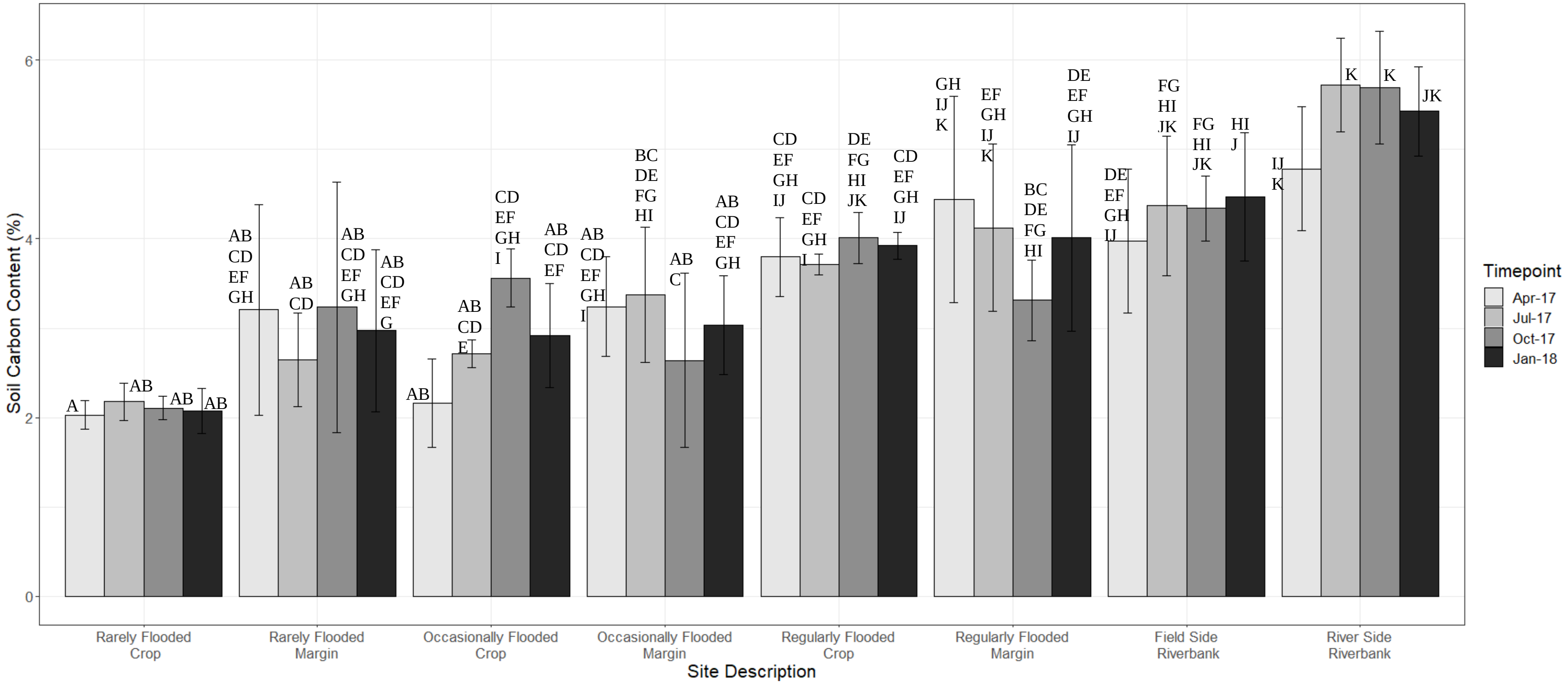

Figure 3B-5. The mean soil carbon content (%) at a depth of approximately 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192). Bars marked with the same letter are not significantly different (*P* < 0.05).

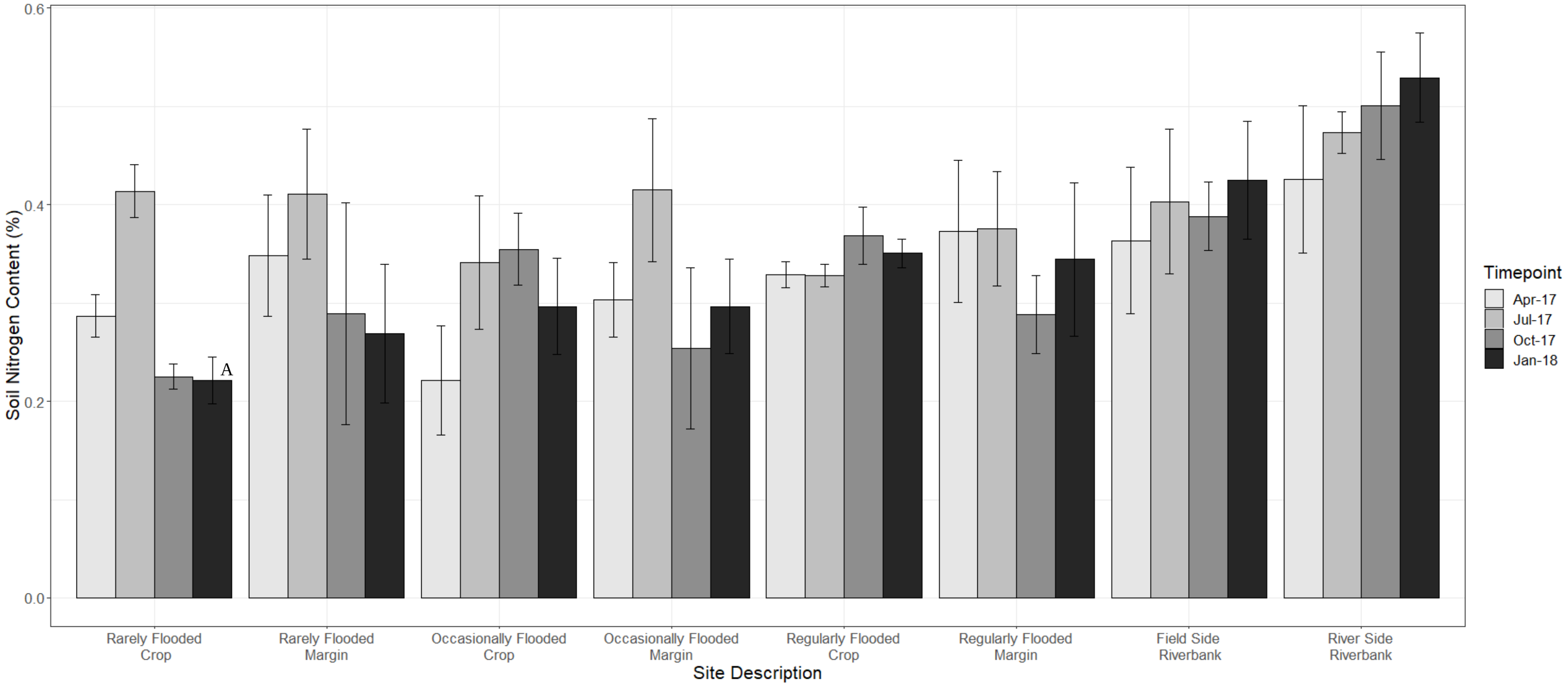

Figure 3B-6. The mean soil nitrogen content (%) at a depth of approximately 10 cm for the different sites over the four sampling timepoints, with error bars showing standard deviation (n = 192) Bars marked with the same letter are not significantly different (*P* < 0.05).

3B.1.2 Population metrics across site and time

A GLM and two way ANOVAs were performed to determine the effects of the time of year and the flooding regime on three measures of population: earthworm abundance m^{-2}, the total biomass (g m^{-2}), and the Shannon diversity index values. A significant effect of site and time was observed for all three population metrics at the level of $P < 0.05$, with the addition of a significant interaction term for earthworm abundance and Shannon diversity index ($P < 0.05$). *P* values are summarised in Table 3B-3.

Table 3B-3. The outcomes of the GLM and two way ANOVAs performed to determine the differences between a number of population metrics across differing flooding regimes and time within the Swale arable site (n=192).

Earthworm population metric	**Variable**	***P* value (DF, F F/X^2)**
Total abundance (indm^{-2})	Timepoint	< 0.0001 (3, 29.71)
(untransformed)	Site	< 0.0001 (7, 88.22)
	Interaction Term	0.020 (21, 36.02)
Total biomass (g m^{-2})	Timepoint	< 0.0001 (3, 9.37)
(square root transformed)	Site	0.0001 (7, 4.49)
	Interaction Term	0.070 (21, 1.54)
Shannon diversity index	Timepoint	0.026 (3, 3.18)
(log10, +1 transformed)	Site	< 0.0001 (7, 7.36)
	Interaction Term	0.012 (21, 1.86)

Following the two way ANOVAs, Tukey post-hoc testing was performed to determine where the significant differences lay within the significant interaction terms.

The total earthworm abundance (Figure 3B-7) was significantly higher in the regularly flooded margin in April 2017 than in any sampling timepoint of the rarely flooded margin, the regularly flooded crop, and in the riverbank sites. The total biomass (Figure 3B-8) did not show a significant interaction term of sampling timepoint and site. The total biomass of earthworms was significantly higher in April 2017 than at the other sampling timepoints, and was significantly lower in the regularly flooded crop than in the regularly and occasionally flooded margins and the rarely flooded crop. For both total earthworm abundance and biomass, there was a general trend that the difference between the crop and margin sites increased with flooding frequency. Earthworm Shannon diversity was highly variable (Figure 3B-9). Post hoc testing showed that broadly there were no significant differences between the flooding frequencies, soil uses, and sampling timepoints. However, the diversity values for the occasionally flooded margin in January 2017 was significantly higher than the diversity in the regularly flooded crop in July 2017.

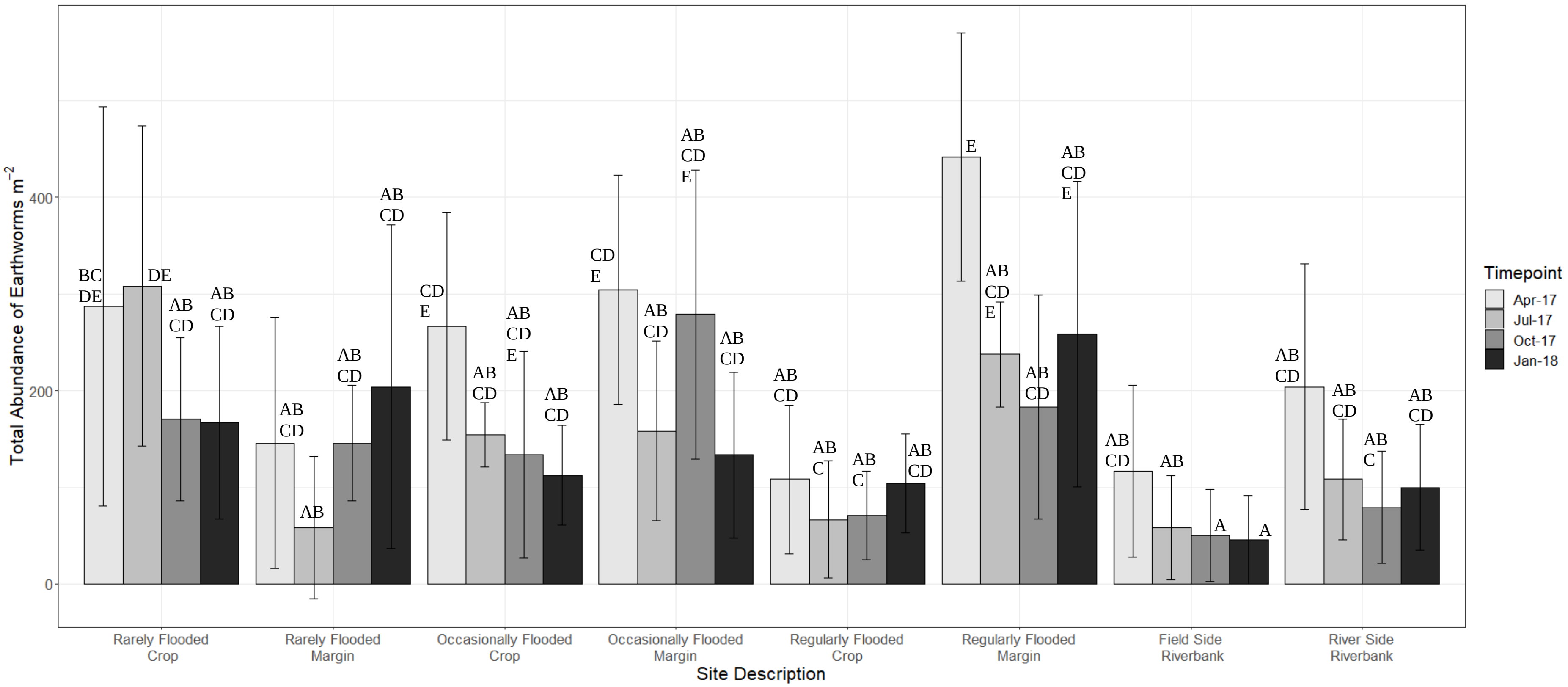

Figure 3B-7. The mean total abundance of earthworms m^{-2} for the different sites over the four sampling timepoints, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

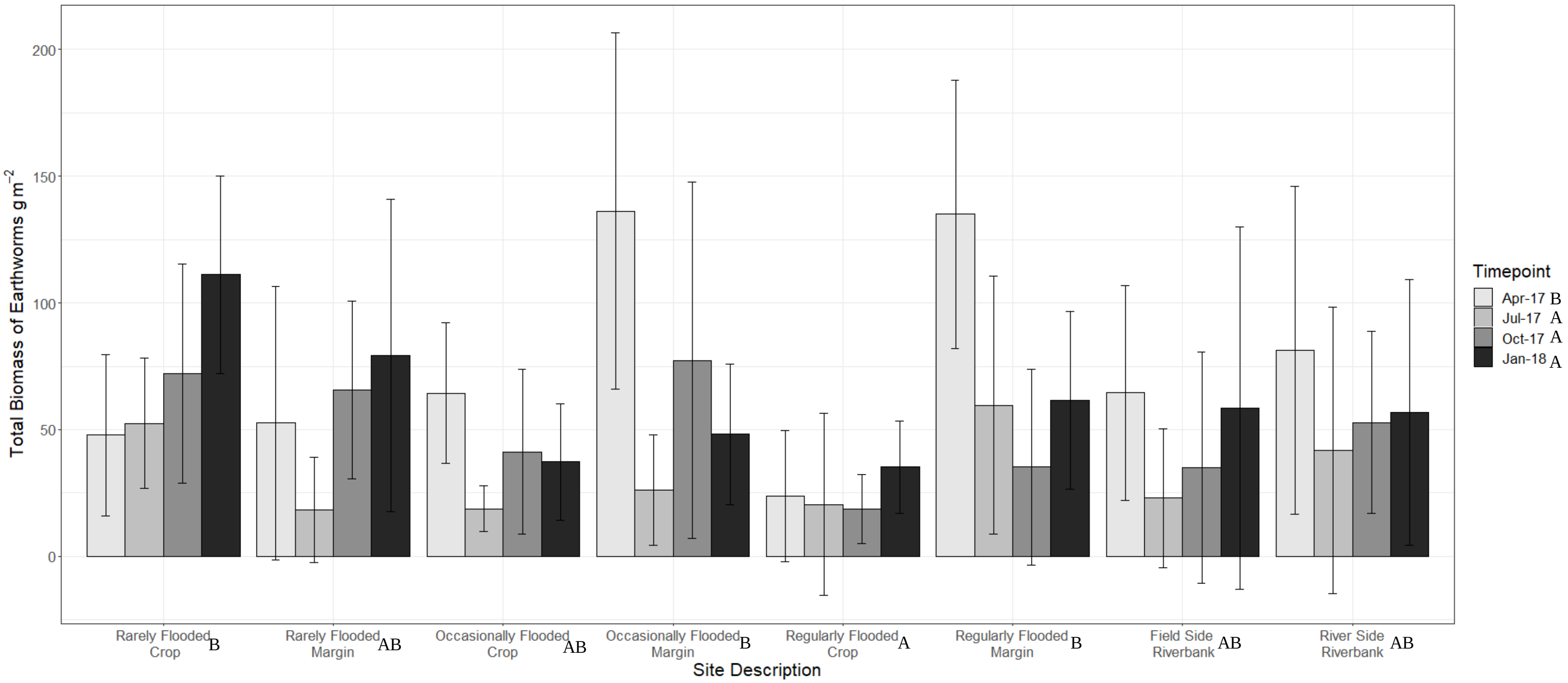

Figure 3B-8. The mean total biomass of earthworms (g m^{-2}) for the different sites over the four sampling timepoints, with error bars showing standard deviation. X axis site descriptions marked with the same letter show no significant difference between sites across all timepoints (*P* < 0.05). Legend labels marked with the same letter are not significantly different across all sites (*P* < 0.05).

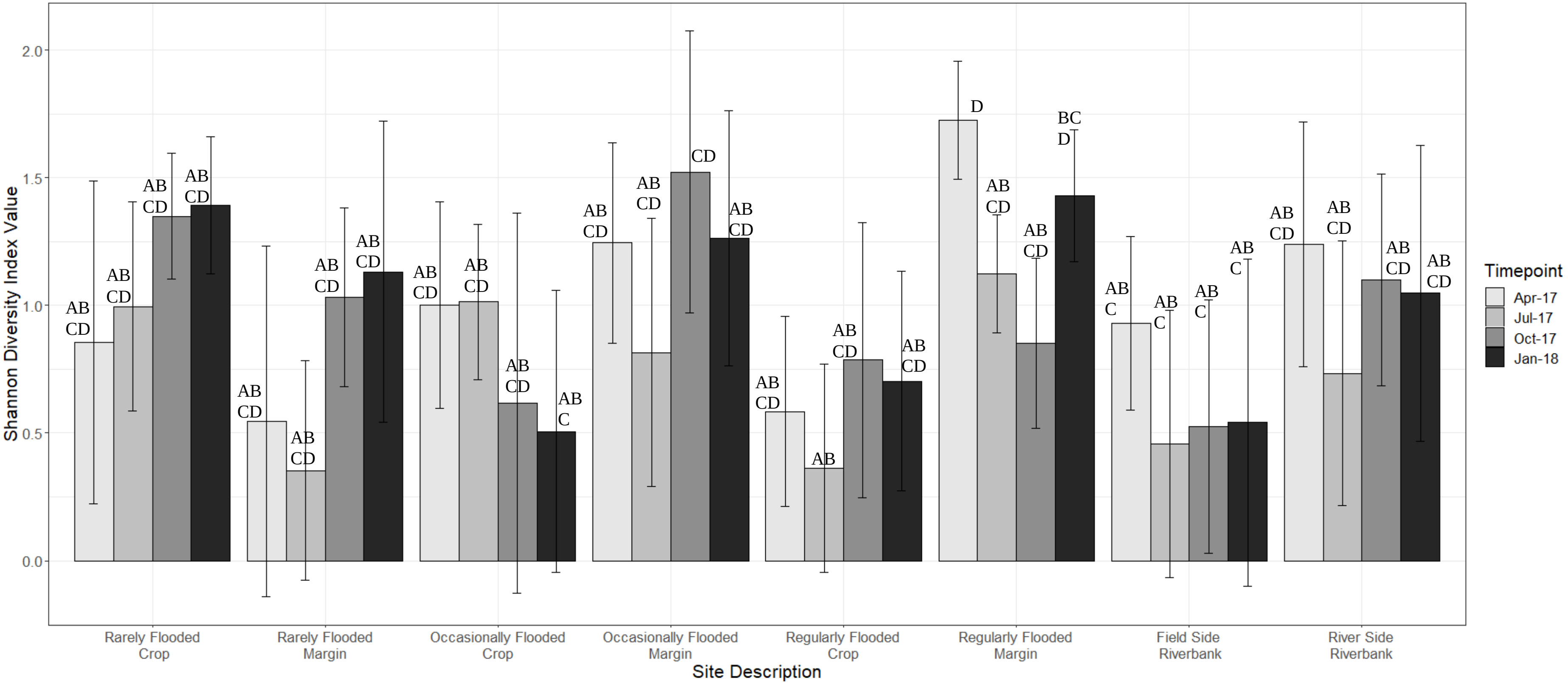

Figure 3B-9. The mean Shannon Diversity Index values of earthworms per pit for the different sites over the four sampling timepoints, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

3B.1.3 Population structure at the ecotype level across sites

Figure 3B-10 shows the relative proportions of the different earthworm ecotypes by the number of adult or juvenile individuals of the anecic, endogeic or epigeic ecotypes for each site at the different sampling time points. GLMs with a Quasipoisson link indicated that the proportion of all categories of adult and juvenile anecic, endogeic, and epigeic earthworms differed significantly between each site ($P < 0.01$). There was an effect of the timepoint for all earthworm ecotypes except juvenile epigeics ($P < 0.05$). Interaction terms were significant for adult anecics, adult endogeics, and adult epigeics and juvenile epigeics ($P < 0.05$). The P values for the significance of the timepoint, site, and interaction term are summarised in Table 3B-4.

Table 3B-4. The outcomes GLMS performed to determine how the proportions of the population composed of adult or juvenile individuals of the anecic, endogeic, or epigeic ecotypes differed across the sampling timepoints and sampling sites.

Ecotype and age of individual (n)	Variable	*P* value (DF, LR Chi sq))
Adult anecics (n = 27)	Timepoint	0.0002 (3, 119.354)
	Site	< 0.0001 (7, 42.844)
	Interaction term	0.0388 (21, 33.720)
Juvenile anecics (n = 89)	Timepoint	0.0308 (3, 8.888)
	Site	< 0.0001 (7, 32.848)
	Interaction term	0.3617 (21, 22.673)
Adult endogeics (n = 257)	Timepoint	< 0.0001 (3, 64.740)
	Site	< 0.0001 (7, 59.826)
	Interaction term	0.0069 (21, 40.278)
Juvenile endogeics (n = 749)	Timepoint	< 0.0001 (3, 34.375)
	Site	0.0394 (7, 14.746)
	Interaction term	0.3307 (21, 120252)
Adult epigeics (n = 39)	Timepoint	< 0.0001 (3, 37.174)
	Site	< 0.0001 (7, 95.523)
	Interaction term	< 0.0001 (21, 77.173)
Juvenile epigeics (n = 102)	Timepoint	0.0678 (3, 7.133)
	Site	< 0.0001 (7, 35.596)
	Interaction term	0.0143 (21, 37.622)

Tukey post hoc testing did not indicate significant differences based on the interaction terms for any of the earthworm ecotypes which indicated a significant interaction term between the sites and timepoints. Likewise, Tukey post hoc testing suggested that there was no significant difference between the timepoints or sites for juvenile anecics, while juvenile endogeics only indicated a significant difference between the timepoint, with no significant effect of site.

Juvenile endogeics represented a significantly smaller proportion of the community in January 2018 than in April and July 2017 (P = 0.05), while there was no significant difference in the percent of the community between October 2017 and January 2018, and between October 2017 and April 2017

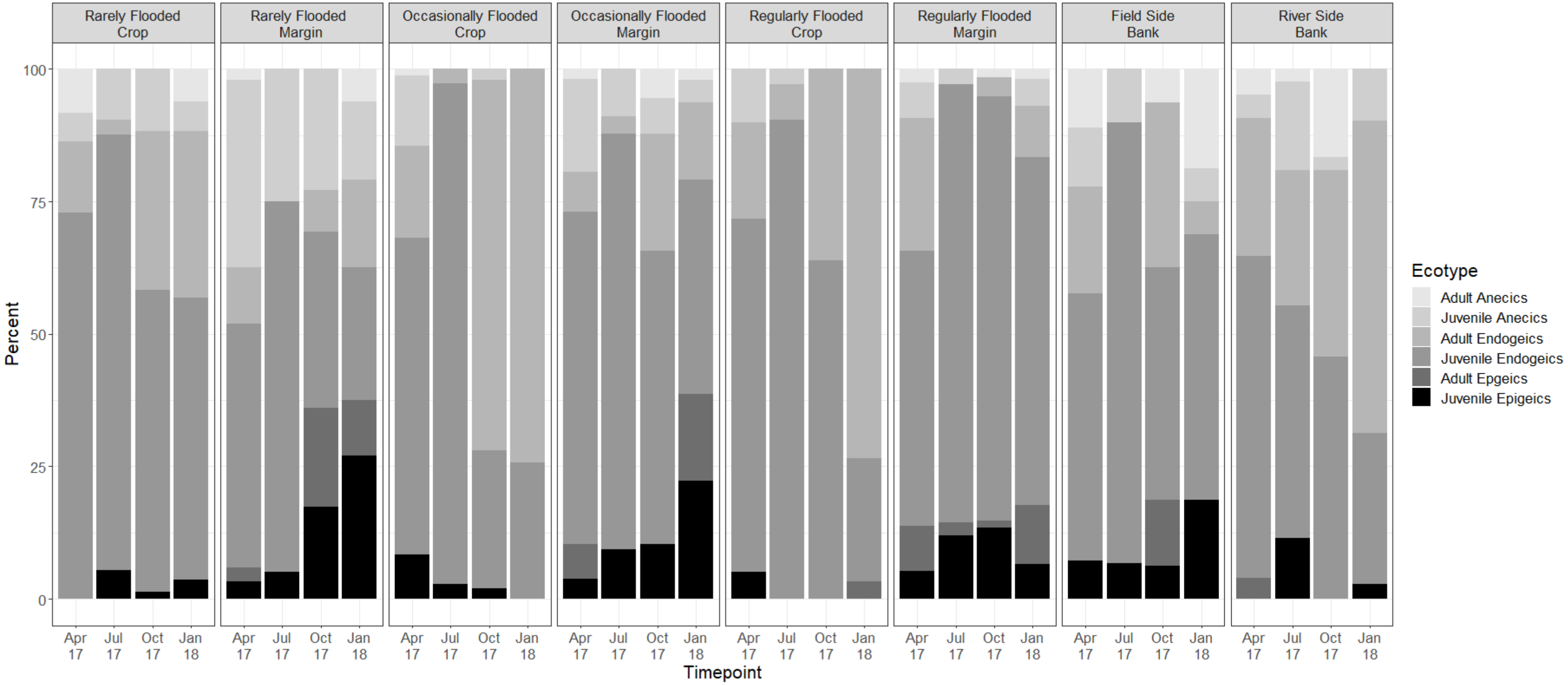

Figure 3B-10. The mean proportions of the population represented by adults and juveniles of the three earthworm ecotypes determined from six sampling pits between the eight sites and four sampling timepoints in the Swale floodplain

3B.1.4 Differences in abundance and mass of species across sites

Adults of seven earthworm species were present across multiple sites and timepoints: *A caliginosa*, *A. chlorotica*, *A. rosea*, *L. castaneus*, *L. rubellus*, *A. longa* and *L. terrestris*. As overall abundances were low for several species, statistical testing was only performed for individuals of *A. chlorotica* (n = 156) and *A. caliginosa* (n = 74), with all other species present at n < 26.

To determine if the abundance of adult earthworms of independent species differed across the sites within the field and the timepoints sampled, GLMs with a Poisson link were performed. The only species to show a significant response was *A. chlorotica*, which significantly differed in abundance between sites (*P* = 0.041). Post hoc testing showed that the abundance of individuals of *A. chlorotica* was significantly higher in the occasionally flooded crop site than in all other sites (*P* < 0.05).

In order to determine if the mass of individual adult earthworms of the seven species differed across the sites (Table 3B-5), two way ANOVAs were performed with the sampling site and the time of sampling. To achieve normality, the mass of individuals of *A. chlorotica* was log transformed.

The biomass of adult individuals of *A. caliginosa* was significantly different between sampling timepoints (*P* = 0.012). Individuals of *A. caliginosa* were significantly higher in biomass in October 2017 and January 2018 than in July 2017, with no significant difference between April 2017 and the other sampling timepoints.

The biomass of adult individuals of *A. chlorotica* significantly differed between timepoints (*P* < 0.0001), with individuals significantly higher in biomass in October 2017 and January 2018 than in April 2017, with no significant difference between July 2017 and the other sampling timepoints. There was also a significant difference between sites (*P* < 0.0001), with individuals generally higher in biomass in the crop sites than in the margins, and with no significant difference between the riverbank sites and all sites within the field.

Table 3B-5. The mean and range of the biomass (g) of individuals of the six species present in statistically valid quantities in the Swale field site. Shaded row and column headings for individual species indicate a significant difference assessed by two way ANOVAs between time periods and sites (lighter shading indicates a significance of P < 0.05; darker shading a significance of P < 0.01). For any given species values, cells with the same letter are not significantly different (P < 0.05). N/A indicates no range, with only one earthworm found in a set of six pits

A. caliginosa

	Rarely flooded crop	Rarely flooded margin	Occasionally flooded crop	Occasionally flooded margin	Regularly flooded Crop	Regularly flooded margin	Field side bank	River side bank
Apr 17 AB	0.39 (0.26 – 0.57)	0.29 (0.07 – 0.53)	0.74 (N/A)	0.58 (N/A)	0.45 (N/A)	0.37 (0.04 – 0.59)	0.41 (0.17 – 0.65)	0.37 (0.27 – 0.45)
Jul 17 B	-	-	0.29 (N/A)	0.72 (0.13 – 0.22)	-	-	-	0.22 (0.19 – 0.29)
Oct 17 A	0.68 (N/A)	0.33 (0.25 – 0.38)	0.32 (N/A)	0.31 (0.15 – 0.51)	0.26 (N/A)	-	0.60 (0.39 – 0.87)	0.42 (0.25 – 0.54)
Jan 18 A	0.34 (0.18 – 0.51)	0.53 (0.41 – 0.72)	-	0.46 (0.45 – 0.48)	-	0.40 (0.27 – 0.46)	0.53 (N/A)	0.60 (0.54 – 0.69)

A. chlorotica

	Rarely flooded crop AC	Rarely flooded margin ABC	Occasionally flooded crop AC	Occasionally flooded margin AB	Regularly flooded crop AC	Regularly flooded margin BC	Field side bank ABC	River side bank ABC
Apr 17 A	0.21 (0.11 – 0.28)	0.20 (N/A)	0.20 (011 – 0.31)	0.24 (0.14 – 0.43)	0.21 (N/A)	0.30 (0.12 – 0.28)	0.31 (0.23 – 0.37)	-
Jul 17 AB	0.23 (N/A)	-	-	-	-	-	-	-
Oct 17 B	0.36 (0.18 – 0.56)	-	0.36 (0.22 – 0.56)	0.23 (0.18 – 0.30)	0.28 (0.19 – 0.38)	0.23 (0.18 – 0.28)	0.29 (N/A)	0.30 (0.22 – 0.37)
Jan 18 B	0.36 (0.23 – 0.66)	-	0.33 (0.15 – 0.53)	0.20 (0.18 – 0.22)	0.37 (0.15 – 0.58)	0.22 (0.19 – 0.26)	-	0.32 (0.25 – 0.39)

A. rosea

	Rarely flooded crop	**Rarely flooded margin**	**Occasionally flooded crop**	**Occasionally flooded margin**	**Regularly flooded crop**	**Regularly flooded margin**	**Field side bank**	**River side bank**
Apr 17	0.16 (0.12 – 0.21)	0.09 (N/A)	0.21 (0.14 – 0.29)	0.25 (N/A)	-	0.19 (0.14 – 0.25)	-	-
Jul 17	-	-	-	-	0.21 (N/A)	-	-	0.30 (N/A)
Oct 17	-	0.21 (N/A)	-	0.24 (0.23 – 0.27)	-	0.22 (N/A)	-	0.35 (N/A)
Jan 18	0.32 (0.31 – 0.33)	-	0.18 (N/A)	0.18 (N/A)	-	-	-	0.53 (0.18 – 0.33)

L. castaneus

	Rarely flooded crop	**Rarely flooded margin ab**	**Occasionally flooded crop**	**Occasionally flooded margin**	**Regularly flooded crop**	**Regularly flooded margin**	**Field side bank**	**River side bank**
Apr 17	-	-	-	0.15 (0.13g – 0.16)	-	0.11 (0.07 – 0.14)	-	-
Jul 17	-	-	-	-	-	-	-	-
Oct 17	-	-	-	-	-	-	-	-
Jan 18	-	0.15g (N/A)	-	0.20 (N/A)	-	-	-	-

L. rubellus								
	Rarely flooded crop	**Rarely flooded margin A**	**Occasionally flooded crop**	**Occasionally flooded margin**	**Regularly flooded crop B**	**Regularly flooded margin**	**Field side bank**	**River side bank**
Apr 17	-	0.17 (0.14 – 0.19)	-	0.23 (0.11 – 0.31)	-	0.29 (0.17 – 0.42)	-	0.13 (0.09 – 0.16)
Jul 17	-	-	-	-	-	0.19 (N/A)	-	-
Oct 17	-	0.18 (0.18 – 0.18)	-	-	-	0.64 (N/A)	-	-
Jan 18	-	0.18 (0.13 – 0.21)	-	0.21 (0.17 – 0.23)	0.69 (N/A)	0.17 (0.13 – 0.23)	-	-

A. longa								
	Rarely flooded crop	**Rarely flooded margin**	**Occasionally flooded crop**	**Occasionally flooded margin**	**Regularly flooded crop**	**Regularly flooded margin**	**Field side bank**	**River side bank**
Apr 17	-	-	2.62 (N/A)	1.30 (N/A)	-	1.71 (1.32 – 2.35)	-	1.72 (N/A)
Jul 17	-	-	-	-	-	-	-	-
Oct 17	-	-	-	3.25 (N/A)	-	-	2.93 (N/A)	-
Jan 18	1.42 (N/A)	-	-	-	-	1.27 (N/A)	-	-

	L. terrestris							
	Rarely flooded crop	**Rarely flooded margin**	**Occasionally flooded crop**	**Occasionally flooded margin**	**Regularly flooded crop**	**Regularly flooded margin**	**Field side bank**	**River side bank**
Apr 17	-	3.23 (N/A)	-	3.65 (N/A)	-	-	3.38 (3.19 – 3.56)	1.65 (N/A)
Jul 17	-	-	-	-	-	-	-	-
Oct 17	-	-	-	2.29 (2.17 – 2.42)	-	1.56 (N/A)	-	2.36 (2.31 – 2.41)
Jan 18	3.15 (2.73 – 3.57)	-	-	1.65 (N/A)	-	-	2.00 (1.34 – 2.39)	-

3B.1.5 Correlations between soil environmental factors and earthworm population metrics

To determine if there was a linear relationship between the soil environmental factors and the earthworm population metrics of abundance, biomass, and Shannon Diversity, Spearman's Rank correlations were performed for the entire dataset, and then for the crop, margin, and riverbank soils (Table 3B-6).

Table 3B-6. Spearman's Rank rho (ρ) values of correlations between soil environmental factors and earthworm population metrics. Lighter shaded cells indicate a statistically significant correlation of *P* < 0.01. Darker shaded cells indicate a statistically significant correlation of *P* < 0.05.

	Abundance per m^2			
	Whole Dataset	**Crop**	**Margin**	**Riverbank**
Temperature	ρ = - 0.09	ρ = - 0.05	ρ = -0.19	ρ = - 0.04
Bulk Density	ρ = 0.26	ρ = 0.50	ρ = - 0.11	ρ = 0.35
Soil Moisture Percentage	ρ = - 0.18	ρ = - 0.46	ρ = 0.29	ρ = - 0.10
pH	ρ = - 0.1	ρ = -0.27	ρ = 0.30	ρ = 0.22
Percent Carbon	ρ = - 0.35	ρ = - 0.27	ρ = 0.04	ρ = 0.02
Percent Nitrogen	ρ = - 0.35	ρ = - 0.21	ρ = - 0.19	ρ = - 0.07

	Total earthworm biomass (g m^{-2})			
	Whole Dataset	**Crop**	**Margin**	**Riverbank**
Temperature	ρ = - 0.28	ρ = - 0.31	ρ = - 0.35	ρ = - 0.27
Bulk Density	ρ = 0.09	ρ = 0.43	ρ = - 0.11	ρ = 0.23
Soil Moisture Percentage	ρ = - 0.03	ρ = - 0.26	ρ = 0.15	ρ = 0.08
pH	ρ = - 0.07	ρ = - 0.16	ρ = - 0.01	ρ = 0.22
Percent Carbon	ρ = - 0.17	ρ = - 0.54	ρ = 0.00	ρ = - 0.07
Percent Nitrogen	ρ = - 0.26	ρ = - 0.41	ρ = - 0.26	ρ = - 0.10

	Shannon Diversity index values			
	Whole Dataset	**Crop**	**Margin**	**Riverbank**
Temperature	ρ = - 0.17	ρ = - 0.02	ρ = - 0.41	ρ = - 0.18
Bulk Density	ρ = 0.11	ρ = 0.34	ρ = - 0.16	ρ = 0.25
Soil Moisture Percentage	ρ = 0.04	ρ = - 0.24	ρ = 0.39	ρ = 0.13
pH	ρ = - 0.02	ρ = - 0.23	ρ = 0.20	ρ = 0.35
Percent Carbon	ρ = - 0.20	ρ = - 0.47	ρ = - 0.08	ρ = 0.04
Percent Nitrogen	ρ = - 0.30	ρ = - 0.40	ρ = - 0.34	ρ = 0.03

While a number of correlations were statistically significant, many were not strongly correlated, with the highest ρ value calculated at 0.54.

Across the whole dataset, total earthworm abundance, total biomass and Shannon diversity were negatively correlated with the soil percent carbon and percent nitrogen, although the correlation coefficient values were low. Earthworm total biomass and Shannon diversity were negatively associated with soil temperature, while total earthworm abundance was negatively associated with the soil moisture percentage and positively associated with bulk density.

In the crop sites, there was a correlation with almost all soil factors for earthworm abundance, total earthworm biomass, and Shannon diversity. Earthworm population metrics were positively associated with bulk density and negatively associated with soil moisture and soil percent carbon, while earthworm abundance and diversity were negatively associated with pH, and earthworm biomass and abundance were negatively associated with the percent nitrogen. In the margin, there was more variability in the correlations; earthworm abundance was positively associated with both soil moisture percentage and pH, earthworm biomass was negatively associated with soil temperature and percent nitrogen, and earthworm diversity was negatively associated with soil percent nitrogen and soil temperature, and positively associated with soil moisture. In the riverbank sites, there were no statistically significant correlations with total biomass, earthworm abundance positively correlated with bulk density, and diversity positively correlated with pH.

To determine which environmental factors were the most influential on earthworm populations, GLMs were produced comparing all environmental factors to the individual earthworm population metric. Using stepwise model selection of GLMs (Zhang, 2016), the environmental factors which explained the most variance within the model, and their statistical significance on the population variable, were selected for each earthworm population variable. Statistical significance, *df*, and *t* values for each variable across the whole dataset, and across the soil uses of crop, margin, and riverbank, are summarised in Table 3B-6.

For earthworm abundance, stepwise model selection of a GLM using a Poisson link determined that the key environmental factors were soil moisture ($P < 0.0001$), soil carbon percentage ($P < 0.0001$), and soil nitrogen percentage ($P = 0.042$). For total biomass, the key significant environmental factors were the mean temperature between 5 cm and 10 cm ($P < 0.032$) and the soil nitrogen percentage ($P = 0.010$). Although not significant, the soil carbon percentage was also determined to be essential to include in the model to explain some of the variance observed

(P = 0.10). For Shannon diversity, the key significant environmental factors were soil moisture and soil nitrogen percentage ($P < 0.0001$). Although not significant, soil bulk density was also determined to be essential to include in the model to explain some of the variance observed (P = 0.79). The key environmental factors for the earthworm populations were then determined for each soil use category of crop, margin and riverbank (Table 3B- 7).

Table 3B- 7. The key factors explaining the most variance for each earthworm population metric across the whole dataset and soil uses of crop, margin, and riverbank, the *df* values, *t* values, and significance level of each factor. Lighter shaded cells indicate a statistically significant correlation of *P* < 0.01. Darker shaded cells indicate a statistically significant correlation of *P* < 0.05.

Soil use	Population metric	Soil Environmental factor					
Whole Dataset (df = 191)	**Abundance**	Soil moisture (*t* = 3.13)	Percent carbon (*t* = -3.49)	Percent nitrogen (*t*= -2.03)	-	-	-
	Total biomass	Mean temperature (*t* = -2.28)	Percent nitrogen (*t* = -2.57)	Percent carbon (*P* = 0.1, *t*= 1.64)	-	-	-
	Shannon Diversity	Soil moisture (*t* = 3.62)	Percent nitrogen (*t* = -3.97)	Bulk density (*P*= 0.08, *t*= 1.77)	-	-	-
Crop (df = 71)	**Abundance**	Soil moisture (*t* = -2.20)	Percent carbon (*t* = -6.55)	Percent nitrogen (*t*= 3.66)	pH (*P* = 0.06, *t* = 1.86))	-	-
	Total biomass	Mean temperature (*t* = -2.46)	Percent carbon (*t* = - 4.97)	-	-	-	-
	Shannon Diversity	Percent carbon (*t* = -4.60)	-	-	-	-	-
Margin (df = 71)	**Abundance**	pH (*t* = 0.34)	Percent carbon (*t* = 1.99)	Percent nitrogen (*t* = -4.64)	Temperature (*P* = 0.10, t = 1.62))	Soil moisture (P = 0.09, *t* = 1.68))	Bulk density (P = 0.10, *t* = -1.62)
	Total biomass	Percent carbon (*t* = 2.72)	Percent nitrogen (*t* = -3.08)	-	-	-	-
	Shannon Diversity	Soil moisture (*t* = 5.01)	Percent nitrogen (*t* = -4.88)	-	-	-	-
Riverbank (df = 43)	**Abundance**	Soil moisture (*t* = 2.37)	Bulk density (*t* = 4.82)	Percent carbon (*t* = 3.28)	Percent nitrogen (*t* = -3.33)		
	Total biomass	Bulk density (*t* = 2.74)	Soil moisture (*P* = 0.06, *t* = 1.93)				
	Shannon Diversity	Soil moisture (*t* = 2.40)	Bulk density (*t* = 2.92)				

3B.2 Discussion

3B.2.1 Soil environmental factors

The environmental factors of soil temperature, soil moisture, soil pH, and soil nitrogen percentage all differed significantly depending on the time of year and the flooding regime of the field site. Soil carbon percentage was not affected by the time of year, only by flooding regime.

3B.2.1.1 Soil temperature

Soil temperatures varied depending on the time of year, with higher soil temperatures observed during the July 2017 and October 2017 sampling, and lower soil temperatures during the spring and winter sampling in April 2017 and January 2018. The soil temperatures increase from the rarely flooded sites of the field to the river bank sites, with the exception of the January 2018 sampling. For the April, July and October 2017 sampling sessions the sampling was completed over one day, and began in the rarely flooded sites before moving down the field through the occasionally flooded and regularly flooded sites. This meant that the ambient temperatures increased with the time of day, and the soil temperature rose accordingly. As seen in Table 3B-8, the increases in temperature throughout the day seen in Fig. 3B-2 coincide with the differences between the temperatures reported at 9 am and 9 pm. The January 2018 sampling period had to be split over two days due to inclement weather conditions, leading to the variability in the temperature pattern.

3B.2.1.2 Soil bulk density and its association with soil carbon, soil nitrogen, and soil moisture

Soil bulk density decreased as flooding increased, and was highly co-correlated with soil moisture and soil percent carbon and nitrogen. As discussed in Section 3A.5.1, accumulation of organic matter in the soil leads to decreases in soil bulk density (Bronic and Lal, 2005). Cultivated soils are typically lower in soil organic matter content than uncultivated soils (Six et al., 1998), with percent carbon and percent nitrogen content increasing both with flooding and with decreased cultivation in the margin and riverbank sites. However, there was a difference in the percentage increase in soil percent carbon with increased flooding between the crop and the margin. Between the rarely flooded and regularly flooded sites, the soil percent carbon increased by an average across all the sampling times of 85.8% in the crop and 44.1% in the margin. The smaller percent increase in organic matter content between the margin sites is likely due to the higher levels of organic matter input to field margin soils from the decomposition of leaf litter. High levels of compaction in the unflooded crop sites due to the use of mechanised equipment (Domżal et al., 1993; Słowińska-Jurkiewicz and Domżal, 1989) can be observed, with the soil in the rarely flooded crop site significantly higher in bulk density than the rarely flooded margin. These already high levels of compaction in the crop soils may make the effect of reduced bulk density occurring with increased organic matter (Bronick and Lal, 2005) and soil moisture more strongly evident in the crop soils than the margin soils. Although the crop sites are dominated by endogeic earthworms, particularly *A. chlorotica*, the pink morph of which acts as a decompacting species (Amossé et al., 2015), it seems likely that agricultural practices are driving the high bulk density values seen in the crop sites.

In addition to the difference between the sites, there is also a temporal difference in the soil bulk density. The bulk density of the soil is higher in the April and July 2017 sampling than in the October and January sampling periods. This corresponds with a period of lower soil moisture in the same months. As soil moisture is lost, some soil shrinkage occurs, resulting in reductions of soil volume (Reeve et al., 1980). Bulk density, as a measure of the dry weight of soil by volume, may be expected to increase as soil moisture decreases. As discussed in Section 3A.5.1, and Table 3B-8 below, the month of April 2017 was very dry, with the UK receiving 47% of the expected April rainfall that year (Met Office, 2017), and the site received a total of 30 mm of rainfall for the month. However, the cycle of land use for that year may also have played a role in the observed bulk density changes within the crop. Following the sampling in July 2017, the cereal crop was harvested and a brassica crop planted before the October 2017 sampling. The disturbance of the soil caused by this activity mostly likely contributed to the lower bulk density. Similarly, previous studies have found that the integration of organic material into topsoil reduces soil bulk density (Zebarth et al., 1999; Guo et al., 2016); the leftover cereal straw was ploughed into the soil before planting of the brassicas took place, with straw residue found in the sampling pits in October 2017.

Table 3B-8. The average daily rainfall (mm), total month's rainfall (mm), and mean and range of air temperatures at 9 am and 9 pm reported at Topcliffe Weather Monitoring Station (SE 39800 78900) (Met Office, 2019). This is the closest weather monitoring station to the field site at a distance of 2.8 miles

Month	Average daily rainfall (mm)	Total month's rainfall (mm)	Mean minimum and maximum air temperature at 9am (°C)	Mean minimum and maximum air temperature at 9pm (°C)
January 2017	2.39	74	0.5 - 4.8	1.2 – 6.0
February 2017	3.57	100	2.9 - 6.7	3.7 – 8.6
March 2017	3.20	99.2	3.5 - 8.5	5.9 – 12.1
April 2017	1.00	30	2.7 - 9.6	7.1 – 13.7
May 2017	2.26	70	7.1 - 14.2	10.9 – 17.8
June 2017	6.55	196.4	10.8 - 16.8	14.3 – 19.8
July 2017	3.70	114.8	11.0 - 16.5	14.2 – 20.5
August 2017	3.69	114.4	9.6 - 16.3	13.5 – 19.9
September 2017	6.48	194.4	8.8 - 13.9	11.5 – 17.3
October 2017	3.54	109.6	8.3 - 13.3	10.6 – 15.4
November 2017	2.77	83.2	2.3 - 7.6	3.5 – 9.6
December 2017	2.50	77.4	0.1 - 4.7	1.2 – 6.9
January 2018	3.23	100.2	2.0 - 5.7	2.7 – 7.1
February 2018	2.09	58.4	-1.5 - 2.9	-0.1 – 5.4

3B.2.1.3 Soil moisture

Soil moisture varied with both the flooding regime and with the time of year. The soil was wetter during the October 2017 and January 2018 sampling than in the April and July 2017 sampling. This association with both the flooding regime and the time of year is not unexpected. As seen in Table 3B-8, the months of October 2017 and January 2018 had higher monthly rainfall than April 2017, but July 2017 had very high monthly rainfall and the lowest soil moisture content. Transpiration of the growing cereal crops may have contributed to this low soil moisture content (Francis and Pidgeon, 1982), as the crops were almost fully grown. The low soil moisture content in this period may also be a result of high soil temperatures. High soil temperatures lead to higher rates of soil moisture evaporation (Wang et al., 2012), and this pattern is reflected in the negative correlations between temperature and soil moisture percentage for the margin and the river bank soils. This correlation is not observed within the crop sites. Higher bulk density (Meek et al., 1992; Bharati et al., 2002), reduced plant residue cover (Mukhtar et al., 1985; Unger and Vigil, 1998) and litter layers (Ossola et al., 2015), and reduced earthworm populations (Ehlers et al., 1975) in crop soil compared to the margin all likely contribute to lower levels of water infiltration, leading to lower soil moisture in crop soil than in margin soil.

In the dataset there is very little significant difference between the soil moisture percentage in the crop and margin in each flooding regime. As flooding increases, the soil moisture content of the soil in both the crop and margin increases, suggesting that the percent carbon and nitrogen content of the soil is associated with soil moisture content. The correlations in Table 3B-1 show that soil moisture is positively correlated with soil carbon in the crop, margin, and riverbank soils. As discussed in Section 3A.2.1, increased soil carbon leads to increased retention of soil moisture (Rawls et al., 2003). With regular flooding leading to increases in soil carbon content, flood- and rainwater are likely to be retained in the soils for longer in the regularly flooded sites.

3B.2.1.4 Soil pH

Soil pH differed between the crop and the margin in the rarely flooded sites, but not in the regularly flooded sites. A correlation between soil moisture and pH is observed in the crop, margin, and river bank sites, with higher soil moistures associated with higher pH. In the rarely flooded margin, the pH ranges from 4.18 to 6.31, while in the regularly flooded margin the pH ranges from 6.13 to 6.90. In contrast, in the crop the pH ranges from 5.37 to 6.36 in the rarely flooded site, and 5.73 to 6.60 in the regularly flooded site. The contrast between the crop and margin in the rarely flooded region is likely not due to the application of fertiliser within the crop, as this tends to acidify soil (Peryea and Burrows, 1999). The lower pH in the margin may be caused by the presence of decomposing leaf litter (van Breemen, 1982; de Cauwer, 2006), which contributes to higher organic matter content of soils (Olson, 1963; Melillo et al., 1989).

3B.2.1.5 Soil carbon and nitrogen content

Both soil carbon and soil nitrogen percentages increased as flooding increased. However, only soil nitrogen also showed variable patterns over time, particularly showing increases in the rarely and occasionally flooded sites in July 2017. The increased percentages of carbon and nitrogen with increased flooding is expected; as discussed above and in Section 3A.2.1, these increases in soil organic matter contents are likely due to the slower rates of organic matter decomposition under wet and waterlogged conditions. However, the increase in percent nitrogen within the rarely and occasionally flooded sites is not explained by this mechanism. The fertiliser handbook RB209 recommends that nitrogen is applied to crops between late February and late April (Agriculture and Horticulture Development Board, 2019). The timing of the soil nitrogen content increase, and the fact that increases occur both in the crop and margin sites, suggest that the increases are not due to the application of NPK fertiliser. At present, the causes for the increases in soil nitrogen content between April 2017 and July 2017 are not understood.

3B.2.2 **Earthworm populations**

3B.2.2.1 Earthworm populations and soil use

Earthworm abundance, biomass and diversity all display differences between the sites. In the unflooded sites, there is little difference in the abundance, biomass and diversity of earthworms between the crop and margin, while the sites with occasional and regular flooding showed greater differences between the crop and margin. This lack of difference in the unflooded sites between the crop and margin is unexpected, as typically earthworm populations are higher in permanent field margins than within conventionally tilled crop soils (Roarty and Schmidt, 2013), with low overall populations in crop soil (Boag et al., 1997). Curry reported the abundance of individuals m^{-2} in crop as 155 ± 46, and 470 ± 47 in field margin soils, and a biomass of 38.3 ± 12.5 g m^{-2} in crop and 126.5 ± 14.4 g m^{-2} in field margin soils. These abundance and biomasses are comparable to those found in the occasionally flooded crop, but higher than in all field margin and river bank soils sampled in this study (Table 3B-9).

3B.1.1.1 Variability in the study

Variability in this study was very high. In this field there was no significant difference between the total earthworm abundance, earthworm biomass and Shannon diversity in the rarely flooded crop and regularly flooded margin. The diversity of earthworms increases from the April and July 2017 sampling to the October 2017 and January 2018 sampling. This is likely due to seasonal patterns of earthworm behaviour, with some species entering an aestivation period during the hot and dry summer conditions (Edwards and Lofty, 1977; Gerard, 1967).

Table 3B-9. The mean and standard deviation of the total abundance of earthworms m^{-2} and the total biomass (g m^{-2}) of earthworms across all timepoints in the different flooding regimes of rarely, occasionally and regularly flooded, and soil uses of crop, margin, and river bank sites.

	Individuals m^{-2}		Total biomass (g m^{-2})	
	Crop	**Margin**	**Crop**	**Margin**
Rarely flooded	233.33 (± 152.81)	138.54 (± 120.68)	70.94 (±41.84)	53.92 (± 48.56)
Occasionally flooded	166.67 (±100.18)	218.75 (± 130.89)	40.39 (± 28.40)	71.96 (± 64.77)
Regularly flooded	87.5 (± 58.98)	280.20 (149.63)	24.53 (± 24.13)	72.88 (± 56.78)
	Field side bank	**River side bank**	**Field side bank**	**River side bank**
Regularly flooded	67.71 (± 64.47)	122.91 (± 97.76)	45.24 (± 49.13)	58.22 (± 51.98)

Previous studies have found that earthworm populations in sites already highly disturbed through activity such as ploughing show a greater reduction in numbers in response to unfavourable conditions compared to populations in less disturbed sites (Barnes and Ellis, 1979). In more generalised studies, other researchers have also found that in arable fields soil properties are less significant predictors of earthworm biodiversity than land management (Frazão et al., 2017). In this study, the differences between the crop and the margin increased as sampling moved further down the field and into sites subject to the dual stresses of field use and flooding, with earthworm population metrics generally lower in the crop than in the margin. The unploughed river bank on both the field and river sides was not statistically dissimilar to either the crop or the margin, suggesting that the addition of the stress of agricultural activity may be driving the differences in earthworm population observed between the crop and margin in the regularly flooded area.

These links between land use and earthworm population metrics can be observed through the model generation process. The model selection showed that earthworm abundance and biomass were negatively correlated with soil nitrogen percentage within the crop and margin sites. In the riverbank sites, there was no strong association between earthworm populations and soil nitrogen content. This suggests that, in the crop and margin, flooding caused accumulation of organic matter but also contributed to reduced earthworm populations which are already reduced within the crop. Within the riverbank sites, which are not subject to any agricultural disturbance, earthworm populations were not significantly different to the margin and crop in the regularly flooded region. This suggests that, while agricultural land use plays a part in reducing earthworm populations, flooding still exerts pressures on earthworm populations. Another factor to consider was the high presence of molehills on the riverbank sites. It may be the case that local mole populations and flooding were providing additional pressure on patchy earthworm distribution, making patterns of earthworm populations and soil environmental conditions in the riverbank hard to identify. These high levels of variations within the earthworm community measures may also explain why there are significant differences observed in the proportion of the population of various ecotypes at the site, timepoint, and interaction terms between the two shown in Section 3B.1.3 show no significant difference in post hoc testing. This suggests that the results for the differences between the proportions of the community represented by the adults and juveniles of the different ecotypes must be treated with caution.

There are high levels of variation in earthworm population throughout the field. Earthworm abundances in pits ranged from 0 to 26, and Shannon diversity index values from 0 to 2.1. This may be due to a number of factors. It may be the case that the variation is due to ‘grouping’ behaviour observed in some earthworm species, with species that typically have larger individuals less likely to clump than species of a smaller average biomass (Poier and Richter, 1992). However, when comparing the coefficient of variation (CV) values (Table 3B-10), there is little difference in the variability across the whole dataset between earthworm species, with the lowest and highest CV values, 95.39 (*A. caliginosa)* and 373.93 (*L. castaneus*), both associated with relatively small bodied earthworm species. The high variability may instead be due to high levels of spatial heterogeneity of soil resources (Ettema and Wardle, 2002). Previous studies have found that variability of resource availability tends to be higher in undisturbed soils than in regularly disturbed soils (Conant et al., 2003; Robertson et al., 1993). However, in this dataset the CV values of the percent carbon and nitrogen values are higher in the crop and margin soils than in the riverbank soils (Table 3B-11), suggesting high variability in resource distribution. It may the case that within the already low earthworm populations of the crop sites, resource distribution creates patchiness in the distribution of earthworm populations, leading to the high variability observed.

Table 3B-10. The mean, standard deviation and coefficient variation of the abundance of individuals of each of the seven species found across the whole dataset

Species	**Mean abundance of individuals of each species**	**Standard deviation**	**Coefficient of Variation** ($\frac{sd}{mean}$ $\boldsymbol{x}$ **100**)
A. caliginosa	2.31	2.21	95.39
A. chlorotica	4.88	6.19	126.96
A. rosea	0.75	0.98	131.17
L. castaneus	0.34	1.29	373.93
L. rubellus	0.81	1.35	166.71
A. longa	0.31	0.64	206.23
L. terrestris	0.5	0.84	168.48

Table 3B-11. The mean, standard deviation and coefficient variation of the soil environmental factors for the whole dataset and soil use categories of crop, margin, and riverbank across all timepoints

Soil environmental factor	Soil use category	Mean values across all sampling timepoints	Standard deviation	Coefficient of Variation ($\frac{sd}{mean}\ x\ 100$)
Soil temperature (°C)	**Whole dataset**	9.90	5.05	51.05
	Crop	9.47	4.97	52.48
	Margin	9.77	4.30	44.00
	Bank	10.75	6.13	57.04
Bulk density (g cm^{-3})	**Whole dataset**	1.61	0.27	16.68
	Crop	1.76	0.25	14.21
	Margin	1.59	0.26	16.40
	Bank	1.41	0.12	8.65
Soil moisture content	**Whole dataset**	36.62	12.75	34.81
	Crop	33.34	12.46	37.36
	Margin	35.18	13.58	38.61
	Bank	43.69	8.45	20.02
pH	**Whole dataset**	6.07	0.61	9.98
	Crop	6.18	0.31	5.05
	Margin	5.70	0.77	13.57
	Bank	6.46	0.23	3.52

Soil percent carbon	**Whole dataset**	3.57	1.19	33.21
	Crop	2.93	0.84	28.62
	Margin	3.35	1.00	29.95
	Bank	4.85	0.87	17.87
Soil percent nitrogen	**Whole dataset**	0.35	0.09	26.16
	Crop	0.31	0.07	21.98
	Margin	0.33	0.08	25.04
	Bank	0.44	0.08	17.38

3B.1.1.2 Earthworm population composition

When considering the populations at the ecotype level, adult epigeic earthworms made up greater proportions of the populations in the margin and riverbank sites, although juveniles were found in all sites. Across all the crop sites, adult epigeics were only found in the regularly flooded region of the field. The ecotype composition observed within the arable site, in particular the differences between the crop and the margin, correspond with observations made in other studies, which found that under conventional cropping and tillage systems the abundance of earthworms (Tebrügge and Düring, 1999), particularly anecic and epigeic earthworms (Chan, 2001), is reduced. Although small bodied individuals, the soil surface habitat of epigeic earthworms leaves them vulnerable to crush or trampling damage (Cluzea et al., 1992), while the large bodies of anecic earthworms may be injured, or burrows destroyed, by ploughing activity (Wyss and Glasstetter, 1992).

The reduction in epigeic and anecic earthworms in cropping systems may also be due to the reduced presence of surface litter, the preferred food source of both epigeic and anecic earthworms. Studies in fields cropped with surface applied organic matter such as mulch had a much higher presence of both epigeic and anecic earthworms than un-mulched fields (Pelosi et al., 2009), suggesting a strong effect of leaf litter cover on abundances of these earthworms within crops. Land use and management, therefore, likely have a stronger impact upon earthworm populations within the arable crop than the overall effects of flooding, especially when it is taken into consideration that the population of epigeic earthworms increased in the riverbank site of the arable field, which is the site most likely to flood.

Endogeic earthworms are some of the most abundant in agricultural soils (Pelosi et al., 2009, Schmidt et al., 2001). In this study, it was found that the proportion of endogeic earthworms in the population, and the abundances of individuals belonging to endogeic species, do not differ between the crop and the margin. This is in accordance with previous studies, which found little to no difference between endogeic abundance between soils under different tillage treatment, including no till (Edwards and Lofty, 1982; Chan, 2001; Stroud, 2019). Wyss and Glasstetter, 1992, theorised that this was because the endogeic earthworms are generally smaller individuals than other earthworm species strongly negatively impacted by tillage, such as anecic earthworms, and so are not injured or destroyed during ploughing activity (Wyss and Glasstetter, 1992). Only individuals of *A. chlorotica* showed a greater biomass within the crop than the margin. The mass of individuals of *A. chlorotica* was significantly higher in the crop sites than in the margins, with the mass of individuals on both sites of the river bank not differing significantly from either the crop or the margin. Additionally, there was no effect of flooding on the biomass of individuals of *A. chlorotica*, with no significant difference in the biomass of individuals between the frequently flooded, occasionally flooded, or rarely flooded crop. As many of the soil environmental conditions are less favourable in the crop than in the margin, with lower percent carbon and nitrogen, lower soil moisture content, and higher bulk density, this higher biomass of individuals in the crop than in the margin is an unexpected result. This may be a result of soil environmental conditions such as pH. The pH trends more acidic within the margin than in the crop. Although earthworms can tolerate pH in the range of 4.5 to 7 in culture (Lowe and Butt, 2005), the lowest pH value recorded was 4.18 in the regularly flooded margin site. Previous studies into earthworm populations and acidic soils have found that *A. chlorotica* tended to occur in soils in the pH range of 6.0 to 7.7 (Piearce, 1972). Although individuals were recorded in the margin sites, these individuals may be stressed, and at a lower biomass than individuals in the crop. There may also be an effect of reduced competition for resources allowing individuals to grow larger in the crop sites, with very few earthworms competing in the same niche. Previous laboratory studies have found that earthworm individuals grow to larger body sizes in earthworm monoculture than in mixed culture (Butt, 1998). While the crop sites of the field were not a monoculture, the low earthworm biodiversity may allowed a similar response in the body size of individuals of *A. chlorotica* in the crop. Alternatively, it may be the case that although the overall carbon and nitrogen percentages in the crop are significantly lower than those of the margins, farming practices are influencing the earthworm biomass. As geophagous earthworms, which consume soil as they move through it, *A. chlorotica* may benefit from the integration of straw into the soil. Other studies have found that abundance of earthworms in arable crops increase when wheat stubble was retained and integrated into soil (Haines and Uren, 1990), although this applies to the earthworm population as a whole, rather than the biomass of one species. If this practice is causing the differences in the biomass of individuals of *A. chlorotica*, the same effect may be expected for individuals of *A. caliginosa*. However, no significant difference in biomass of individuals between the crop and the margin was observed for this species. This lack of effect observed in *A. caliginosa* may be attributed to inter-specific competition with *A. chlorotica.* A meta-analysis found that studies typically report negative effects of and responses to the other species between *A. chlorotica* and *A. caliginosa* (Uvarov, 2009), with endogeic survival generally higher in mixed populations containing both epigeic and anecic earthworms than in endogeic monoculture

(Sheehan, 2006). At the time of writing, no other studies have found this effect of higher biomass individuals of *A. chlorotica* occurring in crop than in pasture.

3B.3 Conclusion

Regular flooding of sites of an arable field led to increased soil moisture content, decreased bulk density, an increase of the soil pH from slight acidity towards neutrality, and increases in the percent carbon and percent nitrogen content of the soil. There was also a strong difference in these environmental factors as a result of the land use, with margin soil more acidic, reduced in bulk density, and higher in soil moisture content, percent carbon and percent nitrogen than the crop soil.

The data suggests that the combination of flooding and agricultural soil use stresses earthworms more than the individual effects of agricultural soil use and flooding. The total abundance, total biomass and diversity of earthworms was lower in the crop than in the margin, supporting existing literature on the populations of earthworms in farmland soil. There was also an effect of flooding on the distribution of earthworms: in the regularly flooded crop, the earthworm population was significantly lower than in the regularly flooded margin. However, there was no significant difference between earthworm populations in the rarely flooded crop site and the regularly flooded margin. This suggests that flooding supressed earthworm populations in the regularly flooded margins to the extent that they were not statistically different to the low, stressed populations in the rarely flooded crop. Populations in the regularly flooded crop, subject to the dual stresses of agricultural soil use and flooding, were significantly lower than the regularly flooded margin.

At the ecotype level, there were significant differences between the proportions the population represented by adults of the different earthworm ecotypes between the sites. Endogeic earthworm species dominated the crop sites. This supports the existing literature, which has found that endogeics are abundant in arable soils as they can mostly avoid mechanical damage from farming equipment. The abundance of epigeics was also greatly influenced mostly by the land use, with higher populations in the margin sites than in the crop sites. The abundance of anecic earthworms were significantly higher in the margins and riverbank sites than the crop sites, but decreased with flooding within the field, increasing again in their proportion of the population in the riverbank. The population dynamics of epigeic and anecic earthworms is expected. Both ecotypes are highly sensitive to the mechanical damage that endogeic individuals avoid, and rely on leaf litter cover for foraging, of which there is very little within the crop.

As populations of earthworms in arable soils are already reduced compared to undisturbed soils, it is important to understand how the additional stress of increased flooding may impact their populations, as earthworms are highly influential on soil porosity and nutrient turnover. This field study suggests that flooding of arable field leads to reduced earthworm populations in cropped soil, with the already stressed populations within the crops less able to recover from flooding events than margin populations. However, as the dominant earthworm ecotype in arable fields are endogeic earthworms, many of which can survive flooded conditions, as discussed in Chapter 3A, the overall effect of flooding on ecosystem services provided by earthworm processing of soil may be low.

3B.4 References

Agriculture and Horticulture Development Board. 2019. Nutrient Management Guide (RB209) Section 4: Arable Crops. Accessed online at https://projectblue.blob.core.windows.net/media/Default/Imported%20Publication%20Docs/RB209%20Updates%202019/RB2091878_Section4_190116_WEB.pdf [10th September 2019)]

Amossé, J., Turberg, P., Kohler-Milleret, R., Gobat, J-M., Le Bayon, R-C. 2015. Effects of Endogeic Earthworms on the Soil Organic Matter Dynamics and the Soil Structure in Urban and Alluvial Soil Materials. *Geoderma* 243: 50 – 57

Barnes, B.T., Ellis, F.B. 1979. Effects of Difference Methods of Cultivation and Direct Drilling, and Disposal of Straw Residues, on Populations of Earthworms. *Journal of Soil Science* 30(4): 669 – 679.

Bharati, L., Lee, K.-H., Isenhart, T.M., Schultz, R.C. Soil-Water Infiltration under Crops, Pasture, and Established Riparian Buffer in Midwestern USA. *Agroforestry Ecosystems* 56(3): 249 – 257

Boag, B., Palmer, L.F., Neilson, R., Legg, R., Chambers, S.J. 1997. Distribution, Prevalence and Intensity of Earthworm Populations in Arable Land and Grassland in Scotland. *Annals of Applied Biology* 130(1): 153 – 165.

Bronick, C.J., Lal, R. 2005. Soil Structure and Management: A Review. *Geoderma* 124(1-2): 3 – 22

Butt, K.R. 1998. Interactions between Selected Earthworm Species: A Preliminary, Laboratory-Based Study. *Applied Soil Ecology* 9(1-3): 75 – 79

Chan, K.Y. 2001. An Overview of Some Tillage Impacts on Earthworm Population Abundance and Diversity – Implications for Functioning in Soils. Soil and Tillage Research 57(4): 179 – 191.

Cluzeau, D., Binet, F., Vertes, F., Simon, J.C., Riviere, J.M., Trehen, P. 1992. Effects of Intesnive Cattle Trampling on Soil-Plant-Earthworms Systems in Two Grassland Types. *Soil Biology and Biochemistry* 24(12): 1661 – 1665

Conant, R.T., Smith, G.R., Paustian, K. 2003. Spatial Variability of Soil Carbon in Forested and Cultivated Sites. *Journal of Environmental Quality* 32(1): 278 – 286

Curry, J.P., Byrne, D., Schmidt, O. 2002. Intensive Cultivation can Drastically Reduce Earthworm Populations in Arable Land. *European Journal of Soil Biology* 38(2): 127 – 130.

de Cauwer, B., Reheul, D., Nijs, I., Milbau, A. 2006. Effect of Margin Strips on Soil Mineral Nitrogen and Plant Biodiversity. *Agronomy for Sustainable Development* 26(2): 117 – 126

Domżal, H., Hodara, J., Słowińska-Jurkiewicz, A., Turski, R. 1993. The Effects of Agricultural Use on the Structure and Physical Properties of Three Soil Types. *Soil & Tillage Research* 27(1-4): 365 – 382

Edwards, C.A., Lofty, J.R. 1977. Biology of Earthworms (2nd Edition). London: Chapman and Hall.

Edwards, C.A., Lofty, J.R. 1982. The Effect of Direct Drilling and Minimal Cultivation on Earthworm Populations. *Journal of Applied Ecology* 19: 723-734

Ehlers, W. 1975. Observations on Earthworm Channels and Infiltration on Tilled and Untilled Loess Soil. *Soil Science.* 119(3): 242 – 249

Ettema, C.H., Wardle, D.A. 2002. Spatial Soil Ecology. *Trends in Ecology & Evolution* 17(4): 177 – 183

Francis, P.E., Pidgeon, J.D. 1982. A Model for Estimating Soil Moisture Deficits under Cereal Crops in Britain: 1. Development. *The Journal of Agricultural Science* 98(3): 651 – 661

Frazão, J., de Goede, R.G.M., Brussaard, L., Faber, J.H., Groot, J.C.J, Pulleman, M.M.P. 2017. Earthworm Communities in Arable Fields and Restored Field Margins, as Related to Management Practices and Surrounding Landscape Diversity. *Agriculture, Ecosystems & Environment* 248: 1 – 8

Gerard, B.M. 1967. Factors Affecting Earthworms in Pastures. *Journal of Animal Ecology* 36(1): 235 – 252

Guo, L., Wu, G., Li, Y., Li, C., Liu, W., Meng, J., Liu, H., Yu, X., Jiang, G. 2016. Effects of Cattle Manure Compost on Topsoil Organic Matter, Bulk Density and Earthworm Activity in a Wheat-Maize Rotation System in Eastern China. *Soil and Tillage Research* 156: 140 – 147

Haines, P.J., Uren, N.C. 1990. Effects of Conservation Tillage Farming on Soil Microbial Biomass, Organic Matter and Earthworm Populations, in North-Eastern Victoria. *Australian Journal of Experimental Agriculture* 30(3): 365 – 371

Lowe, C.N., Butt, K.R. 2005. Culture Techniques for Soil Dwelling Earthworms: A Review. *Pedobiologia* 49(5): 401 – 413.

Met Office (2019): Met Office Integrated Data Archive System (MIDAS) Land and Marine Surface Stations Data (1853-current). NCAS British Atmospheric Data Centre, 2019. Available at http://catalogue.ceda.ac.uk/uuid/220a65615218d5c9cc9e4785a3234bd0

Met Office, 2017. Statistics for April 2017. Available at: https://www.metoffice.gov.uk/news/releases/2017/statistics-for-april-2017 [accessed 15/08/2018].

Meek, B.D., Rechel, E.R., Carter, L.M., deTar, W.R., Urie, A.L. 1992. Infiltration Rate of a Sandy Loam Soil: Effects of Traffic, Tillage, and Plant Roots. *Soil Science Society of America Journal* 56(3): 908 – 913

Melillo, J.M., Aber, J.D., Linkins, A.E., Ricca, A., Fry, B., Nadelhoffer, K.J. 1989. Carbon and Nitrogen Dynamics along the Decay Continuum: Plant Litter to Soil Organic Matter. *Plant and Soil* 115(2): 189 – 198

Mukhtar, S., Baker, J.L., Horton, R., Erbach, D.C. 1985. Soil Water Infiltration as Affected by the Use of the Paraplow. *Transactions of the ASAE* 28(6): 1811 – 1816

Olson, J.S. 1963. Energy Storage and the Balance of Producers and Decomposers in Ecological Systems. *Ecology* 44(2): 322 – 331

Ossola, A., Hahs, A.K., Livesley, S.J. 2015. Habitat Complexity Influences Fine Scale Hydrological Processes and the Incidence of Stormwater Runoff in Managed Urban Ecosystems. *Journal of Environmental Management* 159: 1 – 10

Pelosi, C., Bertrand, M., Roger-Estrade, J. 2009. Earthworm Community in Conventional, Organic, and Direct Seeding with Living Mulch Cropping Systems. *Agronomy for Sustainable Development* 29(2): 287 – 295

Peryea, F.J., Burrows, R.L. 1999. Soil Acidification Caused by Four Commercial Nitrogen Fertiliser Solutions and Subsequence Soil pH Rebound. *Communications in Soil Science and Plant Analysis* 30(3&4): 525 – 533

Piearce, T.G. 1972. Acid Intolerant and Ubiquitous Lumbricidae in Selected Habitats in North Wales. *Journal of Animal Ecology* 41(2): 397 – 410

Poier, K.R., Richter, J. 1992. Spatial Distributions of Earthworms and Soil Properties in an Arable Loess Soil. *Soil Biology and Biochemistry* 24: 1601-1608

R Core Team 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rawls, W.J., Pachepsky, Y.A., Ritchie, J.C., Sobecki, T.M., Bloodworth, H. 2003. Effect of Soil Organic Carbon on Soil Water Retention. *Geoderma* 116(1-2): 61 – 76

Reeve, M.J., Hall, D.G.M., Bullock. P. 1980. The Effect of Soil Composition and Environmental Factors on the Shrinkage of Some Clayey British Soils. *Journal of Soil Science* 31(3): 429 – 442

Roarty, S., Schmidt, O. 2013. Permanent and New Arable Field Margins Support Large Earthworm Communities but do not Increase In-Field Populations. *Agriculture, Ecosystems & Environment* 170: 45 – 55

Robertson, G.P., Crum, J.R., Ellis, B.G. 1993. The Spatial Variability of Soil Resources Following Long-Term Disturbance. *Oecologia* 96(4): 451 – 456

Schmidt, O., Curry, J.P., Hackett, R.A., Purvis, G., Clements, R. O. 2001. Earthworm Communities in Conventional Wheat Monocropping and Low-Input Wheat-Clover Intercropping Systems. *Annals of Applied Biology* 138: 377 – 388

Sheehan, C., Kirwan, L., Connolly, J., Bolger, T. 2006. The Effects of Earthworm Functional Group Diversity on Earthworm Community Structure. *Pedobiologia* 50(6): 479 – 487

Six, J., Elliott, E.T., Paustian, K., Doran, J.W. 1998. Aggregation and Soil Organic Matter Accumulation in Cultivated and Native Grassland Soils. *Soil Science Society of America Journal* 62(5): 1367 – 1377

Słowińska-Jurkiewicz, A, Domżal, H. 1989. The Structure of the Cultivated Horizon of Soil Compacted y the Wheels of Agricultural Tractors. *Soil & Tillage Research* 19(2-3): 215 – 226

Stroud, J.L. 2019. Soil Health Pilot Study in England: Outcomes from an On-Farm Earthworm Survey. *PLoS ONE* 14(2): p.e0203909.

Tebrügge, F., Düring, R.-A. 1999. Reducing Tillage Intensity – A Review of Results from a Long-Term Study in Germany. *Soil and Tillage Research* 53(1): 15 – 28.

Unger, P.W., Vigil, M.F. 1998. Cover Crop Effects on Soil Water Relationships. *Journal of Soil and Water Conservations* 53(3): 200 – 207

Uvarov, A.V. 2009. Inter- and Intraspecific Interactions in Lumbricid Earthworms: Their Role for Earthworm Performance and Ecosystem Functioning. *Pedobiologia* 53(1): 1 – 27

van Breemen, N., Burrough, P.A., Velthorst, E.J., van Dobben, H.F., de Wit, T., Ridder, T.B., Reijnders, H.F.R. 1982. Soil Acidification from Atmospheric Ammonium Sulphate in Forest Canopy Throughfall. *Nature* 299: 548 – 550

Wang, S., Fu, B.J., Gao, G.Y., Yao, X.L., Zhou, J. 2012. Soil Moisture and Evapotranspiration of Different Land Cover Types in the Loess Plateau, China. *Hydrology and Earth System Sciences* 16: 2883 – 2892

Wyss, E., Glasstetter, M. 1992. Tillage Treatments and Earthworm Distribution in a Swiss Experimental Corn Field. *Soil Biology and Biochemistry*. 24(12): 1635 – 1639

Zebarth, B.J., Neilsen, G.H., Hogue, E., Neilsen, D. 1999. Influence of Organic Waste Amendments on Selected Soil Physical and Chemical Properties. *Canadian Journal of Soil Science* 79(3): 501 – 504

Zhang, Z. 2016. Variable Selection with Stepwise and Best Subset Approaches. *Annals of Translational Medicine* 4(7): 136

3C. Comparing and contrasting the effects of flooding on environmental factors and earthworm populations across an arable and pasture site

3C.1 Dataset combination

To understand the effect of increased frequency of flooding on soil environmental factors and on earthworm populations, the datasets from Chapters 3A and 3B were combined. As the patterns of earthworm communities and soil environmental factors behaved as expected over the sampling timepoints, and as the months of sampling were alternated between the pasture and arable sites, the data was pooled over the sampling timepoints. By combining the datasets based on frequency of flooding, and excluding the added complexity of timepoint data, broad comparisons could be drawn between the arable and pasture datasets. Although the flooding regimes were not entirely analogous, patterns with increased frequency of flooding could still be observed within the dataset, and are presented in this chapter.

A conceptual diagram of the relationships between the frequency of flooding and the earthworm populations within the pasture field and the crop and field margin sites of the arable field observed in the dataset is presented in Fig. 3C-1. A faster rate of population depletion was observed in the crop soils than in the field margin soils, although the already low population of the field margin soils are significantly reduced with flooding. In rarely flooded regions, the population of pasture soils is higher than the crop and margin soils, which did not significantly differ. In the frequently flooded soils, there was no significant difference between earthworm populations in the pasture and field margin soils.

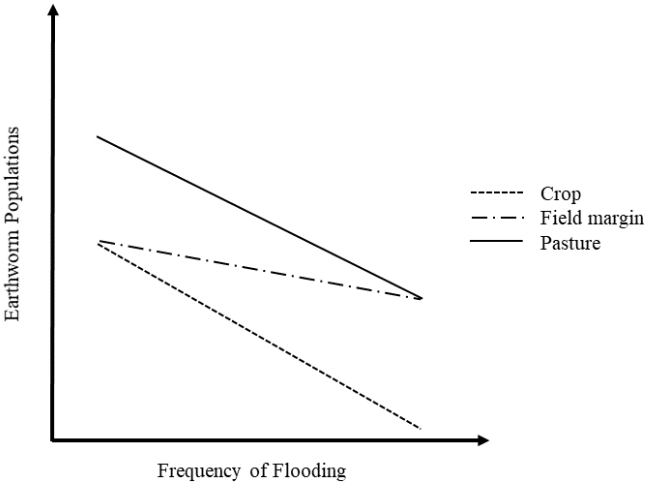

Figure 3C-1. A conceptual diagram of the changes in earthworm populations (eg abundance, biomass, or diversity) observed in this study with increased frequency of flooding in crop, field margin, and pasture sites.

3C.2 Hypotheses

When comparing and contrasting the datasets from the pasture and arable sites, three broad hypotheses were considered:

1. That soil environmental factors would differ based both on the frequency of flooding events and the soil use, with soil bulk density higher, and soil moisture, pH, percent carbon, and percent nitrogen content lower in the crop soils and the rarely flooded regions than in the pasture soils or the frequently flooded regions.

2. That the earthworm population metrics of abundance, biomass, and Shannon diversity index would differ based both on the frequency of flooding events and the soil use, with all population metrics lower in the crop soils and the frequently flooded regions than in the pasture soils or rarely flooded regions.

3. The abundance of adult individuals of different species will differ based both on the frequency of flooding events and the soil use, with all species reduced with increased flooding frequency and within flooding soils, but with abundance varying based on earthworm ecotypes. As discussed in Sections 3A.2.2 and 3B.2.2, earthworms belonging to the endogeic ecotype seem to be the least vulnerable to the stresses of both arable crop soil uses and flooding, with the regularly flooded sites in both the crop, margin, and pasture sites dominated by the earthworm species *A. chlorotica*.

3C.3 Data analysis and statistical methods

Three categories were established for the frequency of flooding: rarely flooded, occasionally flooded, and frequently flooded. Three categories were established for soil use: pasture, crop, and margin. How the sites from Chapters 3A and 3B were categorised is summarised below in Table 3C-1. The riverbank sites from Chapter 3B were not used for this analysis, as they were not comparable to the other field sites surveyed in the study. The datasets were pooled across all sampling time periods.

Table 3C-1. The re-categorisation of the datasets from Chapters 3A and 3B for comparing the effects of flooding regimes and soil use across the whole dataset. The number of samples in each category is reported (n).

	Rarely flooded	**Occasionally flooded**	**Frequently flooded**
Pasture	Chapter 3A: pasture site, rarely flooded (n = 36)	(n = 0)	Chapter 3A: pasture site, fast- and slow-draining (n = 72)
Crop	Chapter 3B: Crop site, rarely flooded (n = 24)	Chapter 3B: Crop site, occasionally flooded (n = 24)	Chapter 3B: Crop site, regularly flooded (n = 24)
Margin	Chapter 3B: Field margin sites, rarely flooded (n = 24)	Chapter 3B: Field margin site, occasionally flooded (n = 24)	Chapter 3B: Field margin site, regularly flooded (n = 24)

When comparing the abundance of species, *A. longa* was excluded from the study, as no individuals of *A. longa* were found in the pasture field.

Three statistical analysis techniques were used for this chapter to address the hypotheses in Section 3C.2. Following testing for normality and heteroscedasticity, and tests of transformed datasets, the decision was made to use non-parametric statistical tests for this dataset. When considering hypothesis 3, that the abundance of adult individuals would differ across the different flooding frequencies and soil uses, only species that had sufficient abundances were used. *A. longa*, *L. terrestris*, and *L. castaneus* were excluded from the analysis, as within the crop, margin, and pasture soils across both datasets, their presence was low ($n > 20$ for each species).

To examine both the changes in the soil environmental factors and the changes in earthworm population metrics of abundance, biomass, and Shannon diversity, Generalised Linear Models (GLMs) were performed as non-parametric two way ANOVAs. To determine the interactions between species abundances with flooding and soil use, a three way GLM was performed on the total abundance of individuals belonging to the species *A. chlorotica*, *A. caliginosa*, *L. rubellus* and *A. rosea* across the species, crop use, and soil flooding regime.

3C.4 Results

3C.4.1 The effects of flooding and soil use on soil environmental factors

The outputs of GLMs performed between soil environmental factors of soil temperature, bulk density, soil moisture, pH, percent carbon, and percent nitrogen and the categories of flooding frequency, soil use, and the interaction term between them are summarised in Table 3C-2. With the exception of soil temperature, there was a significant effect of the frequency of flooding, the soil use, and the interaction term between the two for all soil environmental factors.

Table 3C-2. The outcomes of GLMs, performed as non-parametric equivalents of two way ANOVAs, to determine the differences between a number of soil environmental factors across different flooding frequency and soil use categories (n = 252)

Soil environmental factor	**Variable**	***P* value (DF, X^2)**
Mean temperature (°C)	Frequency of flooding	0.98 (2, 0.04)
	Soil use	0.93 (2, 0.14)
	Interaction term	0.88 (3, 0.65)
Bulk density (g cm^{-3})	Frequency of flooding	< 0.0001 (2, 103.81)
	Soil use	< 0.0001 (2, 697.85)
	Interaction term	0.01 (3, 11.82)
Soil moisture percentage	Frequency of flooding	< 0.0001 (2, 186.21)
	Soil use	< 0.0001 (2, 271.02)
	Interaction term	< 0.0001 (3, 44.39)
pH	Frequency of flooding	< 0.0001 (2, 85.81)
	Soil use	< 0.0001 (2, 90.58)
	Interaction term	< 0.0001 (3, 213.58)
Percent carbon	Frequency of flooding	< 0.0001 (2, 339.62)
	Soil use	< 0.0001 (2, 1157.45)
	Interaction term	< 0.0001 (3, 76.87)
Percent nitrogen	Frequency of flooding	< 0.0001 (2, 286.83)
	Soil use	< 0.0001 (2, 1510.57)
	Interaction term	< 0.0001 (3, 205.01)

Following the GLMs, Tukey post hoc tests were performed to determine where the significant differences lay between the flooding frequencies and soil uses.

The soil bulk density (Fig. 3C-2) was significantly lower with increased frequency of flooding, with the frequently flooded pasture site significantly lower than the rarely flooded pasture site. Crop and margin sites did not significantly differ in bulk density with frequent flooding, but in the occasionally flooded and rarely flooded sites, the bulk density was significantly higher under crop treatment than margin treatment. The soil moisture content (Fig. 3C-3) was significantly lower in the crop and margin soils than in the pasture soils in both the rarely flooded and the frequently sites. Across all flooding regimes, there was no significant difference between crop and margin sites, with the soil moisture content in the frequently flooded crop and margin sites not significantly different to the rarely flooded pasture site. The soil pH (Fig. 3C-4) showed differences across both flooding frequency and soil use. The pasture soils became more acidic with increased flooding, the margin soils moved towards neutrality with increased flooding, and the crop soils increased towards neutrality from rare to occasional flooding, but showed no significant difference between occasional and frequent flooding. The soil carbon percentage (Fig. 3C-5) was significantly higher in the frequently flooded soils than in the occasionally and rarely flooded soils. Soil percent carbon was significantly lower in the crop soils than the margin soils, but only in the rarely flooded soils, with no significant difference in the soil percent carbon between the crop and margin soils in the occasionally flooded and frequently flooded sites. The soil nitrogen percentage (Fig. 3C-6) was significantly higher in the frequently flooded pasture soils than in the rarely flooded pasture soils. Both rarely and frequently flooded pasture soils were significantly higher in soil nitrogen percentage than the crop and margin soils, which did not show a significant difference across any flooding regime.

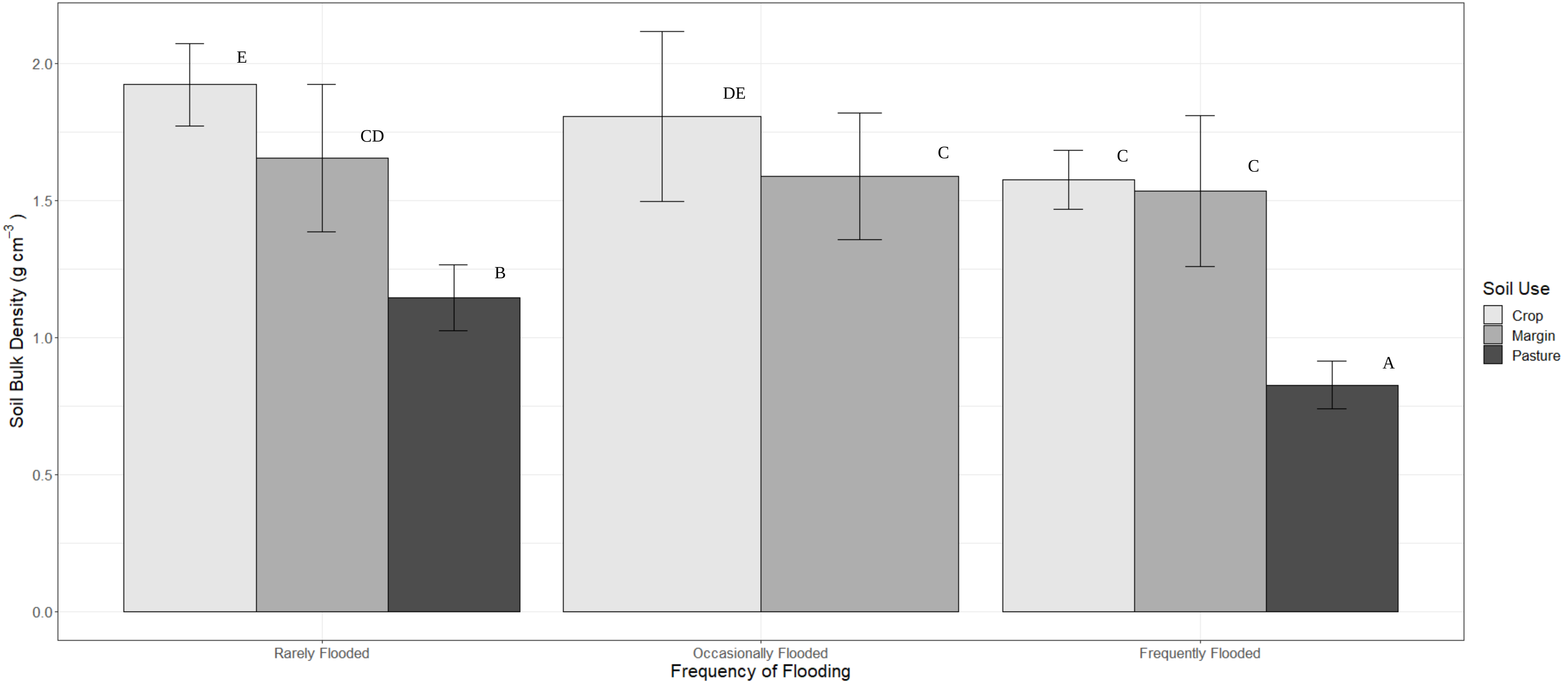

Figure 3C-2. The mean soil bulk densities at a depth of approximately 10 cm (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

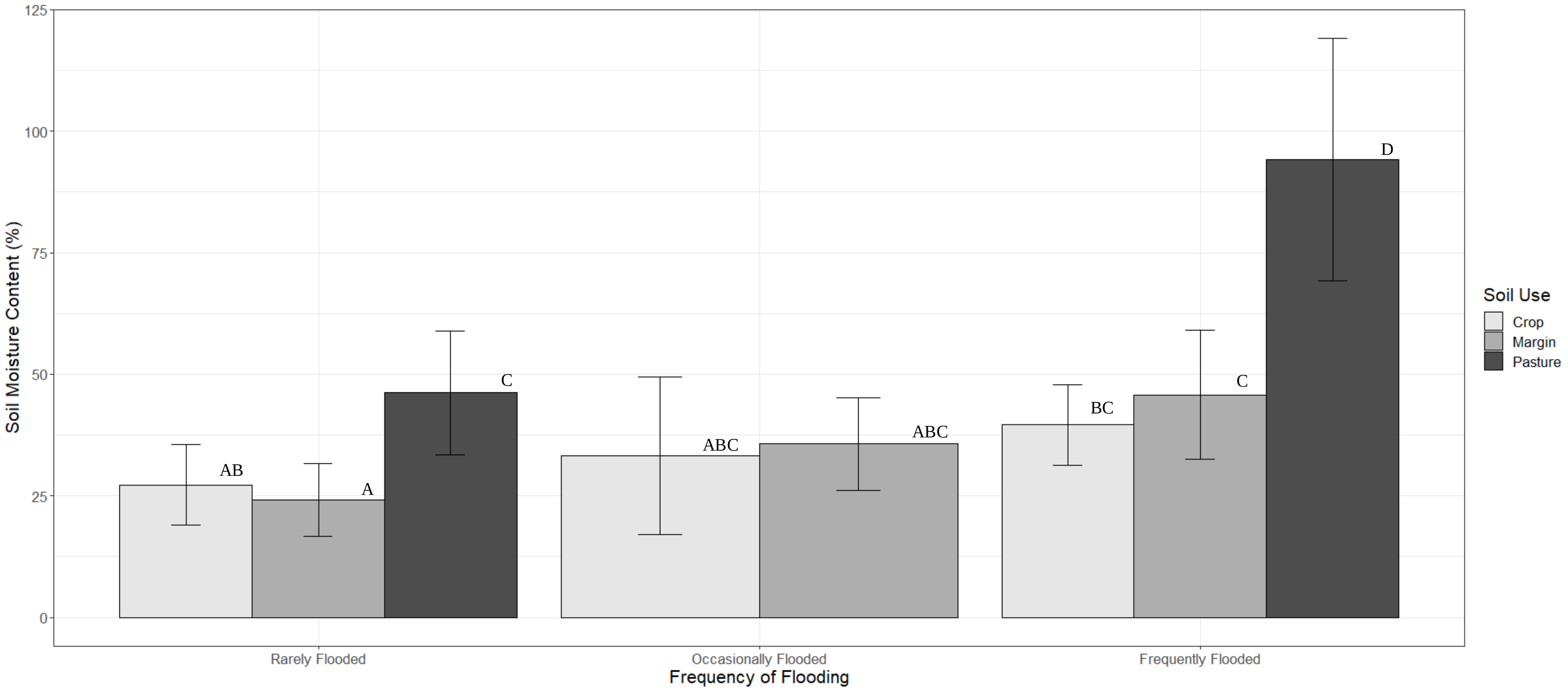

Figure 3C-3. The mean soil moisture content (%) at a depth of approximately 10 cm (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

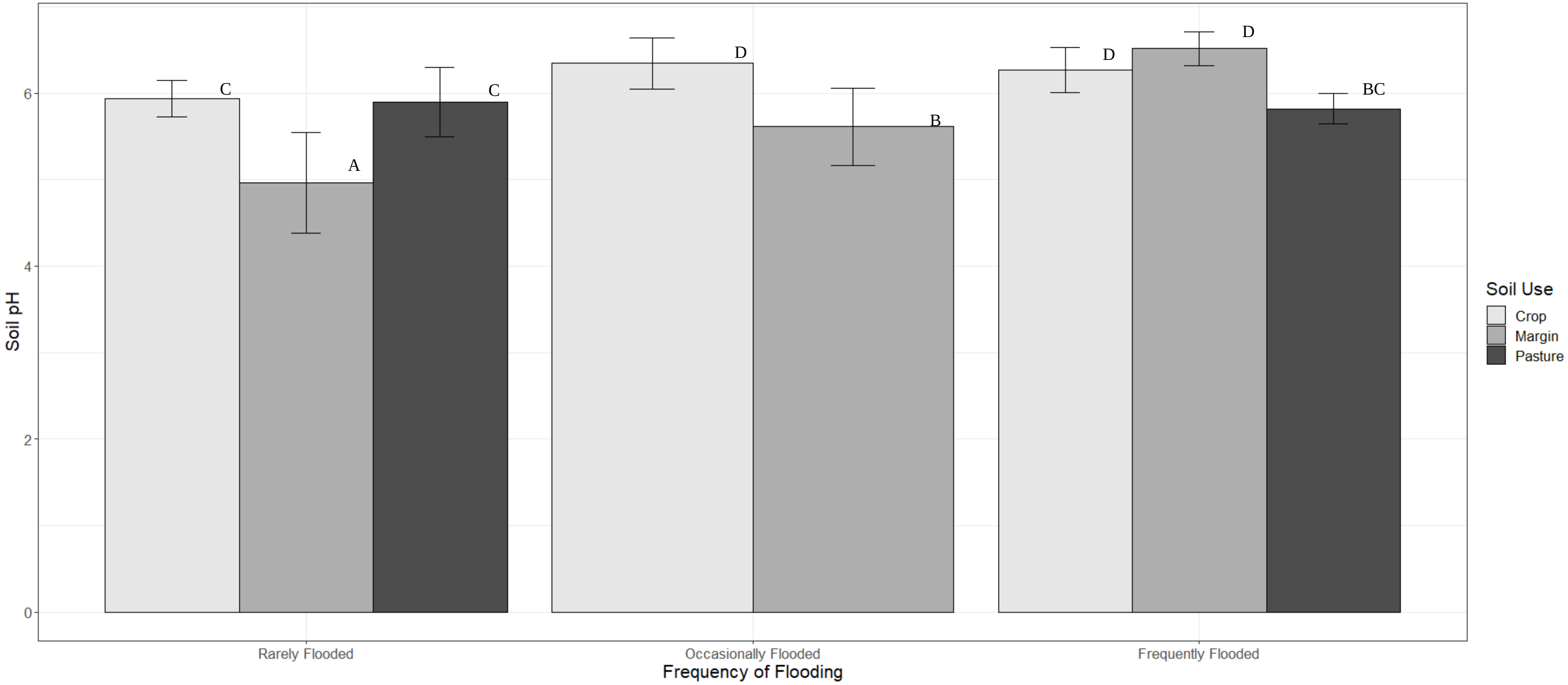

Figure 3C-4. The mean soil pH values at a depth of approximately 10 cm (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

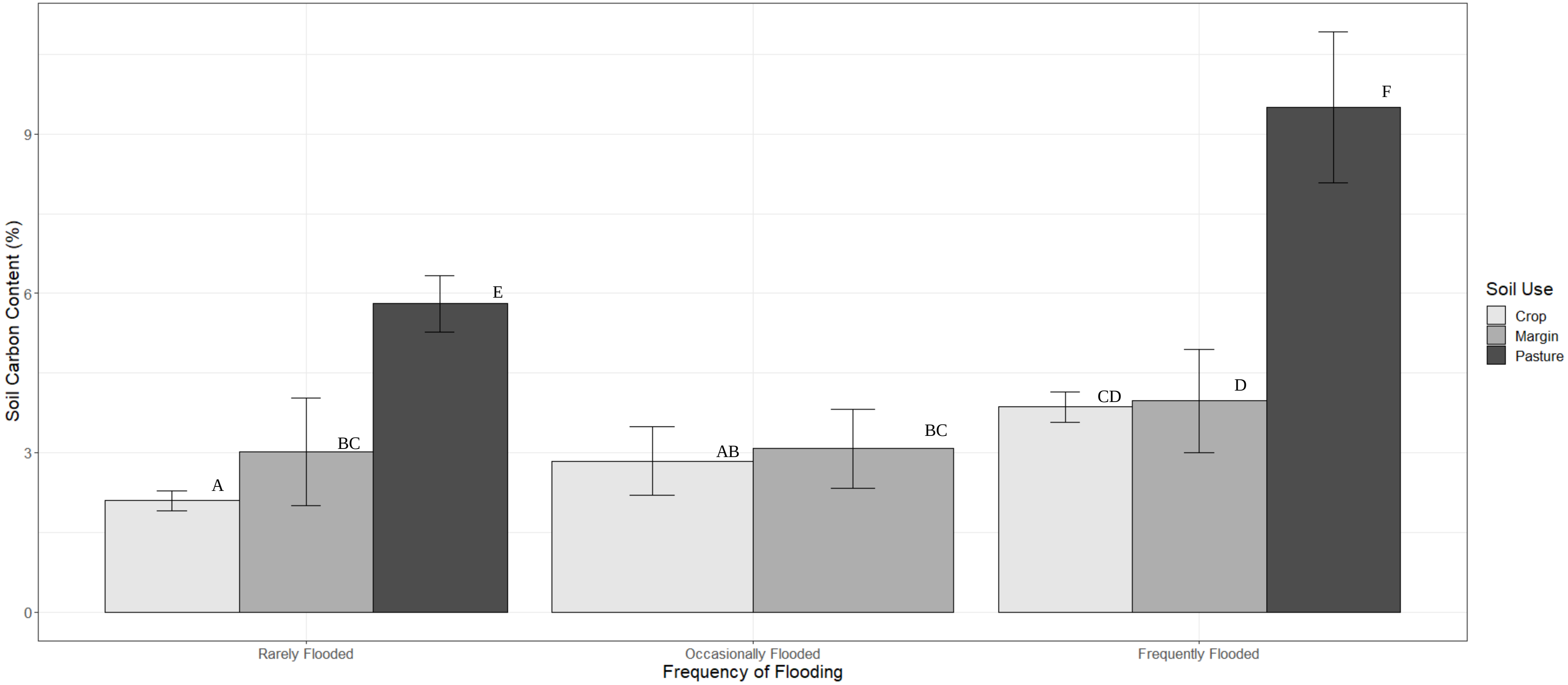

Figure 3C-5. The mean soil carbon content (%) at a depth of approximately 10 cm (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different ($P < 0.05$).

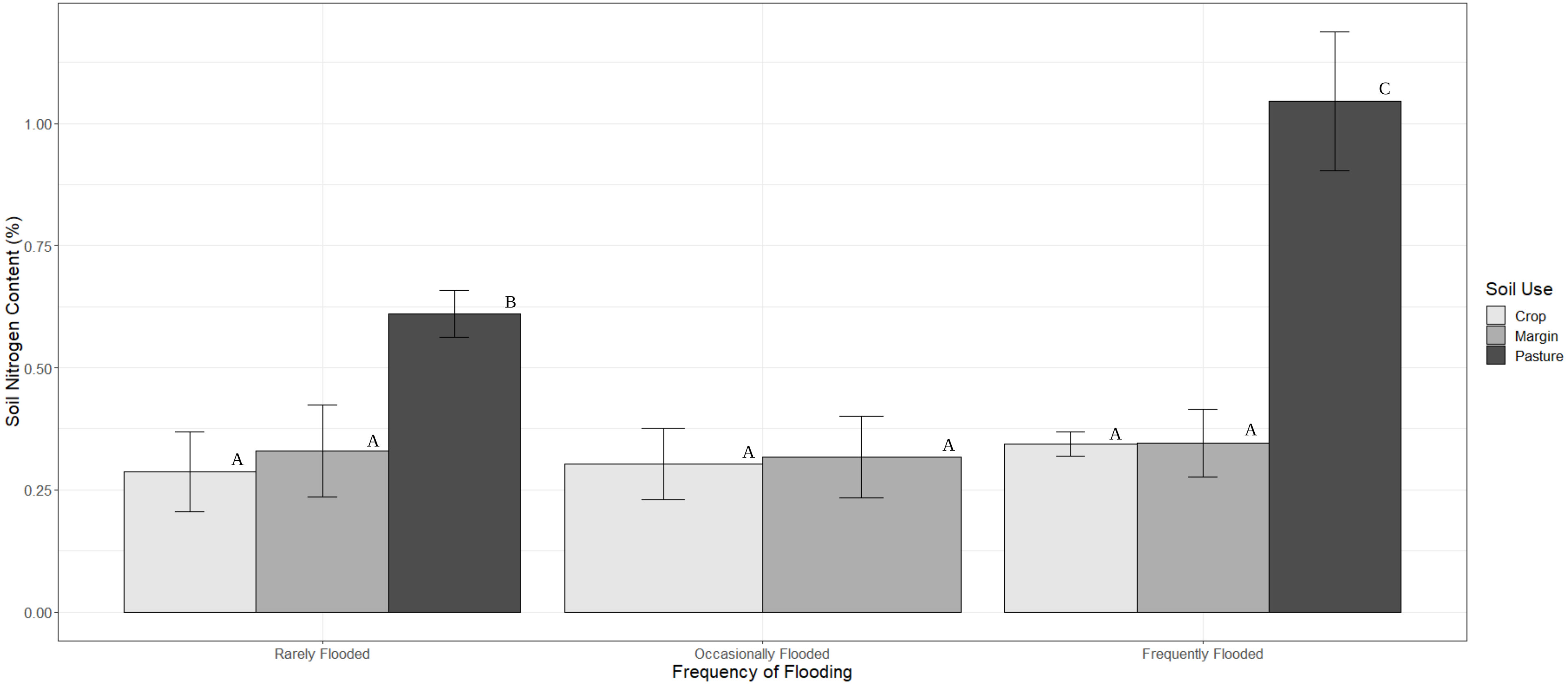

Figure 3C-6. The mean soil nitrogen content (&) at a depth of approximately 10 cm (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different (*P* < 0.05).

3C.4.2 The effects of flooding and soil use on earthworm population metrics

The outputs of GLMs performed between the earthworm community metrics of total abundance, total biomass, and Shannon diversity and the categories of flooding frequency, soil use, and the interaction term are summarised in Table 3C-3. There was a significant effect of the frequency of flooding, the soil use, and the interaction term between the two on the total abundance and total biomass of earthworms m^{-2}. Shannon diversity only showed a significant effect of soil use and the interaction term between the frequency of flooding and soil use categorisation.

Table 3C-3. The outcomes of GLMs, performed as non-parametric equivalents of two way ANOVAs, to determine the differences between a number of earthworm population metrics across different flooding frequency and soil use categories (n = 252)

Earthworm population metrics	**Variable**	***P* value (DF, X^2)**
Total abundance m^{-2}	Frequency of flooding	0.002 (2, 12.01)
	Soil use	< 0.0001 (2, 72.22)
	Interaction term	< 0.0001 (3, 30.26)
Total biomass (g m^{-2})	Frequency of flooding	0.020 (2, 7.62)
	Soil use	< 0.0001 (2, 25.58)
	Interaction term	0.009 (3, 11.66)
Shannon diversity index values	Frequency of flooding	0.210 (2, 3.13)
	Soil use	< 0.0001 (2, 30.43)
	Interaction term	< 0.0001 (3, 32.86)

Following the GLMs, Tukey post hoc tests were performed to determine where the significant differences lay within the interaction terms.

The total earthworm abundance (Fig. 3C-7) was significantly higher in the rarely flooded, pasture soil than in all other soil use and flooding frequency combinations. The lowest abundance of earthworms was observed in the frequently flooded, crop soil. However, this did not significantly differ from the other frequently flooded soils, the occasionally flooded soils, or the rarely flooded margin soils. This is potentially due to the absence of a pasture category for the occasionally flooded frequency category. A GLM performed with the occasionally flooded category removed found that the rarely flooded soils had a higher abundance of individuals than frequently flooded soils (*P* = 0.05).

The total earthworm biomass (Fig. 3C-8) was significantly higher in the rarely flooded pasture soils than in the frequently flooded crop soils, but did not significantly differ from the total earthworm biomass in the occasionally flooded soils or the frequently flooded margin and pasture soils. Within the rarely flooded soils, only the total biomass of earthworms in the margin soils was significantly lower than in the pasture soils. As with the total abundance of earthworms, it may be the case that the lack of a pasture category for the occasionally flooded soils is driving some of the statistical differences observed. A GLM performed with the occasionally flooded frequency removed found that the total earthworm biomass as significantly higher in the rarely flooded soils than the frequently flooded soils (*P* = 0.04).

The Shannon diversity index values (Fig. 3C-9) showed no significant difference between the rarely flooded margin, the occasionally flooded crop, and the frequently flooded pasture. The earthworm diversity of the rarely flooded pasture, the occasionally flooded margin, and the frequently flooded margin and pasture was significantly higher than the frequently flooded crop soil. There was no significant difference between either the occasionally flooded soil or the rarely flooded crop and margin soils.

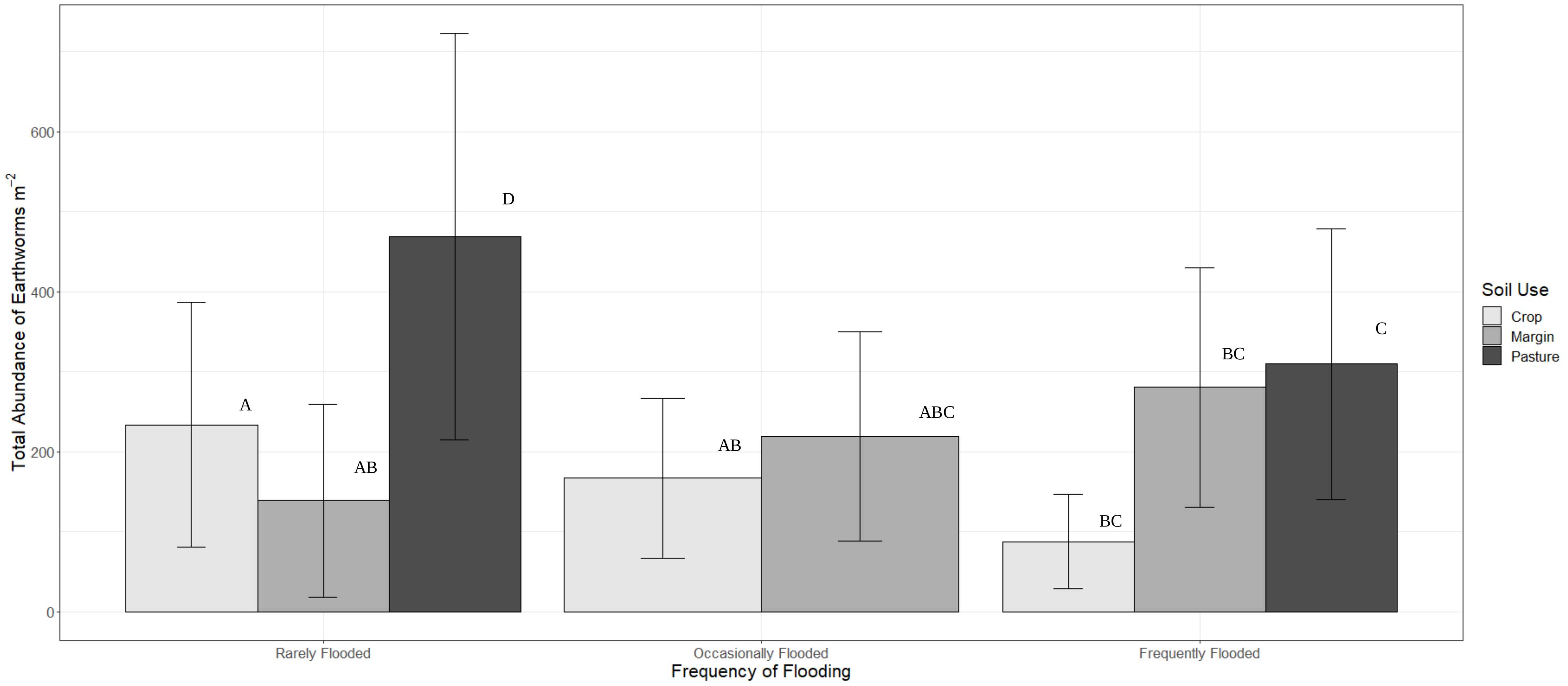

Figure 3C-7. The mean total earthworm abundance m^{-2} (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different (*P* < 0.05).

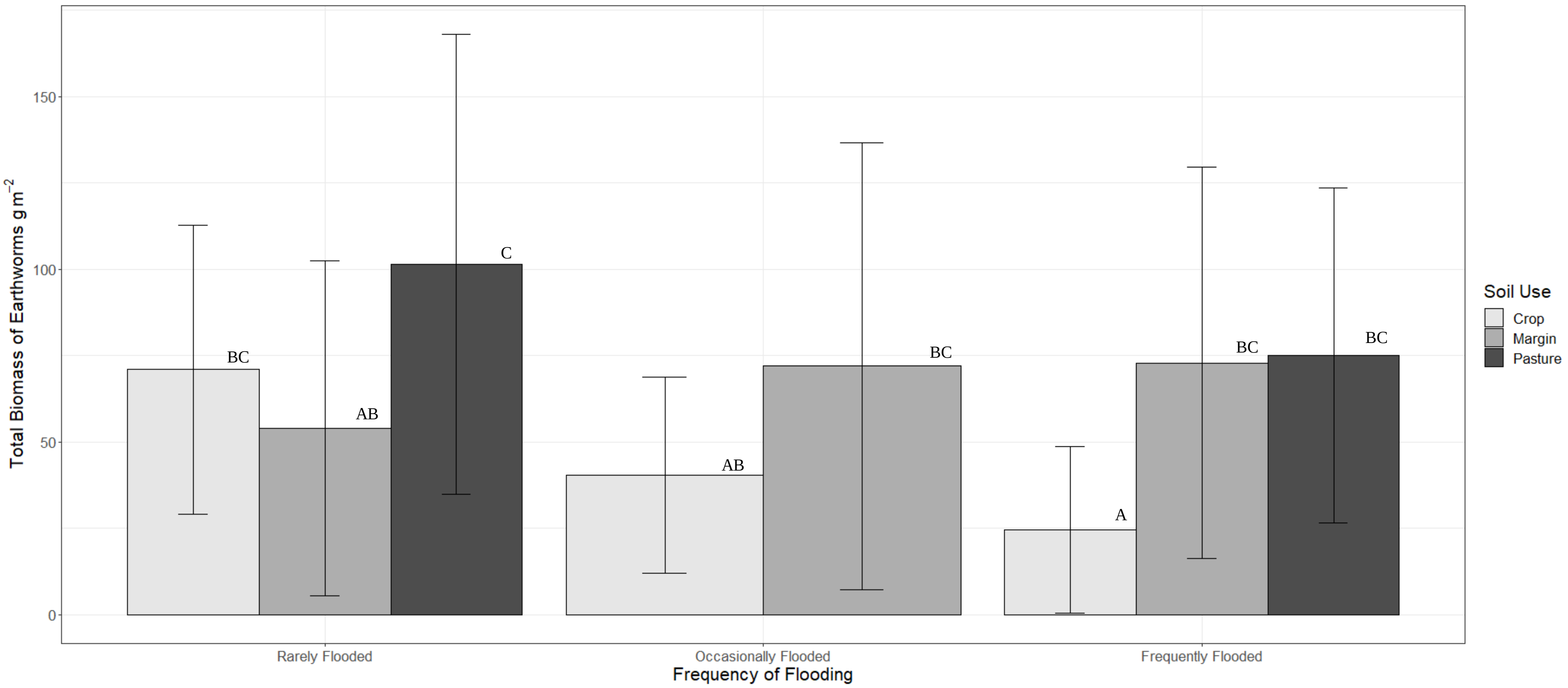

Figure 3C-8. The mean total earthworm biomass (g m^{-2}, n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different (*P* < 0.05).

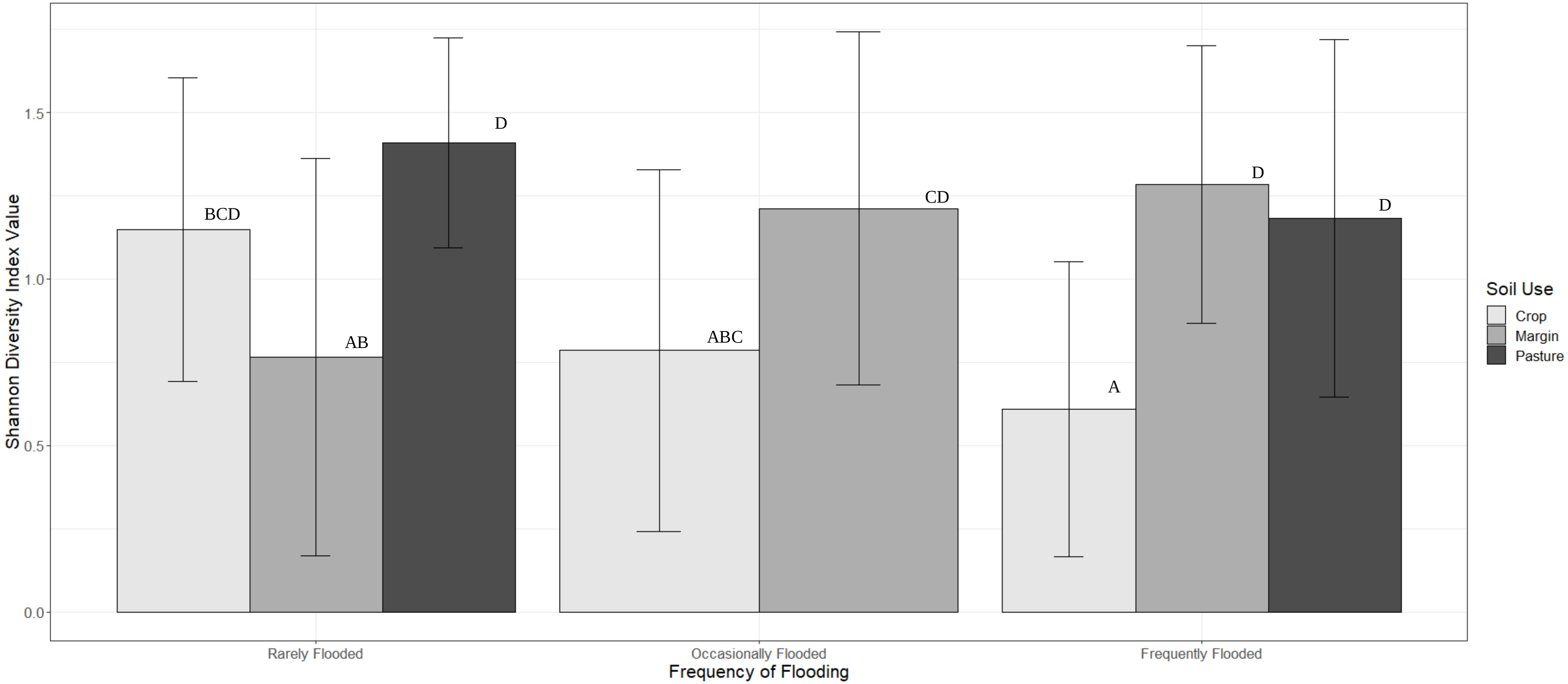

Figure 3C-9. The mean Shannon diversity index values (n = 252) for the different flooding frequencies and soil uses, with error bars showing standard deviation. Bars marked with the same letter are not significantly different (*P* < 0.05).

3C.4.3 The differences in the species composition of the adult earthworm population

A GLM comparing the effects of species, frequency of flooding, and soil use on the total abundance of individuals (Fig. 3C-10) found a significant difference in the abundance of individuals m^{-2} belonging to different species ($P < 0.0001$), between soil use categories ($P = 0.017$), and a significant interaction term between the species and soil use ($P = 0.010$). There was no effect of flooding frequency, and no interactions between flooding and the abundance of adult individuals of different species, flooding and soil use, or flooding, species, and soil use. Post hoc Tukey testing of the interaction term between soil use and species found that the abundance of *A. chlorotica* in occasionally flooded margin soils was significantly higher than all other combinations of soil uses and species. A GLM performed with the occasionally flooded category removed found no effect of flooding on the abundance of individuals of any species, but that the abundance of *A. chlorotica* was significantly higher in pasture soils than in arable soils.

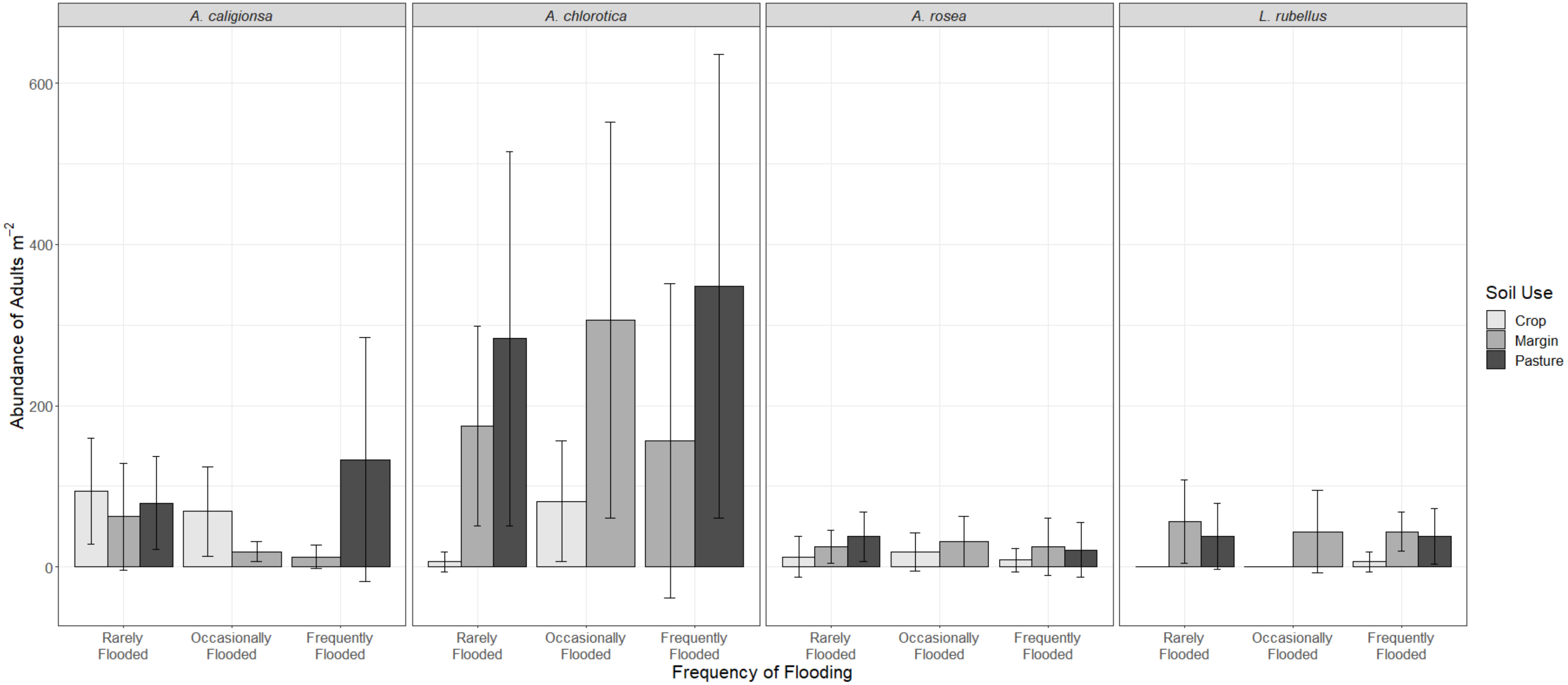

Figure 3C-10. The mean abundances of individuals m^{-2} of *A. caliginosa*, *A. chlorotica*, *A. rosea*, *L. castaneus*, *L. rubellus* and *L. terrestris* across different flooding frequencies and soil uses, error bars showing standard deviation.. The abundance of *A. chlorotica* in the margins soils was significantly higher than all other combinations of soil use and species.

3C.5 Discussion

3C.5.1 Interaction between soil environmental factors

Four key interlinked factors (soil bulk density, soil moisture, and percent carbon and nitrogen) differed significantly with the frequency of flooding, the soil use categorisation, and the interaction term between the two.

In both field sites, these factors are likely highly influenced by flooding. Flooding leads to increases in the organic matter contents of the soils; either through deposition of sediment (Johnston et al., 1984; Venterink et al., 2009) or the reduced rates of organic matter decay that occur under anaerobic saturated conditions, as discussed in Section 2.2.5, and Sections 3A.2.1 and 3B.2.1. This increase in organic matter leads to decreases in bulk density (Bronick and Lal, 2005), and increases in soil water holding capacity (Carter, 2002; Rawls et al., 2003), which leads to higher soil moisture capacity.

Arable fields typically have a lower organic matter content than pasture fields (Bradley, 2005), due to a number of factors such as low leaf litter input (Guo and Gifford, 2002), and the breaking up of aggregates that would otherwise protect soil carbon by farmland machinery (Beare et al., 1994; Follett, 2001). Arable fields also typically have higher bulk density than grazed pasture sites (Bharati et al., 2002), with the use of agricultural machinery leading to high levels of soil compaction even in low traffic crop fields (Hamza and Anderson, 2005). When comparing the values of soil bulk density and soil percent carbon and nitrogen across the dataset, the observed patterns were in agreement with these findings of previous studies, with higher bulk density, and lower percent carbon and nitrogen, in the crop soils than in the pasture soils. Despite these land use induced differences between arable and pasture soils, the same trends in response to flooding are observed. Increased flooding frequencies led to decreases in soil bulk density and increases in soil percent carbon, percent nitrogen, and the soil moisture percentage in all soils.

Soil pH, while significantly different based on the frequency of flooding, the soil use, and the interaction term between the two, did not respond to flooding in the same way in the arable and the pasture fields. In the pasture field, the soil is slightly acidic, and the pH value is significantly higher in the rarely flooded site than in the fast-draining and slow-draining sites. In the arable fields, the soil is acidic in the rarely flooded site, and significantly higher in the crop site than in the margin. As the flooding regime increased, the pH in the margin sites increased significantly, and the pH in the crop sites increased slightly, with the pH in the frequently flooded crop, margin, and river bank sites reaching near neutrality.

In the pasture site, the pH can likely be attributed to the accumulation of organic matter in the soil. In both the fast draining and slow draining site, the regular flooding leads to the accumulation of organic matter, due to the slow rates of organic matter depletion under anaerobic flooded conditions (Reddy and Patrick Jr, 1975). As the frequently flooded sites are not continuously under saturated conditions, when the water drains aerobic decomposition of organic matter begins. The decomposition of organic matter leads to the accumulation of H^+ ions in soil (Porter et al., 1980), leading to more acidic conditions in the frequently flooded regions of the pasture soil. In the arable site, the difference between the margin and the crop in the rarely flooded site can likely be attributed to the same mechanism: higher percent carbon and nitrogen in the margin than in the crop shows a greater content of organic matter available for decomposition, leading in turn to more acidic soil conditions. However, despite the fact that increases in organic matter occur with increased flooding, leading to decreases in bulk density and increases in the soil water holding capacity, the pH in the frequently flooded regions of the arable site increases rather than decreases. The cause of this is unknown, but may be attributed to farming practices of applying lime to neutralise the effect of fertiliser application (Goulding, 2016). This lime is soluble in water (West and McBride, 2005). Following rainfall, the lime may have dissolved and been carried down the slope in surface water, leading to its accumulation at the bottom of the slope in the frequently flooded region, while down slope movement of soil may transport undissolved pieces of lime to the frequently flooded region. This would have a neutralising effect on the expected increased soil acidity with increased soil carbon and nitrogen, and explain the pattern observed.

3C.5.2 Earthworm populations

As expected, the earthworm populations were lower in the arable site than in the pasture site (Section 3B.2.2; Curry et al., 2002; Boag et al., 1997), with significant differences between the total earthworm abundance and biomass across flooding frequency, soil use and the interaction term between the two. Shannon diversity showed a significant effect of soil use and the interaction between soil use and flooding frequency (Figs. 3C-7 – 3C-9). However, this difference between the arable field margin and crop sites and pasture field sites is only evident in the rarely flooded soils. In the frequently flooded soils, there is no significant difference in total earthworm abundance, biomass, or Shannon diversity index value between the field margin and the pasture soils, with both showing a significantly higher earthworm abundance and biomass than the crop soils.

At the ecotype level, both sites were dominated by endogeic earthworms, with the majority of these being individuals of *A. chlorotica*. The abundance of *A. chlorotica* was significantly higher than the abundance of all other earthworm species, with the next most common species being *A. caliginosa*. This high presence of both earthworm species is not unexpected, as a Natural England survey in 2014 found them to be the most common earthworm species in the UK, together comprising 53% of UK earthworm populations (Natural England, 2014).

The total abundance of individuals of *A. chlorotica* did not differ between the flooding regimes, but did show a response to soil use, being most abundant in the occasionally flooded margins. If the occasionally flooded soil category is excluded from the dataset, the abundance of *A. chlorotica* was higher in the pasture soils than in the crop soils, but showed no response to flooding. This distribution suggests that *A. chlorotica* populations were able to persist in the fields largely independent of any stresses caused by agricultural practice or the flooding regime, but showed an expected response to increased soil organic matter and decreased soil disturbance in the pasture sites.

In the arable site, as discussed in Section 3B.2.2, the combination of the stressors of flooding and land use result in lower earthworm populations in the frequently flooded sites. In the pasture site, where earthworm populations are not reduced by agricultural activity, flooding is the key factor affecting earthworm populations. In both Chapters 3A and 3B, it is endogeic earthworm species, particularly *A. chlorotica*, that are able to survive in these stressful conditions and dominate the field sites.

3C.6 Conclusion

Despite the differences in geographical location in the UK, with different environmental conditions and non-analogous flooding regimes, and differences in the soil uses between the arable and pasture field, flooding led to similar effects within the soil environment and on earthworm populations. In both the arable and pasture sites, flooding led to overall decreases in earthworm abundances, biomass, and diversity, and led to an increase in the soil organic matter content and soil moisture content, and decreases in soil bulk density.

Many of the soil environmental factors differed as expected between the arable and pasture field. The bulk density in the arable field was higher that the pasture field, while soil moisture, percent carbon, and percent nitrogen were lower. Soil temperature was closely associated with the time of year of sampling, and so showed no variation across the datasets with flooding frequency or soil uses. Soil pH was the only environmental factor to show a different response in the arable and pasture soils, with regular flooding associated with more acidic soils in the pasture field and more neutral soils in the arable field. At present, the reasons for these different pH responses to flooding frequency are not understood, but are likely a result of agronomic practices such as liming in the arable field.

Earthworm populations showed expected responses to arable and pasture treatments, with total earthworm abundance and biomass greater in pasture soils than arable soils. There was no response of the total abundances of individual species to flooding frequencies, but the endogeic earthworms *A. chlorotica* and *A. caliginosa* were the most abundant across all soil uses. Although the total abundance of individuals of different species did not change with flooding, there was a shift in species distribution, which led to increased proportions of the populations in frequently flooded sites belonging to the endogeic ecotype, as seen in Sections 3A.2.2 and 3B.2.2. This resulted in the dominance of *A. chlorotica* in the frequently regions of all field sites.

For soil environmental characteristics, flooding had an equalising effect on the differences between crop and margin soils. Although these characteristics still differed as expected from the pasture soils, with increased frequency of flooding there was no significant difference in the characteristics of bulk density, soil moisture, pH, percent carbon, percent nitrogen between the field margin and the crop soils. In the rarely flooded crop and margin sites, these variables all significantly differ, suggesting that increased frequency of flooding overrides some of the effects of land use on soil characteristics, likely by increasing the organic matter content of the soils. In contrast, the opposite pattern was observed for earthworm abundances. Total earthworm abundance, biomass, and diversity was not significantly different between the crop and margin soils in the rarely flooded regions. However, with increased frequency of flooding these parameters became significantly different; abundance, biomass, and diversity decreased in both soil uses but substantially more in the crop soils. While in the pasture soils frequent flooding also led to reductions in earthworm populations, the differences were not significant for earthworm diversity and total earthworm biomass. As suggested in Section 3B.2.2, this dataset shows that earthworm populations are reduced the most when subject to the dual stresses of agricultural land use and flooding. In England in 2019, an estimated 47.6% of arable land was cultivated using minimum tillage, with an additional 7% under no-tillage practices (Alskaf et al., 2019). Alskaf et al. (2019) found that a reluctance to take up reduced tillage practices by farmers and land managers was largely driven by a lack of knowledge about its implementation and benefits. Given the evidence in this study, where a combination of the soil stresses of flooding and agricultural land use were shown to reduce earthworm populations, and the evidence that earthworms greatly contribute to soil structure, soil stability, soil organic matter and nutrient cycling (Bertrand et al, 2015), this work may be used to help inform farmers in regularly flooded areas about how changing land management techniques may build a more resilient earthworm population.

Section 3C.5.1 discussed how flooding induces changes in soil environmental factors such as bulk density and soil moisture largely through the accumulation of organic matter. During a flooding event, it is this soil organic matter content that is rapidly broken down by soil microbes, leading to anaerobic conditions in the soil. In the context of this thesis it is important to understand how the application of a soil amendment known to also increase soil organic matter content, in the form of sewage sludge, leads to changes in soil oxygen availability during flooding events. Understanding how soil oxygen in flooded conditions changes with increased organic matter content, and how earthworm species respond to low oxygen environments, may explain the patterns of earthworm populations observed in these chapters.

3C.7 References

Alskaf, K., Sparkes, D.L., Mooney, S.J., Sjögersten, S., Wilson, P. 2019. The Uptake of Different Tillage Practices in England. *Soil Use and Management* 36(1): 27 – 44

Beare, M.H., Hendrix, P.F., Coleman, D.C. 1994. Water-Stable Aggregates and Organic Matter Fractions in Conventional- and No-Tillage Soils. *Soil Science Society of America Journal* 58(3): 777 – 786

Bertrand, M., Barot, S., Blouin, M., Whalen, J., de Oliveira, T., Roger-Estrade, J. 2015. Earthworm Services for Cropping Systems. A Review. *Agronomy for Sustainable Development* 35(2): 553 – 567

Bharati, L., Lee, K.-H., Isenhart, T.M., Schultz, R.C. Soil-Water Infiltration under Crops, Pasture, and Established Riparian Buffer in Midwestern USA. *Agroforestry Ecosystems* 56(3): 249 – 257

Boag, B., Palmer, L.F., Neilson, R., Legg, R., Chambers, S.J. 1997. Distribution, Prevalence and Intensity of Earthworm Populations in Arable Land and Grassland in Scotland. *Annals of Applied Biology* 130(1): 153 – 165.

Bradley, R.I., Milne, R., Bell, J., Lilly, A., Jordan, C., Higgins, A. 2005. A Soil Carbon and Land Use Database for the United Kingdom. *Soil Use and Management* 21(4): 363 – 369

Bronick, C.J., Lal, R. 2005. Soil Structure and Management: A Review. *Geoderma* 124(1-2): 3 – 22

Carter, M.R. 2002. Soil Quality for Sustainable Land Management. *Agronomy Journal* 94(1): 38 – 47

Curry, J.P., Schmidt, O. 2007. The Feeding Ecology of Earthworms – A Review. *Pedobiologia* 50(6): 463 – 477

Follett, R.F. 2001. Soil Management Concepts and Carbon Sequestration in Cropland Soils. *Soil and Tillage Research* 61(1-2): 77 – 92

Goulding, 2016. Soil Acidification and the Importance of Liming Agricultural Soils with Particular Reference to the United Kingdom. *Soil Use and Management* 32(3): 390 – 399

Guo, L.B., Gifford, R.M. 2002. Soil Carbon Stocks and Land Use Change: A Meta-Analysis. *Global Change Biology* 8(4): 345 – 360

Hamza, M. A., Anderson, W. K. 2005. Soil compaction in cropping systems - A review of the nature, causes and possible solutions. *Soil & Tillage Research*, 82: 121-145.

Johnston, C.A., Bubenzer, G.D., Lee, G.B., Madison, F.W., McHenry, J.R. 1984. Nutrient Trapping by Sediment Deposition and Seasonally Flooded Lakeside Wetland 1. *Journal of Environmental Quality* 13(2): 283 – 290

Natural England, 2014. Earthworms in England: Distribution, Abundance and Habitats (NECR145)

Porter, W.M., Cox, W.J., Wilson, I. 1980. Soil Acidity: Is it a problem in Western Australia? *Journal of the Department of Agriculture, Western Australia, Series 4* 21(4): 126 – 133.

Rawls, W.J., Pachepsky, Y.A., Ritchie, J.C., Sobecki, T.M., Bloodworth, H. 2003. Effect of Soil Organic Carbon on Soil Water Retention. *Geoderma* 116(1-2): 61 – 76

R Core Team 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reddy, K.R., Patrick Jr, W.H. 1975. Effect of Alternate Aerobic and Anaerobic Conditions on Redox Potential, Organic Matter Decomposition and Nitrogen Loss in a Flooded Soil. *Soil Biology and Biochemistry* 7(2); 87-94

Venterink, H.O., Vermaat, J.E., Pronk, M., Wiegman, F., van der Lee, G.E.M., van den Hoorn, M.W., Higler, L.W.G., Verhoeven, J.T.A. 2009. Importance of Sediment Deposition and Denitrification for Nutrient Retention in Floodplain Wetlands. *Applied Vegetation Science* 9(2): 163 – 174

West, T.O., McBride, A.C. 2005. The Contribution of Agricultural Lime to Carbon Dioxide Emissions in the United States: Dissolution, Transport, and Net Emissions. *Agriculture, Ecosystems & Environment* 108(2): 145 – 154

4 The availability of oxygen in flooded soil amended with sewage sludge

4.1 Abstract

When soil floods, dissolved oxygen is consumed during respiration by the soil microbial community, reducing the flooded soil to anoxic conditions within twenty-four hours. Sewage sludge, a by-product of the waste water treatment industry, is frequently disposed of in the UK by ploughing into agricultural soils. Sludge leads to many improvements in soil water retention, bulk density, and nutrient availability However, while the rate of oxygen depletion in flooded soils is understood, it is not currently understood if the increased soil organic matter caused by the addition of sewage sludge will lead to a faster rate of oxygen depletion in amended, flooded soil.

Seven soils selected to represent a range of textures (sandy loam, loamy sand, silty clay loam, silty clay, clay sandy loam and clay), pH values (range 6.69 – 7.98) and organic matter percentages (range 4.99% – 15.16%) were collected from around the UK. The soils were air dried, ground, and sieved to < 2 mm. After drying, soils were mixed with air dried anaerobically digested sludge in proportions representing 0, 25, 50 and 100% of the UK annual maximum application rate and added to 600 ml beakers fitted with oxygen sensor spots. Beakers were flooded with deionised water and the dissolved oxygen concentration within the soil and sludge mixtures measured regularly over a period of 24 hours. By calculating the gradient of the slope of the oxygen concentrations over the logged 24 hour period, the differences in the oxygen depletion rates across the soils and the differing biosolid application rates were determined. Repeated measure Analyses of Variance (RM-ANOVAs) indicated that oxygen depletion rates fitted a rapid depletion phase followed by a plateau phase. The effect of sludge addition and soil characteristics on these depletion and plateau phases was determined. The sludge application rate had no significant effect on the rate of oxygen depletion across the twenty-four hour period, but a significant effect during the depletion phase. During the depletion phase, there was no significant effect of pH or soil organic matter content on the gradient of oxygen concentration, but there was a significant effect of soil texture and of the percentage increase in organic matter content. Over the twenty-four hour period, there were significant associations between soil pH, soil organic matter content, and soil texture on the gradient of oxygen depletion.

As sludges have already undergone anaerobic digestion, the majority of the available organic matter has already been broken down, leaving only recalcitrant organic matter. This suggests that while there may be a short term effect of sewage sludge addition on rates of oxygen depletion in flooded fields, over long time periods the effect of field organic matter content is more important in determining rates of oxygen depletion during a flooding event.

4.2 Introduction

Sewage sludge is a by-product of the wastewater treatment industry. This by-product is also known as biosolids and must be disposed of. While options for disposal include landfill, incineration, and, until it was banned in the UK in 1998, discharge into the sea or surface waters, the most common disposal method is the application of sewage sludge to agricultural soil (DEFRA, 2002). In 2010, over one million tonnes of sewage sludge were reused by application to soil and agriculture, accounting for approximately 80% of sewage sludge disposal in the UK (DEFRA, 2012). Similarly, in 2006 in the US and Canada, 60% of sewage sludge was disposed of through application to land (Cogger, 2006). As well as providing a viable route for disposal, the practice of applying sewage sludge to soil provides a number of benefits for soil quality. The application of sewage sludge has been proven to improve soil porosity (Pagliai et al., 1983), reduce the bulk density of soils (Hemmat et al., 2010), and increase the organic matter content of the soil (Tian et al., 2009).

The Sludge (Use in Agriculture) Regulations state that *"The sludge shall be used in such a way that…the quality of the soil and of the surface and ground water is not impaired*" (Public Health, 1989). There are no specific guidelines regarding the application of sludge to fields which are regularly subject to flooding, or in fields that are likely to flood. As UK rainfall patterns begin to shift towards seasonal extremes with anthropogenic climate change (Hulme, 2002), flooding is predicted to increase in both intensity and frequency (Prudhomme, 2003; Kay et al., 2006). There is the possibility that areas which have historically not been at risk of flooding, and have had regular application of sewage sludge, may now be subject to flooding.

Flooded soils can reach anoxic conditions within twenty-four hours (Ponnamperuma, 1984). Anoxia within flooded soils is caused by the rapid consumption of soil oxygen by soil bacteria involved in aerobic respiration of available soil organic matter. As soil oxygen reduces, anaerobic respiration begins to dominate, with bacterial activity moving down the REDOX chain (Bell, 1969; Tanji et al., 2003) once the soils have become anoxic. The rate at which these flooded soils become anoxic is likely to be governed by a number of factors which influence microbial respiration. Microbial respiration in the upper layers of soil increases with temperature (Smith et al., 2003), particularly when soil water content is above the range of 14 – 20% (Xu and Qi, 2001; Rey et al., 2002), a relationship that continues to be observed in flooded soil (Devêvre and Horwáth, 2000).

Considering that the application of sludge increases the organic matter content of soils, and that soil organic matter content is one of the controlling variables on the rate of oxygen depletion when soils flood, it may be expected that the increased organic matter caused by the addition of sludge leads to soils becoming anoxic more rapidly when flooded. Extended periods of anoxia in soil causes flooding injury to plants, inhibiting growth of both above and below ground plant matter (Kozlowski, 1984). Although many terrestrial invertebrates are able to tolerate anoxic conditions to some extent (Schmitz and Harrison, 2004), extended periods of soil anoxia induced by flooding do lead to changes in soil invertebrate community structure (Plum, 2005). The addition of organic matter in the form of sludge to these soils may mean that the soils reach an anoxic stage earlier than soils unamended with organic matter, causing these soils to experience a longer period of anoxia. In turn, this may lead to greater degrees of flooding injury in plants, more extreme changes to the soil invertebrate communities, and a longer recovery time for these communities after flooding events.

The field observations in Chapter 3 found that earthworm populations in both pasture and arable sites varied depending on the flooding regime, but that there were also differences in some soil properties between the regularly flooded and rarely flooded regions of the soil, such as organic matter content, bulk density, and pH. These differences in soil properties are likely to have been caused by the differing regular flooding regimes across the sites. Earthworm populations differed across the two sites, potentially due to earthworm death from the flooding events, differing reproductive rates of earthworm populations between the sites, or due to earthworms evacuating the flooded soils. There are two potential hypotheses for the differences in the soil organic matter content and the earthworm populations with the differing flooding regimes. The first is that the reduced earthworm populations in the regularly flooded regions leads to a preservation of the soil organic matter content. The second is that the higher soil organic matter content may lead to a longer period of anoxia in a flooding event, which would in turn lead to a reduced rate of earthworm survival, with the soil organic matter causing the effect seen in the earthworm populations. This chapter tests the first portion of the second alternative hypothesis, whether an increased organic matter content leads to a more rapid rate of oxygen depletion. The chapter goes on to test whether artificially increasing the soil organic matter content through the application of sludge leads again to a faster rate of oxygen depletion in flooded soil.

In order to examine the rates of oxygen depletion in flooded soils with increased organic matter content, soils from locations around the UK were characterised, amended with varying levels of sewage sludge and then flooded. Two broad hypotheses were considered:

1. A higher sludge loading percentage leads to a higher rate of oxygen depletion in flooded soil, due to the increased organic matter content of the soil that sludge addition causes.

2. The rate of oxygen depletion in flooded soil is also affected by soil characteristics such as pH, soil texture, and soil organic matter content.

4.3 Methods

4.3.1 Sewage sludge

Anaerobically digested sewage sludge was collected from the Esholt water treatment works in West Yorkshire (postcode BD10 0TW) and kept in a field moist state at 4°C until use. The maximum field application rate of sewage sludge to soil is 250 kg of nitrogen per hectare (ADAS, 2014). In order to scale this to the surface area of the beakers, the nitrogen content of the sludge was determined. Three sludge samples were air-dried for twenty four hours. Sludges were air dried rather than oven dried to prevent loss of organic material through drying at high temperature (Jager and Bruins, 1975). The samples were weighed before and after drying in order to determine a dilution factor due to the sludge moisture content. The dried sludge was finely ground in a ball mill, and the carbon and nitrogen content of the samples determined using a Vario Macro C/N Analyser. A certified organic analytical standard of Peaty soil from Elemental Microanalysis Ltd (B2176 – batch 133519), gave recoveries of 97% (± 2.21) and 100% (± 2.94) percent of 15.95% C and 1.29% N respectively. The carbon and nitrogen values were then averaged to find the carbon and nitrogen quantities of the sludge. Sludge properties of percent carbon, percent nitrogen, and pH are summarised in Table 4-1. Triplicate samples of sludge were air dried, and mixed with deionised water at a 1:5g ratio. Sludge pH was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers.

Table 4-1. Mean and standard deviation of the measured percent carbon, percent nitrogen, and pH values of the sludge collected from the Esholt water treatment works.

Sludge property	Mean (Standard deviation)
Percent carbon	30.99 (± 0.41)
Percent nitrogen	4.16 (± 0.09)
pH	7.99 (± 0.27)

In order to determine the percent N of the wet weight sludge, the percent N of the dry weight was divided by the dilution factor: (Equation 4-1)

Equation 4-1. The mathematical determination of the percent nitrogen present in wet weight sludge

$$\frac{\mathbf{4.156}\ (\%\ \mathbf{Nitrogen})}{\mathbf{3.0747}\ (\mathbf{Dilution\ factor})} = \mathbf{1.35\%\ N\ in\ wet\ sludge}$$

Using this value, a metric tonne of the wet sludge would contain 0.00135 tonnes of nitrogen (13.5 kg).

Having determined the nitrogen content of the wet sludge, it then had to be determined how many tonnes of wet sludge from this sample would need to be applied to a hectare to achieve the maximum application rate (250 kg N/ha) (Equation 4-2)

Equation 4-2. The mathematical determination of the tonnes per sludge to be applied per hectare to achieve 250kg N ha^{-1}

$$\frac{250\ (\text{maximum kilograms N per Ha})}{13.5\ (\text{kilograms N per metric tonne})} = 18.5\ \text{tonnes sludge per ha}$$

Finally, by knowing the surface area of the soil used in the experiment, determined by measuring the inner diameter of the beakers, the experimental application rate of the sludge was determined. (Equation 4-3)

Equation 4-3. The mathematical determination of the grams of sludge to be applied to the surface area of the beaker to achieve the equivalent of 250kg N ha^{-1}

$$18.5\ \text{(tonnes per hectare)} \times\ 6.939\ x\ 10^{-7}\text{(hectares per beaker)}$$
$$= 12.8\text{g sludge per beaker}$$

12.8 g of sludge per beaker achieved the maximum rate of application, allowing for the equivalent of 250 kg ha^{-1}. This value was then scaled to achieve application rates of 50% (6.4 g per beaker), 25% (3.2 g per beaker) and 0% (0 g per beaker).

4.3.2 The soils

Soils were obtained from a number of locations around the UK. Details of the locations and the soil properties are listed below in Table 4-2. The soils were selected to represent a range of soil textures and organic matter contents. As soil organic matter content could not be determined until the soils were back in the laboratory, soils from a range of arable, allotment, and pasture uses were selected to ensure that a range of organic matter contents would be represented. Soil texture was determined by hand texturing. Soil pH was determined by mixing 10 g of dried soil with 40 ml of deionised water and using a shaker to thoroughly mix. After allowing sediment to settle, soil pH was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers. Soil organic matter percentage was determined through loss on ignition of triplicate samples.

Table 4-2. The description, location of origin, soil type, pH and soil organic matter content of the experimental soils used in the flooding experiments

Soil description and origin	Grid reference	Soil texture	Soil pH (mean, range)	Soil organic matter (mean, range)	Percent increase in organic matter at 100% application rate of sludge
Arable soil - Spen Farm, near Leeds	SE 44300 41700	Clay sandy loam	7.69 (7.51 – 7.79)	6.56% (6.26% - 6.84%)	33.14%
Arable soil - Loddington, Leicester	SK 78900 02400	Clay	7.82 (7.71 – 7.89)	7.86% (7.81% - 7.92%)	38.34%
Arable soil – Heslington, York	SE62800 49600	Silty clay	7.62 (7.59 – 7.67)	4.29% (4.26% - 4.32%)	40.23%
Allotment soil – Mansfield, Nottinghamshire	SK 55500 61100	Loamy sand	7.25 (7.22 – 7.27)	13.49% (12.86% - 14.26%)	12.79%
Allotment soil – Newcastle	NZ 24800 66900	Sandy loam	7.26 (7.24 – 7.78)	11.20% (7.92% - 19.28%)	15.41%
Allotment soil – Cusgarne, Cornwall	SW 76300 40900	Loamy sand	6.69 (6.57 – 6.83)	15.16% (14.93% - 15.62%)	11.38%
Pasture soil – Heslington East Campus, University of York	SE 63900 50300	Silty clay loam	7.98 (7.94 – 8.01)	4.99% (4.86% - 5.13%)	34.58%

Following the determination of soil properties, soils were air dried over a number of days in the laboratory. The dried soils were ground by hand in a large pestle and mortar to break up any very large aggregates. The ground soils were then sieved to < 2 mm. Any large stones, plant matter or litter were removed and discarded, with large soil aggregates being returned to the pestle and mortar for further breakdown before being sieved again.

4.3.3 Experimental design

Sixteen 600 ml glass beakers were each prepared with two Presens Oxygen Sensitive Spots on opposite sides of the beaker at a height of 10 cm; approximately half way up the soil layer when the beakers were filled. The Presens Oxygen Sensitive Spots are spots approximately 5 mm in diameter consisting of a fluorescent indicator dye, sensitive to oxygen, embedded in a polymer matrix. Light emitted from an LED causes emission of fluorescence from the spot. Oxygen molecules near the sensor causes the excess energy to transfer to the molecule, leading to a decrease of the fluorescence signal. By using a fibre optic cable connected to a computer and the bespoke software, the signal from the sensor spot is translated into a measure of the oxygen concentration within the beaker (Presens, 2016).

Four replicates each of 100%, 50%, 25% and the control of 0% sludge loading were prepared. For each beaker, 400 g of dried soil was weighed out into a separate container. The finely ground dried sewage sludge was added and thoroughly mixed by hand into the dried soil. The soil and dried soil mix was moistened with 100 ml of deionised water, and the soil-sludge mixture thoroughly stirred again. Finally, the rewetted soil and sludge mixture was transferred to the beaker, where it was stirred for a final time to ensure that the sludge was as well distributed through the mixture as possible. Controls were treated in an identical fashion, but without the addition of sewage sludge.

Once the mixtures were in the beakers, care was taken to ensure that, despite some aggregation having begun with the rewetting of the soils and the stirring, there were no large air pockets behind the sensor spots, as this could result in air pockets when the beakers were flooded.

The filled beakers were transferred to a controlled temperature room maintained at 15°C and left for approximately 18 hours in their unflooded, moist state. This was to allow for the soil to settle, as dried soil can exhibit a ‘burst’ of microbial activity when rewetted (Stevenson, 1956).

4.3.4 Measurements

Measurements began at 9 am, when the beakers were each flooded with 300 ml of deionised water. The dissolved oxygen within the soil mass was recorded using the sensor spots, while the dissolved oxygen concentration of the surface water and the surface water temperature was measured using a Thermo Scientific Orion Star A223 and Star A23 Portable Dissolved Oxygen Meter. Once flooded, measurements of the dissolved oxygen and surface water temperature were taken every two hours for the following ten hours until 7 pm, with a final measurement taken at 9 am the following morning, 24 hours after flooding. Following the final measurement, a sample of surface water was siphoned from the beaker, and a pore water sample extracted by centrifuging a sample of the flooded soil. The pH of the surface and the pore water samples was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers, and the samples frozen for potential later analysis.

4.3.5 Data analyses and statistics

Data were analysed using R version 3.4.2.

Data were investigated to try and identify a linear relationship between the concentration of oxygen in the flooded soil and time. Of the transformations investigated, plots of oxygen concentration against log time gave the best approximation of linear trends. Initially the entire dataset was considered; the gradient of plots of oxygen concentration against log time was calculated using a linear model for each replicate of each combination of soil origin and sludge loading level. A subsequent analysis performed repeated measure analyses of variance (RM-ANOVAs) on the dissolved oxygen concentration over time for each soil replicate at each sludge loading percentage. The time point at which the mean dissolved oxygen concentrations ceased to statistically differ was selected as the transition point between a ‘depletion’ phase and a ‘plateau’ phase. After the transition point had been identified, linear models were performed for each repetition of each soil origin and sludge loading level for both the depletion and the plateau phases, over the log transformed hour value, giving the gradient of the oxygen concentration over log time. When interpreting the data, a more negative gradient was indicative of a faster rate of oxygen depletion. A gradient closer to zero indicated a slower rate of oxygen depletion.

The soils were grouped into three categories based on the original land use: allotment, arable, or pasture. As the Cusgarne soil had been obtained from a section of garden regularly used for growing vegetables and flowers, it was classified as allotment soil, as it had not been continuously under grass as expected of a pasture.

To determine if soil characteristics varied between the soils, the data were first assessed for intercorrelation, with the characteristics of pH, texture and organic matter percentage compared to each other using one way ANOVAs, Spearman’s Rank Correlations, and linear models respectively. Initial testing of the datasets showed that, while the data met normality testing assumptions for the logged oxygen depletion rates, there were high levels of intercorrelation between the soil characteristics of pH, organic matter and soil texture. Because of this, the data could not be combined into one large multivariate model, but instead each soil characteristic was separately compared against the logged oxygen depletion rate. To compare the soil characteristics of pH and organic matter percentage between the soil uses of allotment and arable soils, Mann-Whitney U tests were performed. As only one measure of pH or organic matter percentage was present for the pasture soil, it was not included in this portion of the statistical analysis. To address the two hypotheses listed in Section 4.2, the following statistical analyses were performed:

1. To determine the effect of sludge loading percentage on the gradient of oxygen concentration depletion, the 24 hour dataset and depletion and plateau phase datasets were approached separately. Within each dataset, two way ANOVAs were performed comparing the gradient of oxygen concentration to the soil origin and sludge loading percentage, and comparing the gradient of oxygen concentration to the soil use and sludge loading percentage. To determine if the gradients between the ‘depletion’ and ‘plateau’ phases of the exponential curve were significantly different, a Wilcoxon Signed Ranks test was performed comparing the two datasets.

2. To determine the effect of soil characteristics such as pH, soil texture, and soil organic matter content on the gradient of oxygen concentration depletion, the gradients of oxygen data in the 24 hour dataset, depletion phase and plateau phases were also considered separately. Within each dataset, linear models were performed on the gradient of oxygen concentration and the soil pH, soil organic matter content, and the percentage increase in soil organic matter that the application of sewage sludge represented. One way ANOVAs were then performed to determine if the gradient of oxygen concentration varied with soil texture.

4.4 Results

Plots illustrating the oxygen depletion for the soils for the different sludge loading percentages can be found in the supplementary information for this chapter, Section 4.8. Section 4.8.1.1 contains the untransformed oxygen depletion curves, while Section 4.8.1.2 contains the oxygen depletion curves over log time.

4.4.1 Relationships between soil characteristics

All the soil characteristics are highly intercorrelated. Significant relationships between the soil characteristics are summarised in Table 4-3.

Table 4-3. A summary of the statistical test outputs of intercorrelated soil characteristics, showing the characteristic, test performed, *P* values and other test values

	Soil pH	**Soil organic matter percent**	**Percent increase in soil organic matter content**
Soil texture	ANOVA $P < 0.01$, $df = 5$, $F = 127.5$	ANOVA $P < 0.01$, $df = 5$, $F = 1558$	ANOVA $P < 0.01$, $df = 5$, 5.315
Soil pH	▨	Spearman's rank correlation $P < 0.01$, $r_s = -0.76$	Spearman's rank correlation $P < 0.01$, $r_s = 0.32$
Soil organic matter percent	▨	▨	Linear model $P < 0.01$, $df = 107$, $F = 25.17$

A Mann-Whitney U test found a significant difference between the pH values of the different soil uses ($P < 0.0001$, Figure 4-1), with the pH of allotment soils significantly lower than the arable soils.

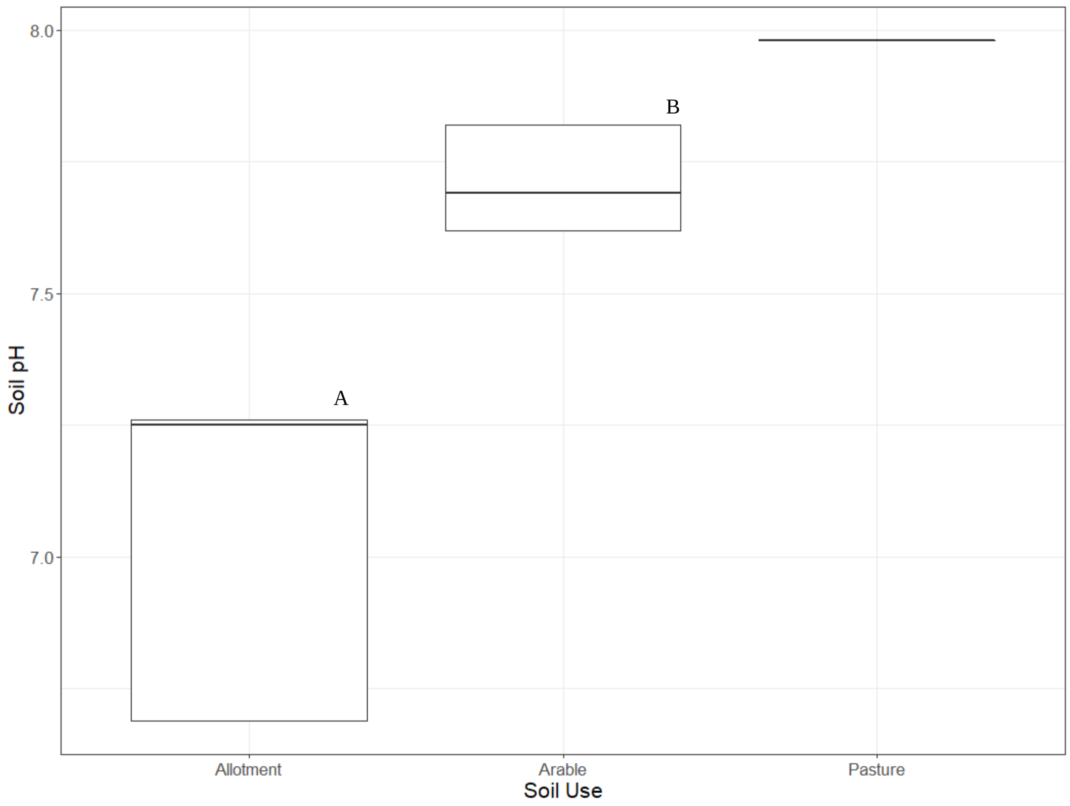

Figure 4-1. The pH measures of all soils, grouped by soil uses of allotment (n=3) and arable (n=3), and pasture (n=1), with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. As there was only one measurement for the pasture soils, they were not included in this portion of the statistical analysis. Soil uses that do not differ significantly (*P* < 0.05) are marked with the same letter.

A Mann-Whitney U test found a difference between the organic matter percentage of the soils (*P* < 0.0001; Figure 4-2), with the percent organic matter content of the allotment soils significantly higher than the arable soils.

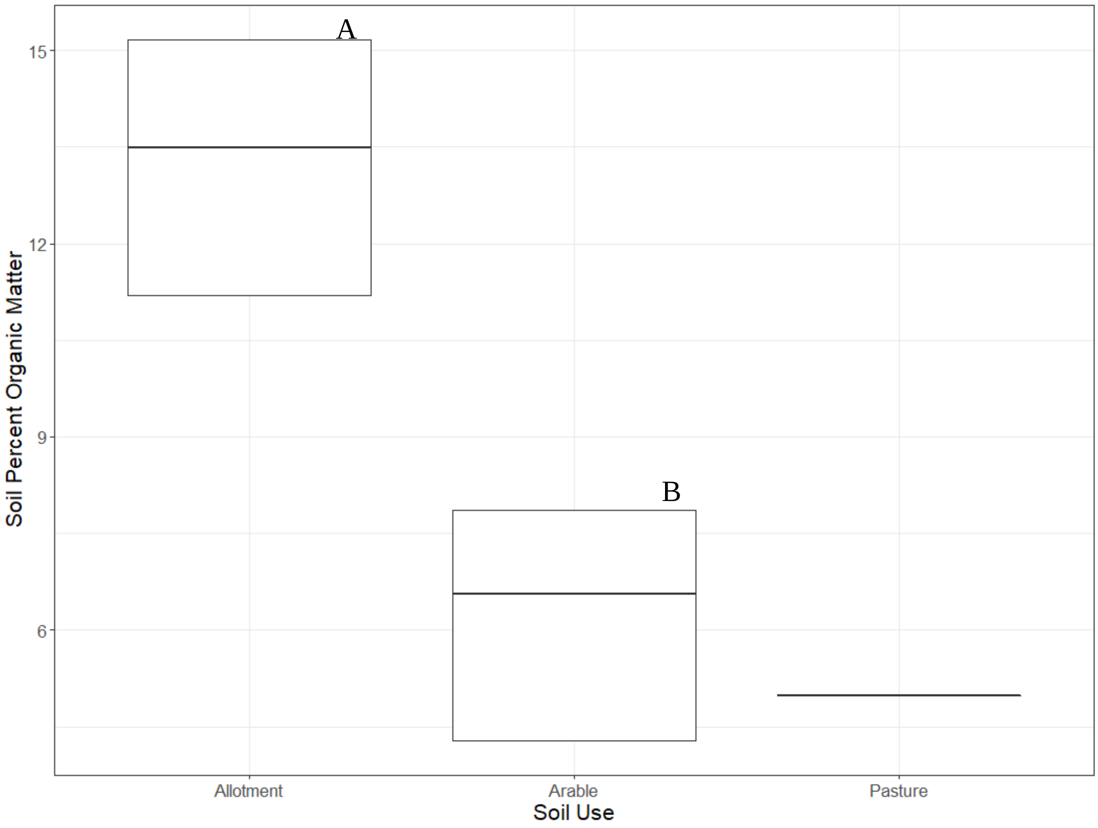

Figure 4-2. The triplicate percent organic matter measures of all soils, grouped by soil uses of allotment (n=9), arable (n=9), and pasture (n=1), with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. As there was only one measurement for the pasture soils, they were not included in this portion of the statistical analysis. Soil uses that do not differ significantly (*P* < 0.05) are marked with the same letter.

4.4.2 Twenty-four hour period

4.4.2.1 The effects of sludge loading and soil on oxygen concentration

The first hypothesis tested was whether the sludge loading percentage, the soil origin, or an interaction of the two affected the gradient of oxygen concentration.

A two way ANOVA examining the effect of both the soil's origin location and the sludge loading percentage on the gradient of oxygen concentration over log time found no significant effect of sludge loading and no significant interaction term, but did indicate a significant effect of the soil's origin ($P < 0.0001$; Figure 4-3). Tukey post hoc testing showed that soils from the Heslington East (n = 15) site had a significantly less negative gradient, indicating a slower rate of oxygen concentration decline, than the other soils used in the study. Spen Farm (n = 15) and Cusgarne (n = 16) soils had a significantly more negative gradient, indicating a faster rate of oxygen concentration decline, than the other soils, while there was no significant difference between the Loddington (n = 15), Heslington (n = 16), Mansfield (n = 16), and Newcastle (n = 16) soils.

.

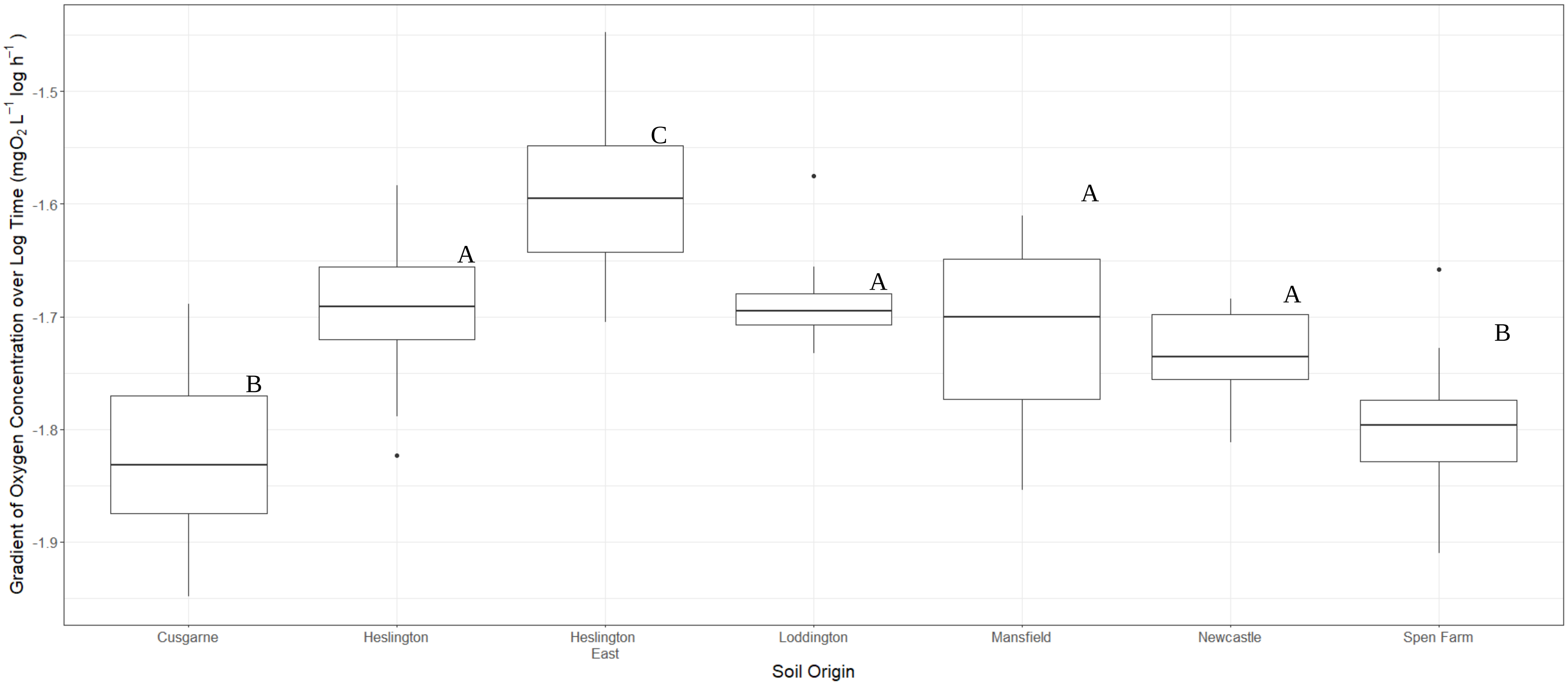

Figure 4-3. The gradients of oxygen concentration for all treatments of each soil across the twenty-four hour dataset, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Soils that do not differ significantly ($P < 0.05$) are marked with the same letter.

The soils were subdivided into three categories based on the uses of the soil at their points of origin; three arable soils, three allotment soils, and one pasture soil. A second variant of the first hypothesis, that the sludge loading and the soil use would affect the rate of oxygen depletion, was tested. A two way ANOVA examining the effect of both soil use and the sludge loading percentage on the gradient of oxygen concentration over log time found no significant effect of sludge loading and no significant interaction term, but did indicate a significant effect of soil use ($P < 0.0001$; Figure 4-4).

Tukey post hoc testing found that the gradient of oxygen concentration was significantly less negative in pasture ($n = 15$) soils than in the arable ($n = 46$) and allotment ($n = 48$) soils, indicating a slower overall rate of oxygen depletion in this soil.

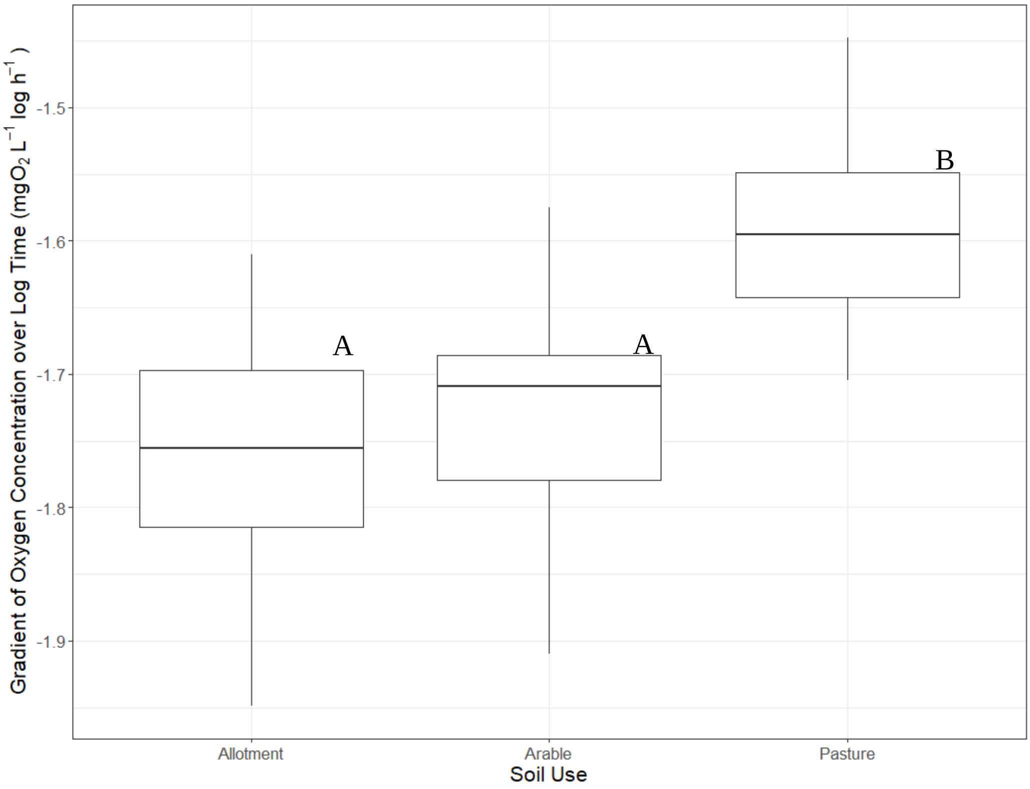

Figure 4-4. The gradients of oxygen concentration for all treatments of the soil use categories of allotment, arable, and pasture across the twenty-four hour dataset, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Soils that do not differ significantly (*P* < 0.05) are marked with the same letter.

As there was only one soil classified as pasture, this significant effect may not be representative of all pasture soils.

4.4.2.2 The effect of soil characteristics on oxygen concentration

The second hypothesis tested was that different soil characteristics were driving the differences in the gradients of the oxygen concentration curves. As the sludge loading percentage had been found to have no statistically significant effect on the rate of oxygen depletion, the results from the different sludge loadings for each soil were bulked to increase replicate numbers. Linear regressions showed a significant relationship between the gradient of the oxygen concentration curve over log time and the pH ($R^2 = 0.31$, $P < 0.0001$; Figure 4-5), the organic matter content ($R^2 = 0.20$, $P < 0.0001$; Figure 4-6), and the percentage increase in organic matter content ($R^2 = 0.07$, $P = 0.005$; Figure 4-7), although the R^2 values for all associations were very low.

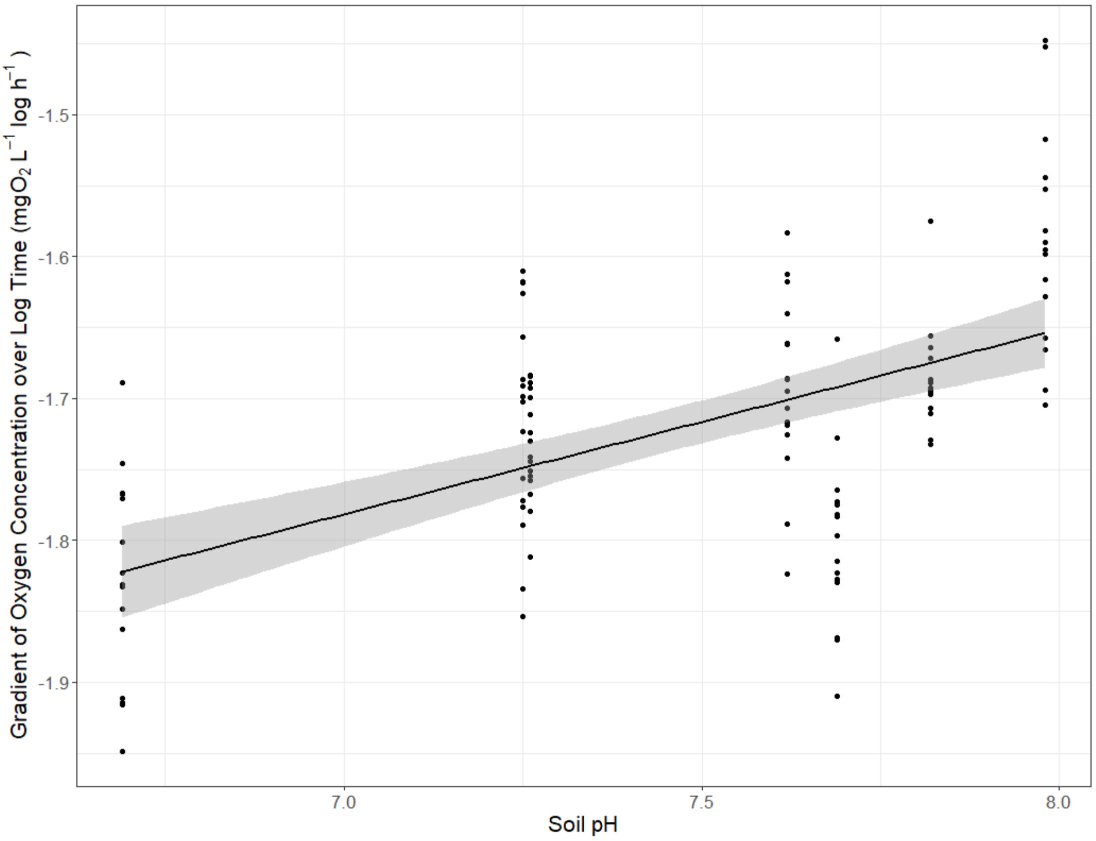

Figure 4-5. The relationship between the gradient of oxygen concentration within the beakers over log time and the soil pH across the twenty-four hour dataset. Trend line added based on linear model, with shading indicating 95% confidence intervals. R^2 = 0.31; *P* < 0.0001.

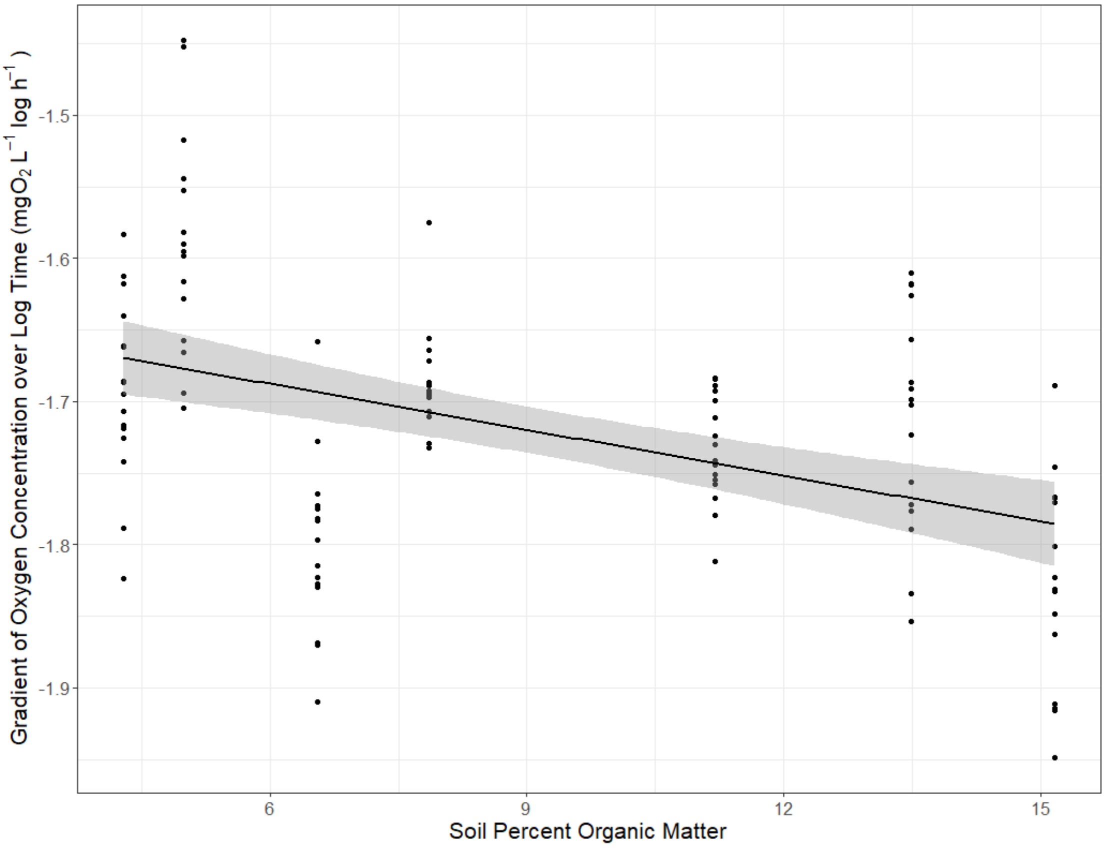

Figure 4-6. The relationship between the gradient of oxygen concentration over log time and the soil organic matter percentage across the twenty-four hour dataset. Trend line added based on linear model, with shading indicating 95% confidence intervals. R^2 = 0.20; *P* < 0.0001.

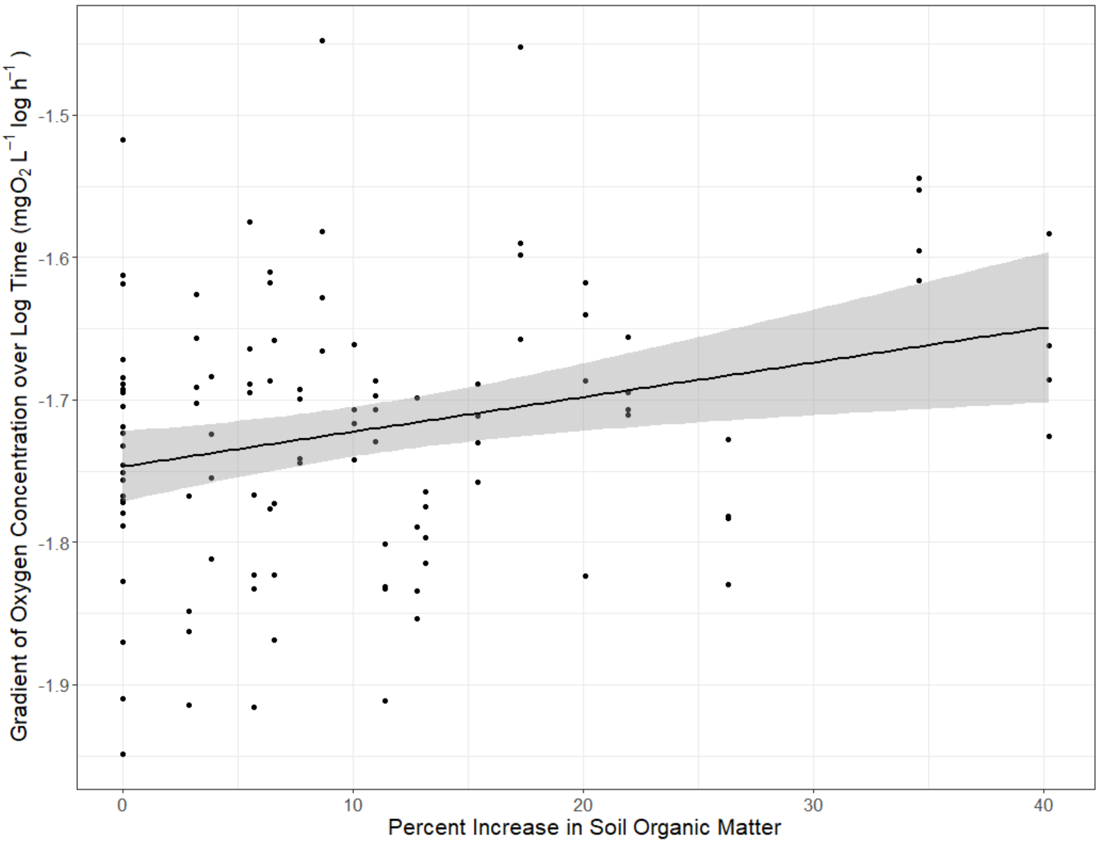

Figure 4-7. The relationship between the gradient of oxygen concentration over log time and the percentage increase in soil organic matter content over the twenty-four hour dataset. Trend line added based on linear model, with shading indicating 95% confidence intervals. $R^2 = 0.07$; *P* = 0.005.

A one way ANOVA found a significant effect of soil texture on the gradient of oxygen concentration depletion ($P < 0.0001$; Figure 4-8). Tukey post hoc testing found that the gradients of oxygen concentration over log time were significantly less negative in silty clay loam (n = 15), silty clay (n = 16) and clay (n = 15) soils than in loamy sand (n = 32) and sandy clay loam soils (n = 15), indicating slower rates of oxygen depletion. The gradient of oxygen concentration over log time in sandy loam soil was not significantly different to any other soil textures.

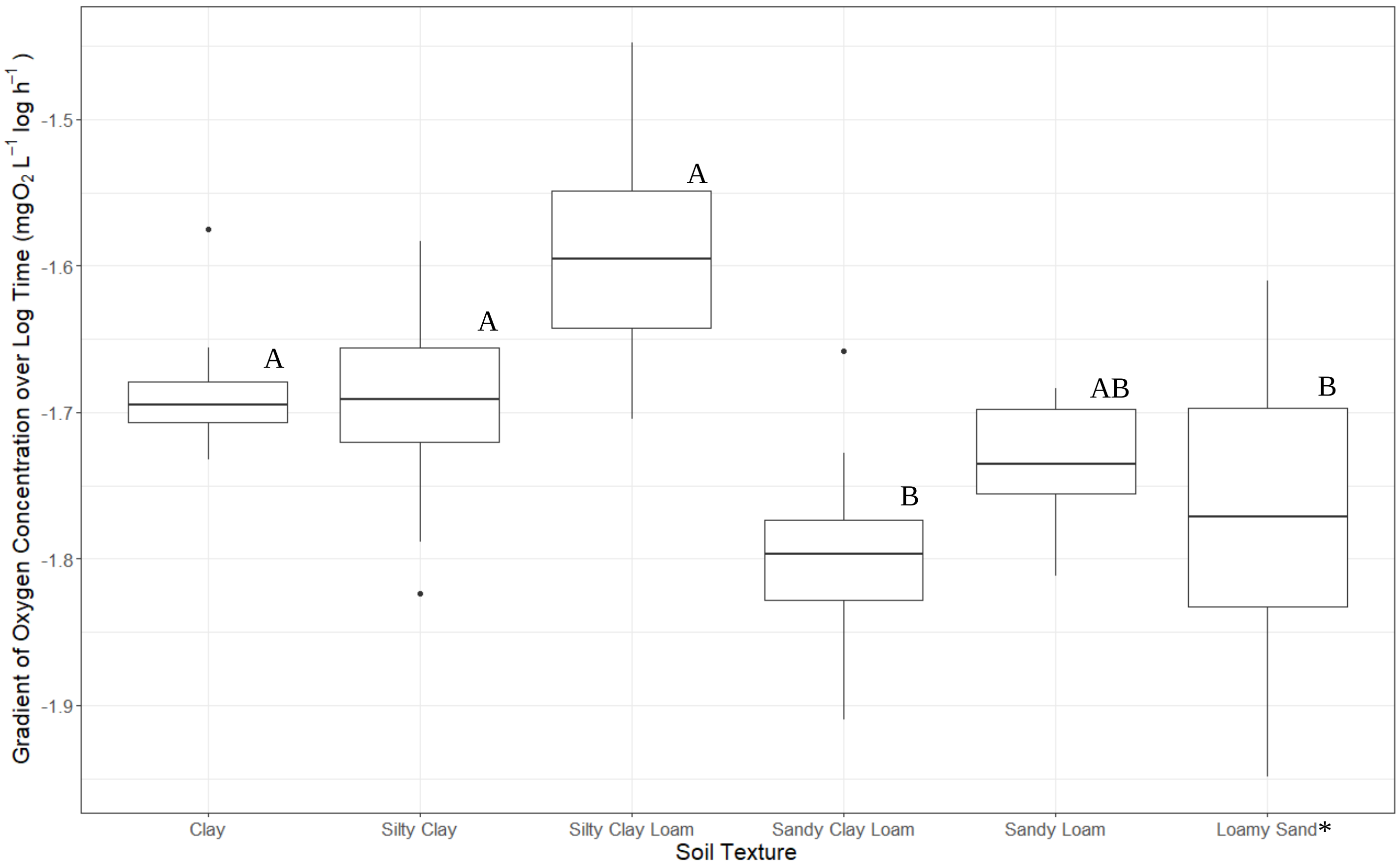

Figure 4-8. The gradients of oxygen concentration for all treatments of each soil texture across the twenty-four hour dataset, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Soils that do not differ significantly ($P < 0.05$) are marked with the same letter. Soil texture marked with an asterisk on the x axis contains two soils; all other soil textures contain one replicate.

4.4.3 Depletion and plateau periods

A Wilcoxon Signed Ranks test on the gradient values for the depletion and the plateau phases of the oxygen concentration gradients over log time found that the gradients in the plateau phase (n = 106) were significantly less negative than the gradients in the depletion phase (n = 110) ($P <$ 0.0001; Figure 4-9). As the gradients are negative, a less negative gradient in the plateau phase is closer to zero, indicating that the plateau phases are ‘flatter’ than the depletion phases.

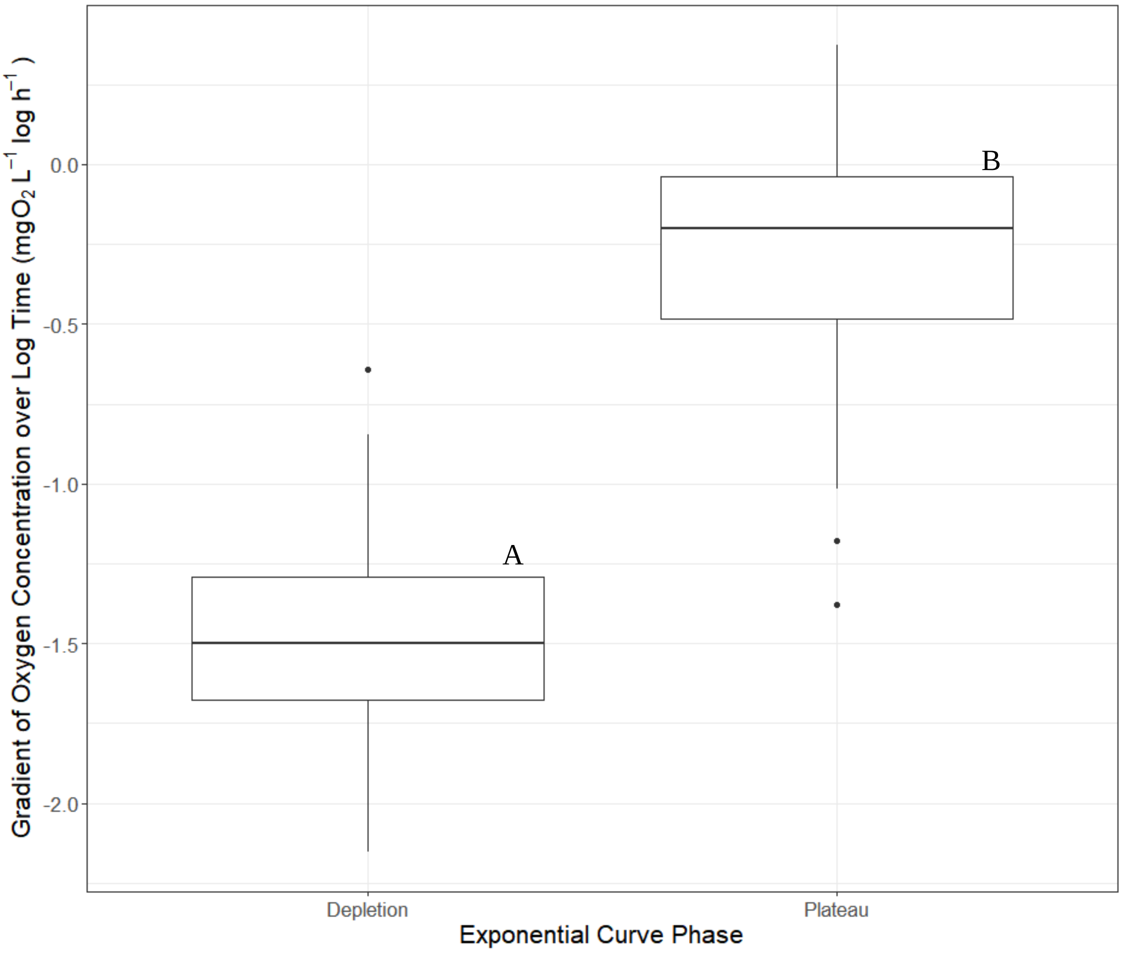

Figure 4-9. The gradients of oxygen concentration over log time for the depletion and plateau phases of the exponential curve, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Gradient values are negative, therefore the significantly higher value for the gradients in the plateau phase indicate that the gradient is closer to zero, indicating a very shallow gradient. Bars marked with the same letter are not significantly different (P < 0.05)

As these two phases have significantly different gradients, they were considered separately when addressing the hypotheses.

4.4.3.1 The effects of sludge loading and soil on oxygen concentration

Within the depletion phase and plateau phases, the first hypothesis tested was whether the sludge loading percentage, the soil origin, or an interaction of the two affected the gradient of oxygen concentration.

A two way ANOVA of the depletion phase data showed a significant effect of the soil origin ($P = 0.04$), of the sludge loading percentage ($P < 0.0001$) and of the interaction term between the two ($P = 0.004$) (Figure 4-10).

Post hoc testing of the interaction term found a general trend within the Loddington and Spen Farm sites that the gradient of oxygen concentration was significantly less negative in the 0% sludge addition soils than in the 100% addition soils, indicating a slower rate of oxygen depletion in the unamended soils. Within the Cusgarne, Heslington, Heslington East, Mansfield and Newcastle site there was no significant difference in the oxygen concentration gradient between sludge percent loadings.

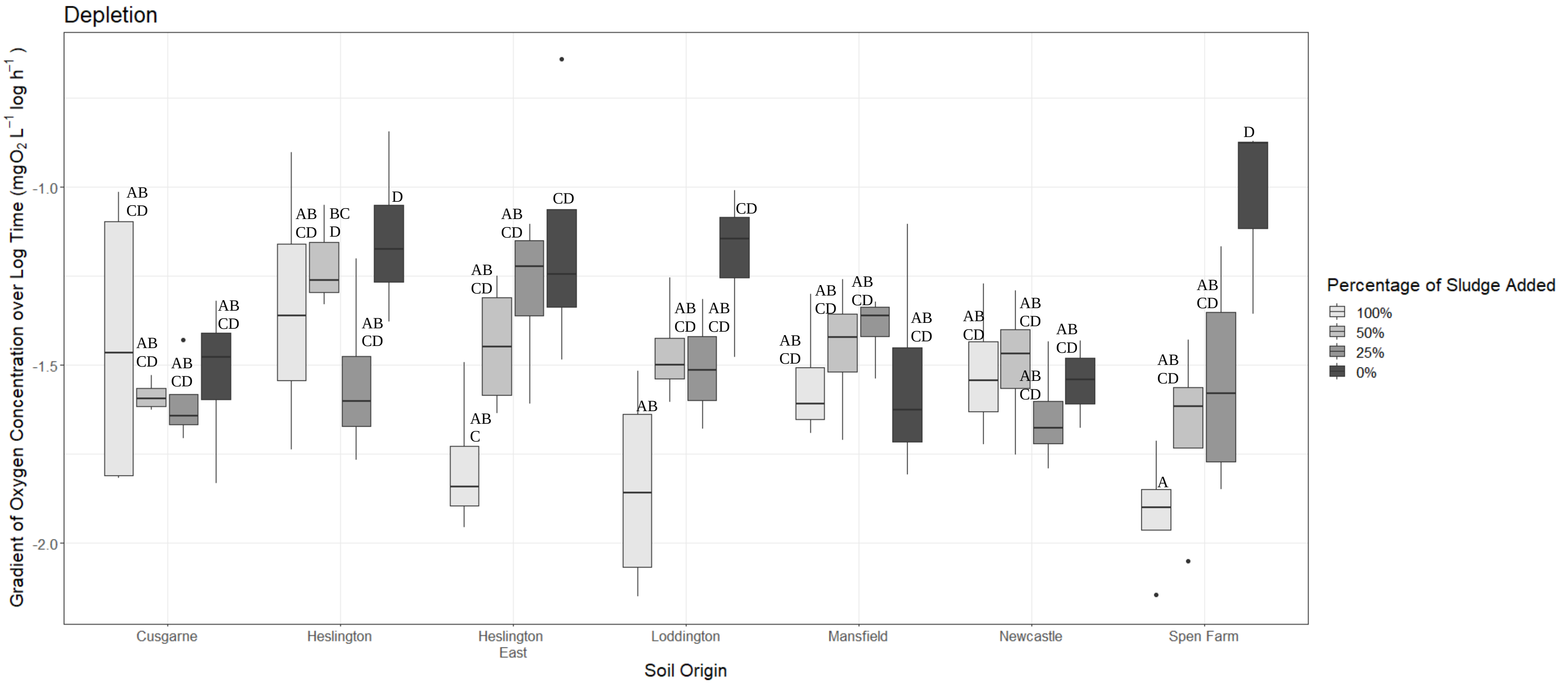

Figure 4-10. The gradients of oxygen concentration over log time for the depletion phase of the exponential curve across the different soil origins and sludge loading percentages, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Bars marked with the same letter are not significantly different (*P* < 0.05).

A two way ANOVA of the plateau phase data showed a significant effect of the soil ($P = 0.03$), but no significant effect of the sludge loading percentage or an interaction term. As there was no significant effect of the sludge loading percentage, the sludge loadings have been bulked (Figure 4-11).

Tukey post hoc testing showed that the gradient of oxygen concentration was significantly more negative in the Heslington East (n = 16) than in the Newcastle (n = 16) soil, with no significant difference between the Loddington (n = 16), Spen Farm (n = 12), Cusgarne (n = 16), Heslington (n = 15) and Mansfield (n = 15) soils.

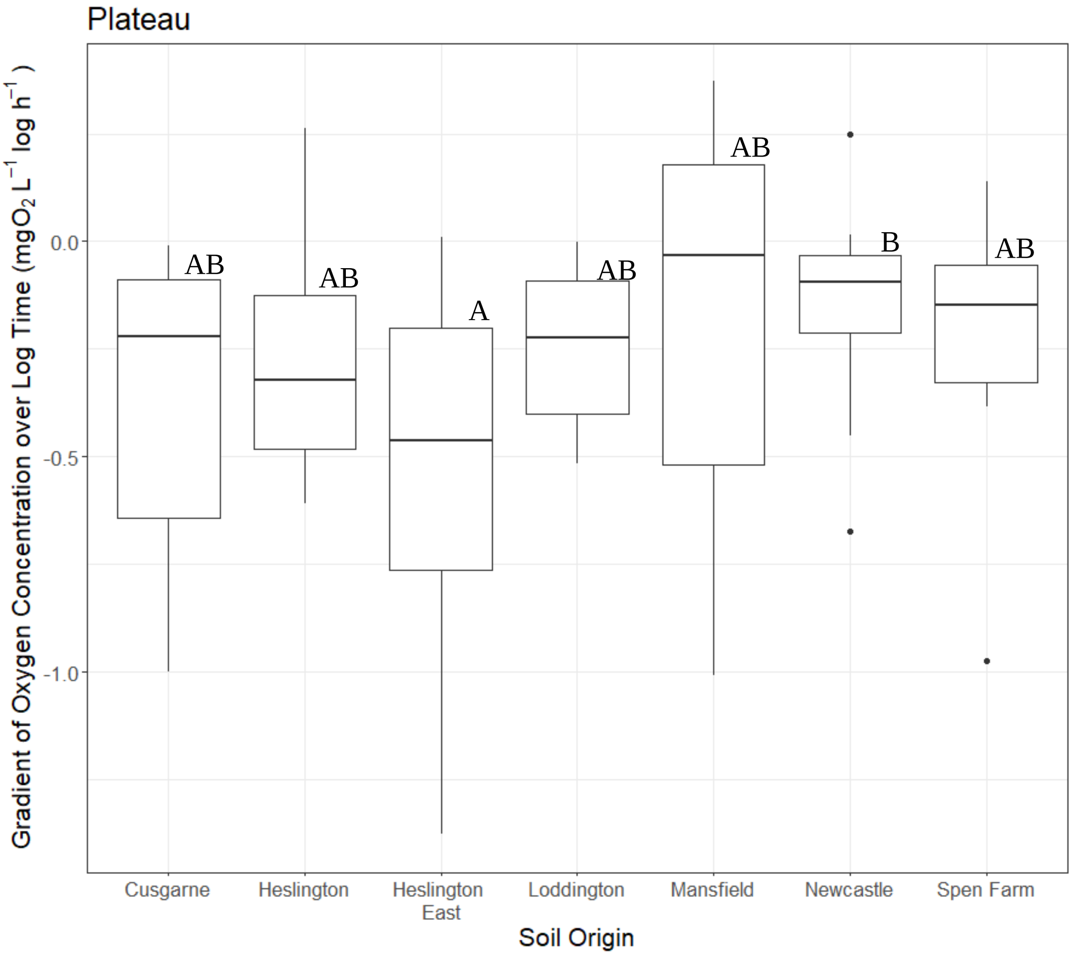

Figure 4-11. The gradients of oxygen concentration for each soil origin, with sludge loading percentages bulked together, over log time for the plateau phase of the exponential curve, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Soils that do not differ significantly ($P < 0.05$) are marked with the same letter.

A two way ANOVA of the depletion phase data showed no significant effect of the soil use, but a significant effect of the sludge loading percentage (P = 0.0001) and of the interaction term between the two (P = 0.001) (Figure 4-12).

Post hoc testing showed that the gradients of oxygen concentration depletion were significantly more negative in the 100% sludge loading pasture and arable soils than in the same soils with 0% sludge loading. Arable soil 0% sludge loading soils were also significantly less negative than all combinations of soil use and sludge loading except arable and pasture at 50% and pasture at 25% sludge loading. The number of replicates for each combination of soil texture and sludge loading are summarised in Table 4-4.

Table 4-4 – The number of replicates of each combination of soil use and sludge loading percent in the depletion phase used in the two way ANOVA and Figure 4-12.

Soil use	**Sludge loading percent**				**Total**
	100%	**50%**	**25%**	**0%**	
Allotment	12	12	12	12	48
Arable	12	11	12	11	46
Pasture	4	4	4	4	16
Total	28	27	28	27	-

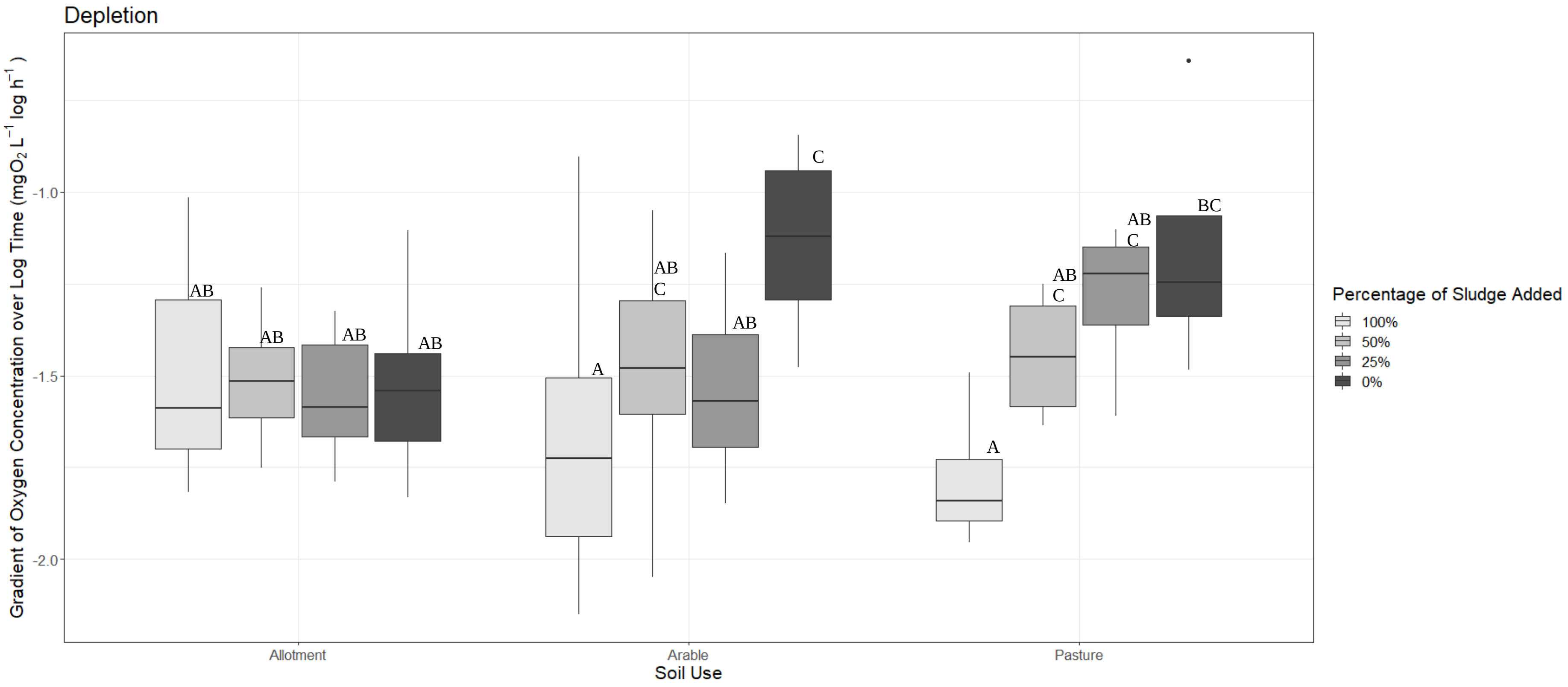

Figure 4-12. The gradients of oxygen concentration for the depletion phase of the exponential curve for all sludge loadings bulked across soil origins, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Bars marked with the same letter are not significantly different between soil uses and sludge loading percentages together (P < 0.05).

A two way ANOVA of the plateau phase data showed a significant effect of the soil use (P = 0.006), but no significant effect of the sludge loading percentage or of the interaction term between the two (Figure 4-13). Tukey post hoc testing found that the gradient of oxygen concentration depletion over log time was significantly more negative in the pasture (n = 16) soil use than in the allotment (n = 47) and arable (n = 43) soil uses, indicating a faster rate of oxygen depletion in the pasture soil.

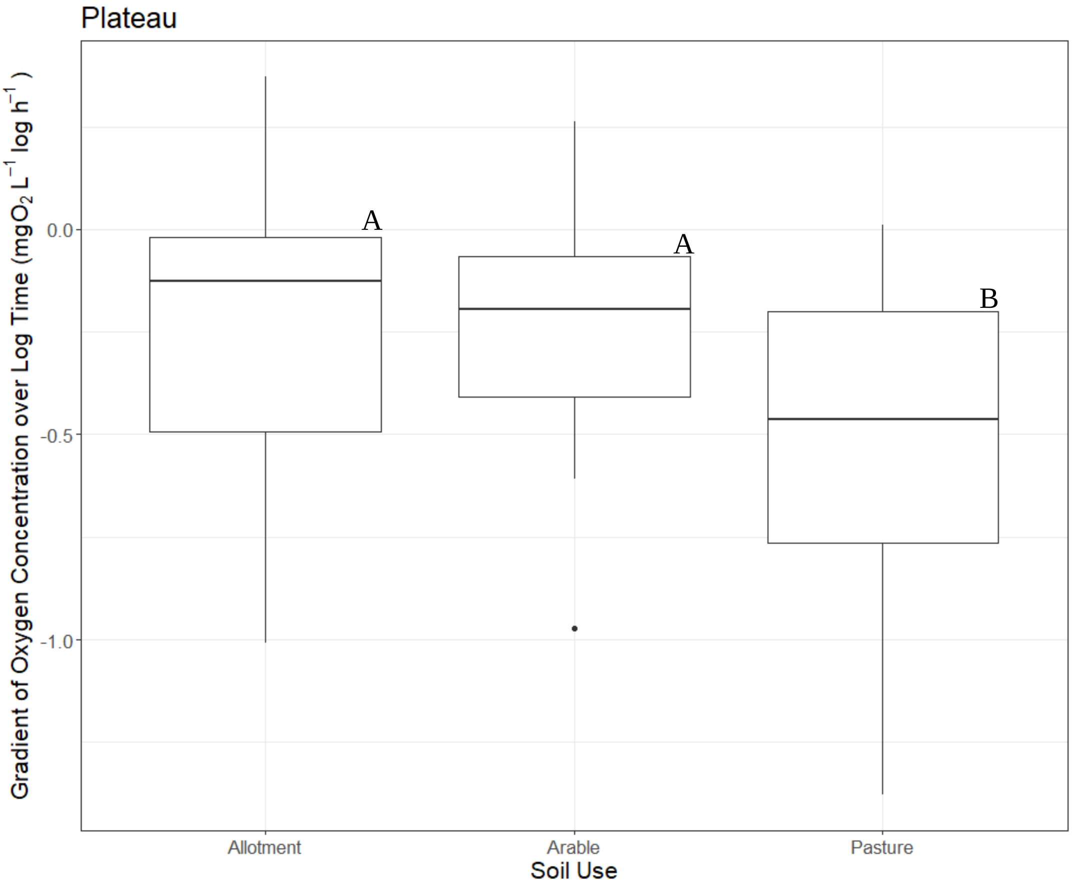

Figure 4-13. The gradients of oxygen concentration over log time for the plateau phase of the exponential curve for all soil uses bulked over across sludge loadings, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Soils that do not differ significantly ($P < 0.05$) are marked with the same letter

4.4.3.2 The effects of soil characteristics on oxygen concentration

The second hypothesis tested was that different soil characteristics were driving the differences in the gradients of the oxygen depletion curves.

For the depletion phase, a one way ANOVA found no significant effect of the soil texture on the rate of oxygen depletion over log time. Linear models found no significant effect of the pH or the organic matter contents on the rate of oxygen depletion. A linear model found a significant effect of the percentage increase of organic matter on the rate of oxygen depletion over log time (P = 0.003; Figure 4-14).

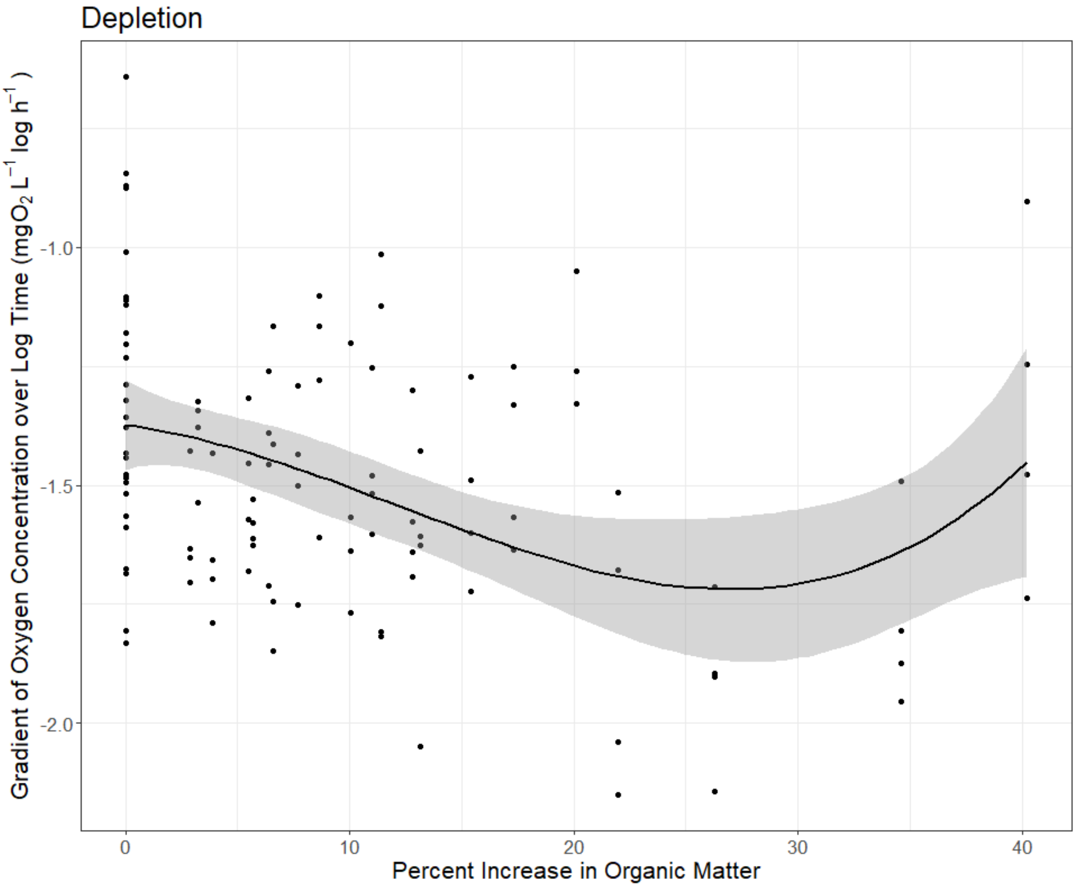

Figure 4-14. The relationship between the percentage increase in the organic matter content caused by application of sewage sludge to soil and the gradient of oxygen concentration over log time during the depletion phase of the oxygen depletion curve. Trend line added using a polynomial linear model of the relationship, with shading indicating 95% confidence intervals. R^2 = 0.07; *P* = 0.003.

However, as Figure 4-14 indicates, the relationship is decidedly non-linear (R^2 = 0.07). Because of this, a generalised linear model (GLM) was performed to examine of the relationship between the logged percentage increase in organic matter (log +1 transformation) and the gradient of oxygen concentration over log time. The GLM found a significant relationship between these factors (*P* = 0.0002; Figure 4-15), although the R^2 value was still low (R^2 = 0.12).

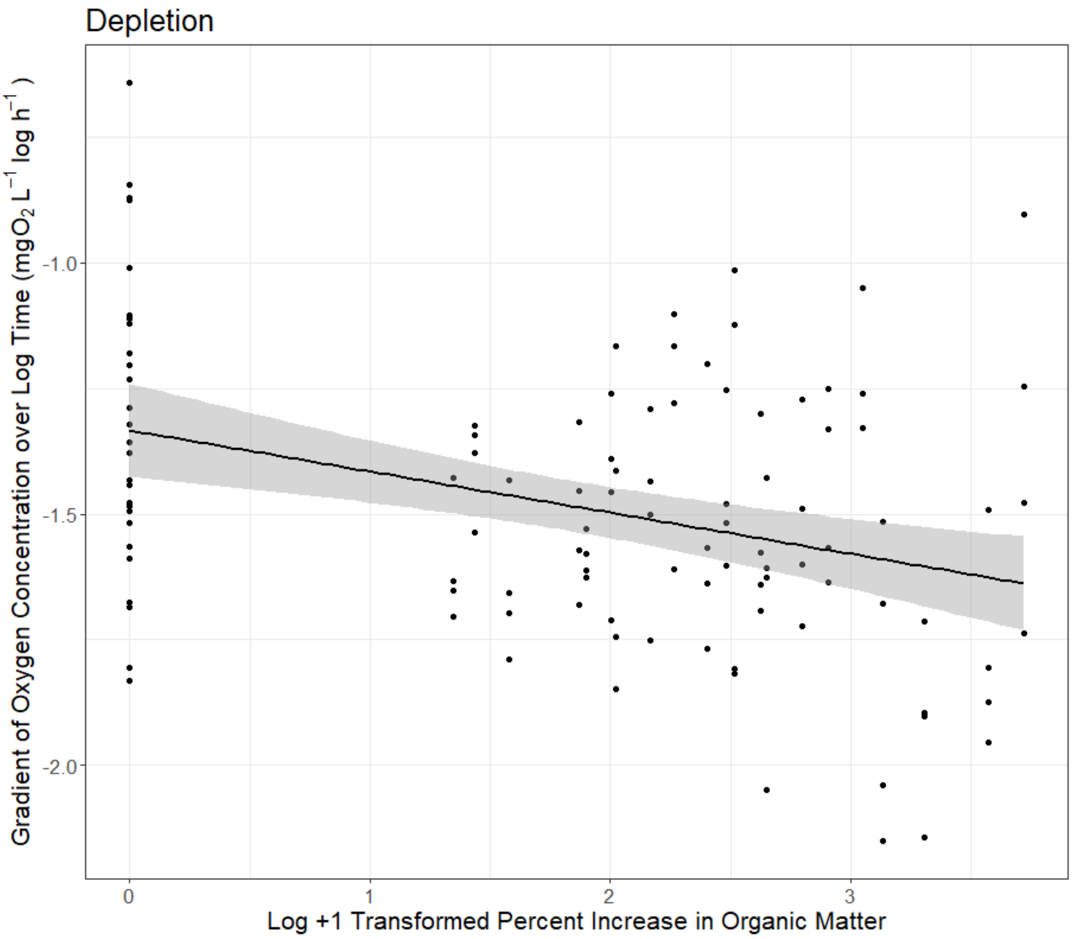

Figure 4-15. The relationship between the logged percentage increase in the organic matter content caused by application of sewage sludge to soil and the gradient of oxygen concentration over log time during the depletion phase of the oxygen depletion curve. Trend line added using a generalised linear model of the relationship, with shading indicating 95% confidence intervals. R^2 = 0.12, *P* = 0.0002)

For the plateau phase, linear models found no significant effect of the pH, organic matter content, or the percentage increase in organic matter content on the rates of oxygen depletion over log time.

A one way ANOVA found a significant effect of the soil texture on the gradient of oxygen concentration over log time (*P* = 0.04; Figure 4-16). Tukey post hoc testing showed that the gradient of oxygen concentration was significantly more negative in the silty clay loam (n = 16) textured soil, indicating a faster rate of depletion, than in the sandy loam (n = 16) textured soil. There was no significant difference between the soil textures of clay (n = 16), loamy sand (n = 32), sandy clay loam (n = 12), and silty clay (n = 15) soils.

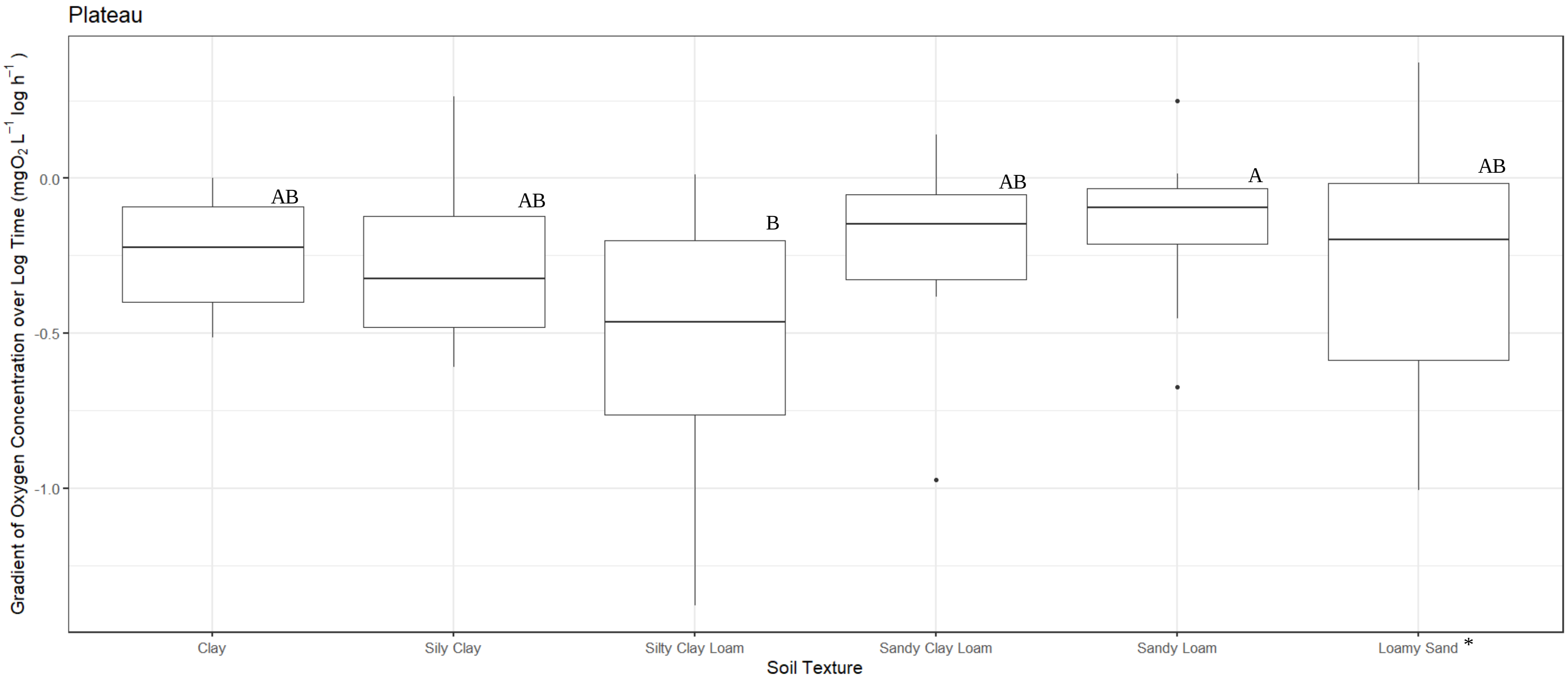

Figure 4-16. The median, 25th and 75th quartile, and range of gradients of oxygen depletion over log time for the plateau phase of the exponential curve for all soil uses. Soils that do not differ significantly (*P* < 0.05) are marked with the same letter. Soil textures indicated on the x axis with an asterisk consist of two soils; all other soil textures contain one.

4.5 Discussion

4.5.1 Intercorrelation of Soil Factors

The strong significant differences between soils, both by individual site and by soil use, suggests that there are factors that vary across the soils which are influencing the rate of organic matter decomposition, and thus the rates of oxygen depletion in soil. During preliminary statistical analysis, the factors of soil organic matter content, soil texture, and soil pH were found to be highly intercorrelated.

There was a significant relationship between the soil texture and the soil organic matter content. This is likely an artefact of the land use, rather than an expected effect of soil texture; by chance the allotment soils were all sandy, whilst the arable soils were all clay-rich. While previous studies have found that organic matter is more likely to be retained in clay soils than in sandy soils (Jenkinson, 1977; Burke, 1989), other studies have found that soil texture is not always likely to be the dominant factor in organic matter retention, and that the quality and frequency of organic matter additions are more likely to be the factors affecting soil organic matter content across different soil textures (Hassink, 1994). In the case of these soils, the regular addition of organic matter to allotment soils in the form of composts, and the typically low organic matter content of agricultural soils (Beare et al.; 1994; Six et al., 1998), is far more likely to have been the impacting factor on the organic matter content of these soils.

The pH and organic matter percentages were negatively correlated, with lower pH values associated with higher percentages of soil organic matter. The pH values observed in this study for each site do not differ from the expected ranges recorded in the UK Soil Observatory survey of topsoil soil pH (UKSO, 2018), and lower pH values are associated with the accumulation of organic matter in soils (Williams and Donald, 1957), caused by the release of H^+ ions by decomposing organic matter (Porter et al., 1980), although this effect is likely to be highly dependent on soil characteristics and agricultural practices (Ritchie and Dolling, 1985). In this study, the lower pH values are associated with the higher organic matter content, with the organic matter content of the soils dictated by their uses at source.

4.5.2 The effect of sewage sludge application on soil oxygen concentration

The hypothesis that there was a significant effect of the sludge loading and soil properties on the rate of oxygen depletion in flooded soil was partially supported. While there was a significant effect of the soil, either based on the soils' origin or use, there was no significant effect of the sludge loading when considered over the entire 24 hour period of the experiment. However, when the 24 hour period was broken down into separate depletion and plateau phases, there was an effect of the sludge loading percentage during the initial depletion phase. The hypothesis was formed under the assumption that the addition of sewage sludge to soil increased the organic matter content of the soil, and that when the soil flooded the additional organic matter would be rapidly broken down, leading to a faster rate of oxygen depletion in the soil with high levels of sludge addition. To an extent, this effect was observed in the experiment, but it was captured only as a brief effect during the depletion phase. While the increase in the organic matter content caused by the sludge was sufficient to drive a short term increase in the rate of oxygen depletion, over the long term period of the experiment other soil factors became more influential on the rate of oxygen depletion. The importance of other soil factors such as soil organic matter content are evident in the significant differences in oxygen concentration gradients still observed in the plateau phases, with pasture soil still showing greater rates of decrease in oxygen concentration compared to the allotment and arable soils during the plateau phase.

To explain the observed effects, the nature of sewage sludge must be considered. The sludge is produced as a by-product of the waste water treatment industry, and is formed by the anaerobic digestion of the solids left after water is drained off. The anaerobic digestion process takes place over a minimum of twelve days, during which easily accessible organic matter is broken down and pathogens are destroyed (FAO, accessed online 2019). This process renders the sewage sludge suitable for disposal, either through application to agricultural land or in landfill. This breakdown of accessible organic matter leaves sludges composed of approximately 60% organic matter, while the remaining portion of the sludge is a complex heterogeneous mixture of nitrogen and phosphorous containing inorganic compounds, inorganic and organic pollutants, pathogens, and water (Rulkens, 2008). As this c. 60% organic matter fraction of the sludge is the remaining product following anaerobic digestion, up to 80% of this organic matter in the sludge may be resistant to decomposition (Terry et al., 1979a). This led to the observed effects of increasing sludge loading having minimal effect on the rate of oxygen consumption in flooded soil, as the very small percentage of available organic matter is rapidly consumed, but no breakdown of the recalcitrant organic matter occurs.

The ratio of carbon to nitrogen within the sewage sludge is not optimal for decomposition, likely as a result of the anaerobic digestion that has already occurred. The sludge used in this work had a percent carbon to percent nitrogen ratio of 7.7:1, with a high nitrogen availability relative to the carbon content. Optimally, composts used for soil enrichment have a carbon to nitrogen ratio of 20 or 30:1, which provides optimal conditions for growth of microorganisms (de Bertoldi et al., 1983) and leads to the greatest rates of seed germination (Gao et al., 2010; Guo et al., 2012). The low C:N ratio of sewage sludge suggests that it may not be optimal for improving soil organic matter content, due to the higher nitrogen content in comparison to the carbon in the organic matter, and may be more impactful on soil properties such as porosity and bulk density.

Previous studies have found that the beneficial effects of sewage sludge addition to soil persist for at least four years after application (Wallace et al., 2009; Lindsay and Logan, 1996). In arid conditions, where the breakdown of organic matter is restricted by the availability of water, one study found evidence that a substantial portion of a single application of sludge persisted in the soil as fragments > 2mm in diameter for a period of 18 years, in part due to the recalcitrant nature of sewage sludge (Walton et al., 2001). While Walton’s study demonstrates the longevity of the material in extreme conditions, it does illustrate that the breakdown of recalcitrant organic matter in sewage sludge is likely governed by a number of soil environmental factors. A study by Devêvre and Horwáth (2000) found that recalcitrant organic matter such as cellulose and lignin was more likely to be broken down under anaerobic than aerobic conditions, which suggests that the breakdown of sludge may be more likely to occur once the soil has reached anoxic conditions, rather than drive the rate of oxygen depletion in flooded soil

4.5.3 Percentage increase of organic matter

As some of the soils were already very low in organic matter percentage, the percent increase in organic matter may have been expected to strongly impact upon the rates of oxygen depletion; in the arable soil from Heslington, York, the percentage increase in organic matter was as high as 40.2% (Table 4-2). However, as the majority of organic matter within the sludge is resistant to bacterial breakdown (see Section 4.5.2), this increase in organic matter content did not represent an increase in the available organic matter content, and so there was no observed effect of the percentage organic matter increase on the rate of oxygen depletion in the flooded soil across the twenty-four hour period of the experiment. When the oxygen depletion curves were broken down into the depletion and plateau phases, there was a significant effect of the percentage increase of organic matter content on the depletion phase. However, the relationship between the percentage increase in the organic matter and the rate of oxygen depletion was not linear, with the greatest increase in the rate of oxygen consumption occurring at an increase in organic matter content of approximately 30% (Fig. 4-16). When the percentage increase in organic matter was logged, a linear relationship with the depletion of oxygen became more evident, with rates of oxygen depletion over log time increasing with the log percentage increase in organic matter content. When viewed over the entire twenty-four hour time scale, it may be the case that while there has been a large increase in the total organic matter, the increase in the available organic matter has been much lower, due to the recalcitrant nature of the sewage sludge. This may lead to the effects of the percentage increase in organic matter only reaching noticeable levels at very high levels, with the smaller increases in organic matter percentages representing negligible increases in available organic matter.

4.5.4 The effect of soil characteristics

4.5.4.1 Soil pH

Across all soil uses and across all time scales, there was no significant effect of pH on the rate of oxygen depletion. Studies have found little effect of soil pH on the rate of decomposition of sewage sludge in soil (Terry et al., 1979b), but across all the soils sampled there was very little variability in the pH. Sample soil pH values ranged from 6.69 in the Cusgarne soil to 7.98 in the Heslington East soil (Table 4-2). It may be the case that a wider range of soil pH values would have affected the rate of soil respiration, as studies have found that soil microbial activity is increased at both extremes of the pH scale, potentially as a response to stressful conditions (Pietri and Brookes, 2008).

4.5.4.2 Soil texture

There was a significant effect of soil texture across the twenty-four hour dataset and for the plateau phase of the oxygen depletion curve, but as discussed in Section 4.5.1, this was likely due to the coincidental grouping of soil textures and soil uses, with the higher organic matter soils generally being sourced from soils with a higher sand content. While this would explain the faster rates of oxygen consumption in the high organic matter, sandy soils, there are characteristics of clay particles in soils that may have contributed to the lower respiration rates observed in the high clay content soils. Clay particles in soils exert a protective effect against the decomposition of organic matter by microorganisms (Allison, 1973) through sorption between complex organic molecules and clay particles (Oades, 1984). Studies examining the breakdown of sewage sludge found that there was no effect of soil texture independently on its decomposition, but in conjunction with saturated conditions the decomposition was slowed in silt loam soil and almost completely halted in clay soils (Miller, 1974). This suggests that there may be an effect of soil texture on the breakdown of sewage sludge, when considered in conjunction with other soil conditions. In both the twenty-four hour dataset and the plateau phase dataset, the rates of oxygen depletion are slower in soils with a high clay component than in the other soil textures. While the data for the twenty-four hour and the plateau phases correspond with Miller's (1974) findings, the results of this experiment found no effect of soil texture in the depletion phase. This may be because labile carbon was readily consumed during the depletion phase, with the protection effects of clay on soil carbon contents only evident once this labile pool has been consumed. However, while there was a significant difference in the gradient of oxygen concentration between soil textures in the plateau phase, the fastest rates of oxygen concentration depletion occurred in the clay-containing silty clay loam, which suggests that the protective effect of clay in soils on organic matter is highly dependent on other soil factors, such as soil organic matter content.

4.5.4.3 Percent organic matter

There was a significant effect of the organic matter content on the rate of oxygen depletion in the soils over the 24 hour period dataset used in the study. In general, the higher organic matter content soils had higher rates of oxygen depletion, indicative of bacterial respiration, than the lower organic matter content soils. However, it is important to note that the relationship is not linear. Fig. 4-8 shows that the fastest rates of oxygen consumption are observed at approximately 12% organic matter content, followed by an apparent plateauing or decrease of the rate of oxygen consumption. It may be the case that the metabolic activity occurring within the soil has been controlled by the organic matter content to an extent, but begins to plateau when other soil properties become the limiting factors of microbial respiration. While it may be expected that temperature is the key restricting factor in governing rates of organic matter decomposition, studies have found that respiration is strongly linked to the both soil temperature (Lloyd and Taylor, 1994), soil moisture (Orchard and Cook, 1983), and the interaction of the two (Yuste et al., 2007; Smith et al., 2003). The relationship of the effect of soil temperature and moisture is not linear, with studies finding that soil respiration is likely to be higher under conditions of lower temperature and higher soil moisture than under conditions of higher temperature and lower soil moisture (Yan et al., 2019). However, the linear effect of soil moisture on soil respiration does not continue indefinitely. Soil respiration increases with water content until it reaches a threshold, either due to the restrictions of soil temperature or soil reaching saturated conditions, before declining (Wang et al., 2006). The interaction of temperature and moisture on rates on breakdown of soil organic matter is also dependent on the pool of organic matter. A high proportion of organic matter in soils, much like the sludge applied, will not be readily available for microbial respiration and is decoupled from the relationship between soil moisture content and soil temperature on microbial respiration (Bosatta and Ågren, 1999; Davidson and Janssens, 2006). It may be the case, therefore, that while there was an increase in the soil organic matter content from 12% to 15% in the high organic matter content soils, the proportion of labile organic matter did not increase, meaning that respiration rates would not differ as greatly across these soils as the increase from 5% to 12% organic matter.

4.5.5 Further Experiments

4.5.5.1 Sewage sludge application methods

The decision to air dry and finely grind the sewage sludge used in the experiments was made in order to ensure that the organic matter was distributed as evenly as possible through the soil. By grinding the sludge to a powder, the surface area of the sludge was greatly increased. If there had been an effect of the sludge loading percentage on the rates of oxygen consumption within the soil, this may have been a cause. However, in the case of this study there was only an effect of sewage sludge application to soil in the short term depletion phase; when considered over the twenty-four hour period of the experiment, soil organic matter content had a stronger effect on the oxygen concentration gradient than the addition of sewage sludge.

The application method of the sewage sludge in this study was not representative of the application of the sludge in agricultural fields, but was used as it provided more reproducible results in the laboratory. Further experiments would need to examine the dynamics of oxygen depletion when the sludge is applied wet and un-ground, as these may differ from the results observed in these microcosm beaker environments; while the rate of decomposition is likely to remain low, other research has found that the decomposition of sludges was greater in soil samples which received surface applied sludge (Terry et al., 1979b). The authors of the study suggested that this may have been due to a protective effect against microbial breakdown that is formed when clay particles bind with organic matter in soil (Allison, 1973).

4.5.5.2 Sequential application of sludge

The applications of sludge are limited to a maximum of 250 kg of nitrogen per hectare a year, but there are no restrictions in the UK on the number of sequential years for which sludge can be applied. Studies have found that the sequential application of sludge leads to an increase in soil organic matter content and in carbon sequestration, with one study finding, after 34 years of sequential sludge application, that a net average of 1.73 Mg C ha^{-1} yr^{-1} was sequestered in amended soils, compared to a range of -0.07 to 0.17 Mg C ha^{-1} yr^{-1} in untreated control soils (Tian et al., 2009). Based on one application, the percentage increase in organic matter could be relatively low, ranging from an increase of 11.4% in the Cusgarne soil to 40.2% in the Heslington soil. While the absolute yearly accumulation may not be high in soils already rich in organic matter content, it is evident that it will rapidly become high in soils initially low in organic matter content. Over a decade of yearly applications of the maximum rates of sludge, this would hypothetically result in a maximum increase 47.9% in the total grams of organic matter in the Cusgarne soil, and of 72% of total organic matter in the Heslington soil, assuming no decomposition of the sludge over time.

Further work could investigate the rates of oxygen depletion during flooding in soils obtained from fields with a history of sewage sludge application, or the establishment of long term plots for sludge application under a variety of crop types or land uses. The establishment of long term plots would require a large commitment of time and resources, while the sampling of historical plots may be more feasible.

4.6 Conclusion

This work shows that while there are differences in the rate of soil oxygen depletion based on soil properties, the addition of organic matter in the form of anaerobically digested sewage sludge has no impact on the rates of oxygen consumption over a long term period. However, there is an effect during the initial depletion phase. The short term effect from the sewage sludge is likely due to the fact that the sludge has already undergone bacterial breakdown during the anaerobic digestion process, leaving only the organic matter that is most recalcitrant and resistant to breakdown. The low percentage of readily available organic matter is rapidly consumed, leading to the short-term effect of added sewage sludge observed. Over the longer time period, the depletion of soil oxygen is most influenced by the organic matter already present in the soil, a factor highly influenced by the soil use at origin.

When considering how these results inform the observations made in the field in Chapter 3, the effect of the organic matter content of the parent soil seems to have been the key factor determining the rates of oxygen depletion observed. The organic matter content of the fields varied significantly depending on the flooding regime, with the more regularly flooded regions of the fields having a higher organic matter content, but also with a differing earthworm population. It may be expected that this would drive changes in the earthworm populations in a flooding event, with the higher organic matter content soils expected to become anoxic more rapidly, creating an environment where fewer earthworms are likely to survive. However, it is also important to consider the fact that the available soil oxygen dropped to anoxic levels within 24 hours, regardless of the organic matter content, and frequently reached anoxic levels within 10 hours. The next question to consider, therefore, is whether there is a critical oxygen level below which earthworms do not survive, and whether this varies across species, thus explaining the population patterns observed in the field.

4.7 References

ADAS, 2014. Biosolids Nutrient Management Matrix. Leaflet. [Accessed online 22/03/2019. Available at http://www.adas.uk/Downloads/Biosolids_Nutrient_Management_Matrix.pdf].

Allison, F.E. 1973. Soil Organic Matter and its Role in Crop Production. Elsevier Scientific Publishing Company. New York.

Beare, M.H., Hendrix, P.F., Coleman, D.C. 1994. Water-Stable Aggregates and Organic Matter Fractions in Conventional- and No-Tillage Soils. *Soil Science Society of America Journal* 53(3): 777 – 789

Bell, R.G. 1969. Studies on the Decomposition of Organic Matter in Flooded Soil. *Soil Biology and Biochemistry* 1(2): 105 – 116.

Bosatta, E., Ågren, G.I. 1999. Soil Organic Matter Quality Interpreted Thermodynamically. *Soil Biology and Biochemistry* 31(13): 1889 – 1891.

Burke, I.C., Yonker, C.M., Parton, W.J., Cole, C.V., Schimel, D.S. Flach, K. 1989. Texture, Climate, and Cultivation Effects on Soil Organic Matter Content in US Grassland Soils. *Soil Science Society of America Journal* 53(3): 800 - 805

Cogger, C.G., Forge, T.A., Neilsen, G.H. 2006. Biosolids Recycling: Nitrogen Management and Soil. *Canadian Journal of Soil Science* 86: 613 – 620.

Davidson, E.A., Janssens, I.A. 2006. Temperature Sensitivity of Soil Carbon Decomposition and Feedbacks to Climate Change. *Nature* 440: 165 – 173.

de Bertoldi, M., Vallini, G., Pera, A. 1983. The Biology of Composting: A Review. *Waste Management and Research* 1: 157 – 176.

DEFRA. 2002. Sewage Treatment in the UK - UK Implementation of the EC Urban Waste Water Treatment Directive. Leaflet [accessed online 08/01/2018].

DEFRA. 2012. Waste Water Treatment in the United Kingdom – 2012. Implementation of the European Union Urban Waste Water Treatment Directive – 91/271/EEC. Leaflet [accessed online 08/01/2018].

Devêvre, O.C., Horwáth, W.R. 2000. Decomposition of Rice Straw and Microbial Carbon Use Efficiency under Different Soil Temperatures and Moistures. *Soil Biology and Biochemistry* 32(11-12): 1773 – 1785.

FAO, Website. Agricultural Uses of Sewage Sludge. Food and Agricultural Organisation of the United Nations. [Accessed online March 2019 - http://www.fao.org/3/T0551E/t0551e08.htm).

Gao, M., Liang, F., Yu, A., Li, B., Yang, L. 2010. Evaluation of Stability and Maturity During Forced-Aeration Compositing of Chicken Manure and Sawdust at Different C/N Ratios. *Chemosphere* 78(5): 614 – 619.

Guo, R., Li, G., Jiang, T., Schuchardt, F., Chen, T., Zhao, Y., Shen, Y. 2012. Effect of Aeration Rate, C/N Ration, and Moisture Content on the Stability and Maturity of Compost. *Bioresource Technology* 112: 171 – 178.

Hassink, J. 1994. Effects of Soil Texture and Grassland Management on Soil Organic C and N and Rates of C and N Mineralization. *Soil Biology and Biochemistry* 26(9): 1221 – 1231.

Hemmat, A., Aghilinategh, N., Rezainejad, Y., Sadeghi, M. 2010. Long-Term Impacts of Municipal Solid Waste Compost, Sewage Sludge and Farmyard Manure Application on Organic Carbon, Bulk Density and Consistency Limits of a Calcareous Soil in Central Iran. *Soil and Tillage Research* 108(1-2): 43 – 50.

Hulme, M. 2002. Climate Change Scenarios for the United Kingdom: the UKCIP02 Scientific Report. Tyndall Centre for Climate Mental Sciences University

Jager, G., Bruins, E.H. 1975. Effect of Repeated Drying at Different Temperatures on Soil Organic Matter Decomposition and Characteristics, and on the Soil Microflora. *Soil Biology and Biochemistry* 7(2): 153 – 159

Jenkinson, D.S. 1977. Studies on the Decomposition of Plant Material in Soil. V. The Effects of Plant Cover and Soil Type on the Loss of Carbon from ^{14}C Labelled Ryegrass Decomposing Under Field Conditions. *Journal of Soil Science* 28(3): 424 – 434.

Kay, A.L., Jones, R.G., Reynard, N.S. 2006. RCM Rainfall for UK Flood Frequency Estimation. II. Climate Change Results. *Journal of Hydrology* 318(1-4): 163-172.

Kozlowski, T.T. 1984. Plant Responses to Flooding of Soil. *Bioscience* 34(3): 162 – 167.

Lindsay, B.J., Logan, T.J., 1998. Field Response of Soil Physical Properties to Sewage Sludge. *Journal of Environmental Quality*, 27: 534-542.

Lloyd, J., Taylor, J.A. 1994. On the Temperature Dependence of Soil Respiration. *Functional Ecology* 8(3): 315 – 323

Miller, R.H. 1974. Factors Affecting the Decomposition of an Anaerobically Digested Sewage Sludge in Soil. *Journal of Environmental Quality* 3(4): 376 – 380.

Oades, J.M. Soil Organic Matter and Structural Stability: Mechanisms and Implications for Management. *Plant and Soil* 76(1-3): 319-337.

Orchard, A.V, Cook, F.J. 1983. Relationship between Soil Respiration and Soil Moisture. *Soil Biology and Biochemistry* 15(4): 447 – 453

Pagliai, M., Bisdom, E.B.A., Ledin, S., 1983. Changes in Surface-Structure (Crusting) After Application of Sewage-Sludge and Pig Slurry to Cultivated Agricultural Soils in Northern Italy. *Geoderma:* 30, 35-53.

Pietri, J.C.A., Brookes, P.C. 2008. Relationships between Soil pH and Microbial Properties in a UK Arable Soil. *Soil Biology and Biochemistry* 40(7): 1856 – 1861.

Plum, N. 2005. Terrestrial Invertebrates in Flooded Grassland: A Literature Review. *Wetlands* 25(3): 721 – 737.

Ponnamperuma, F.N. 1984. Effects of Flooding on Soils. *In*: Flooding and Plant Growth. London; Academic Press, Inc.

Porter, W.M., Cox, W.J., Wilson, I. 1980. Soil Acidity: Is it a problem in Western Australia? *Journal of the Department of Agriculture, Western Australia, Series 4* 21(4): 126 – 133.

Presens, 2016. Presens. [Online]. [20 February 2019]. Available from: https://www.presens.de/knowledge/basics/detail/measurement-principle-of-chemical-optical-sensors-901.html

Prudhomme, C., Jakob, D., Svensson, C. 2003. Uncertainty and Climate Change Impact on the Flood Regime of Small UK Catchments. *Journal of Hydrology* 277(1-2): 1-23.

Public Health, 1989. The Sludge (Use in Agriculture) Regulations 1989. Available at: http://www.legislation.gov.uk/uksi/1989/1263/introduction/made [accessed online 08/01/2018].

R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rey, A., Pegoaro, E., Tedeschi, V., De Parri, I., Jarvis, G., Valentini, R. 2006. Annual Variation in Soil Respiration and its Components in a Coppice Oak Forest in Central Italy. *Global Change Biology* 8(9): 851 – 866.

Ritchie, G.S.P., Dolling, P.J. 1985. The Role of Organic Matter in Soil Acidification. *Soil Research* 23(4): 569 – 576.

Rulkens, W. 2008. Sewage Sludge as a Biomass Resource for the Production of Energy: Overview and Assessment of the Various Options. *Energy and Fuels* 22: 9-15.

Schmitz, A., Harrison, J.F. 2004. Hypoxic Tolerance in Air-Breathing Invertebrates. *Respiratory Physiology and Neurobiology* 141(3): 229 – 242.

Six, J., Elliott, E.T., Paustian, K., Doran, J.W. 1998. Aggregation and Soil Organic Matter Accumulation in Cultivated and Native Grassland Soils. *Soil Science Society of America Journal* 62(5): 1367 – 1377

Smith, K.A., Ball, T., Conen, F., Dobbie, K. E., Massheder, J., Rey, A. 2003. Exchange of Greenhouse Gases between Soil and Atmosphere: Interactions of Soil Physical Factors and Biological Processes. *European Journal of Soil Science* 54(4): 779 – 791.

Stevenson, I.L. 1956. Some Observations on the Microbial Activity in Remoistened Air-Dried Soils. *Plant and Soil* 8(2): 170 – 182

Tanji, K. K., Gao, S., Scardaci, S. C., Chow, A. T. 2003. Characterizing Redox Status of Paddy Soils with Incorporated Rice Straw. *Geoderma*, 114: 333-353.

Terry, R.E., Nelson, D.W., Sommers, L.E. 1979a. Carbon Cycling During Sewage Sludge Decomposition in Soils. *Soil Science Society of America Journal* 43(3): 494 – 499.

Terry, R.E., Nelson, D.W., Sommers, L.E. 1979b. Decomposition of Anaerobically Digested Sewage Sludge as Affected by Soil Environmental Conditions. *Journal of Environmental Quality* 8(3): 342 – 347.

Tian, G., Granato, T.C., Cox, A.E., Pietz, R.I., Carlson Jr, C.R., Abedin, Z. 2009. Soil Carbon Sequestration Resulting from Long-Term Application of Biosolids for Land Reclamation. *Journal of Environmental Quality* 38(1): 61 – 74.

UKSO, 2018. UK Soil Observatory. [Online]. [22 February 2019]. Available from: http://mapapps2.bgs.ac.uk/ukso/home.html

Wallace, B. M., Krzic, M., Forge, T. A., Broersma, K., Newman, R. F. 2009. Biosolids Increase Soil Aggregation and Protection of Soil Carbon Five Years after Application on a Crested Wheatgrass Pasture. *Journal of Environmental Quality*, 38: 291-298.

Walton, M., Herrick, J.E., Gibbens, R.P., Remmenga, M.D. 2001. Persistence of Municipal Biosolids in a Chihuahuan Desert Rangeland 18 Years after Application. *Arid Land Research and Management* 15: 223 – 232.

Wang, C., Yang, J., Zhang, Q. 2006. Soil Respiration in Six Temperate Forests in China. *Global Change Biology* 12(11): 2103 – 2114

Williams, C.H., Donald. C.M. 1957. Changes in Organic Matter and pH in a Podzolic Soil as Influenced by Subterranean Clover and Superphosphate. *Australian Journal of Agricultural Research* 8(2): 179 – 189.

Xu, M., Qi, Y. 2001. Soil-Surface CO_2 Efflux and its Spatial and Temporal Variations in a Young Ponderosa Pine Plantation in Northern California. *Global Change Biology* 7(6); 667 – 677.

Yan, Y., Song, H., Wang, Z., Teramoto, M., Wang, J., Liang, N., Ma, C., Sun, Z., Xi, Y., Li, L., Peng, S. 2019. Temperature Sensitivity of Soil Respiration Across Multiple Time Scales in a Temperature Plantation Forest. *Science of the Total Environment* 688: 479 – 485

Yuste, J.C., Baldocchi, D.D., Gershenson, A., Goldstein, A., Misson, L., Wong, S. 2007. Microbial Soil Respiration and its Dependence on Carbon Inputs, Soil Temperature and Moisture. *Global Change Biology* 13(9): 2018 – 2035

4.8 Supplementary information

4.8.1 Untransformed oxygen depletion curves

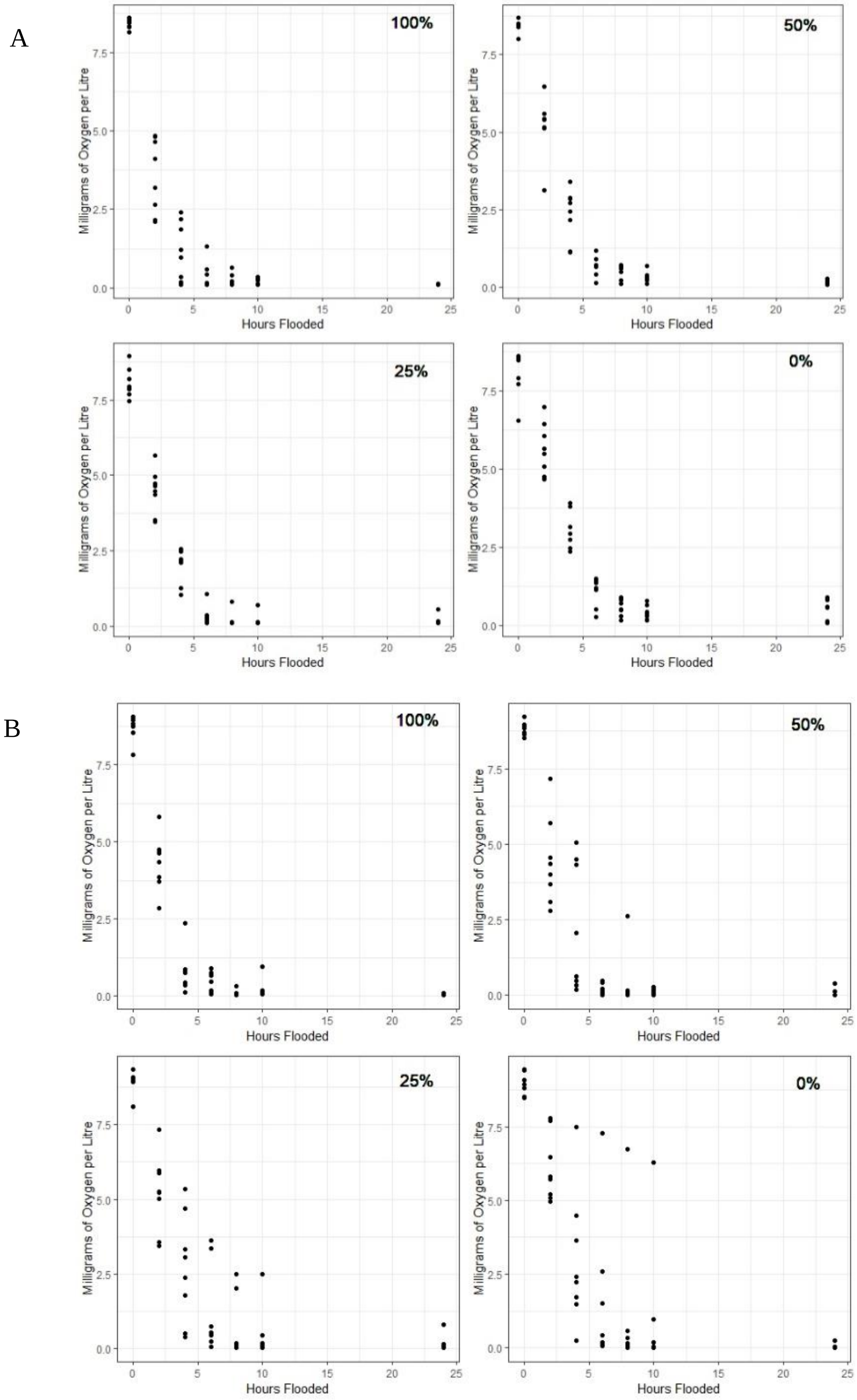

Figure 4-17. The oxygen concentration depletion curves over unlogged time for the A. Loddington and B. Spen Farm soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

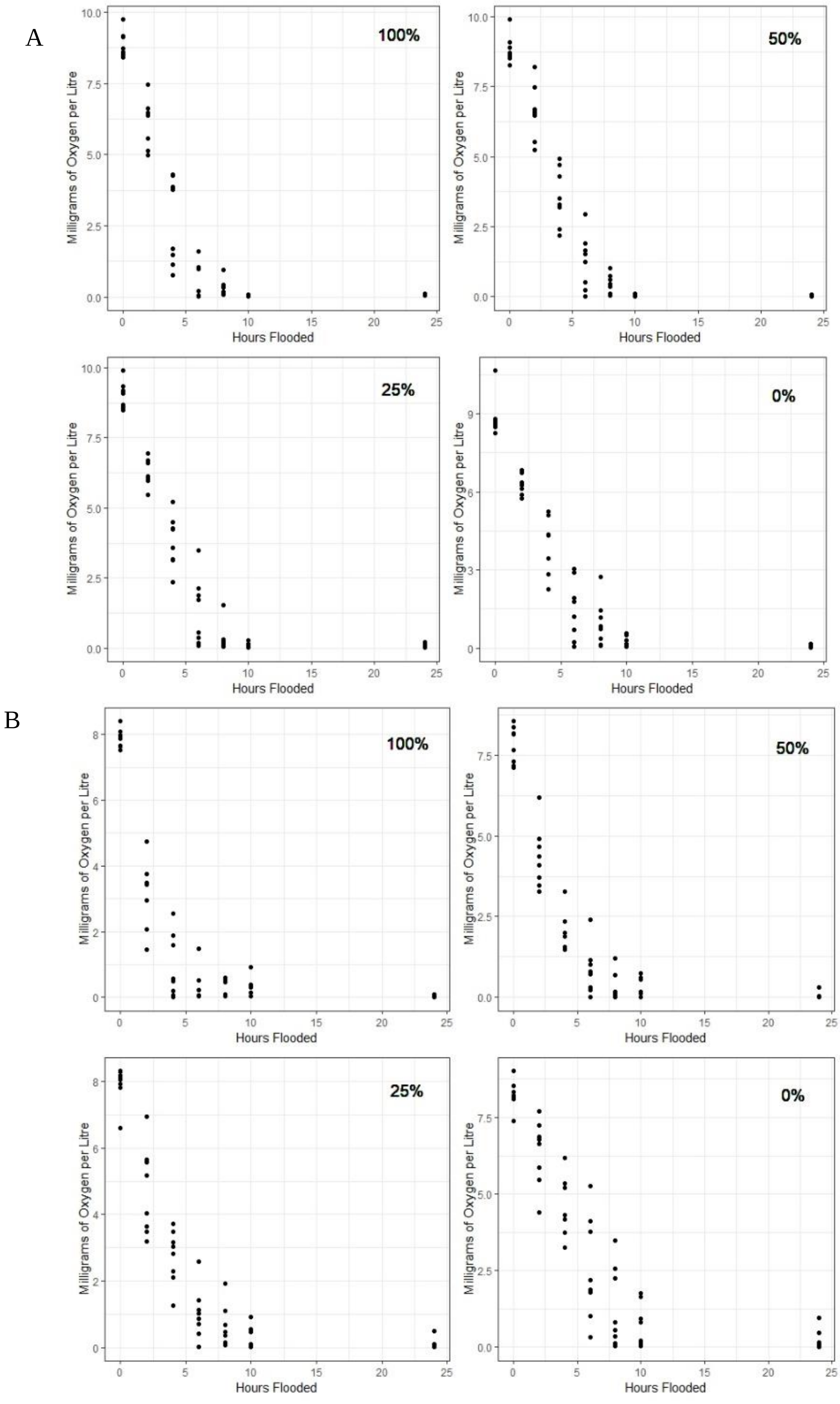

Figure 4-18. The oxygen concentration depletion curves over unlogged time for the A. Cusgarne and B. Heslington East soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

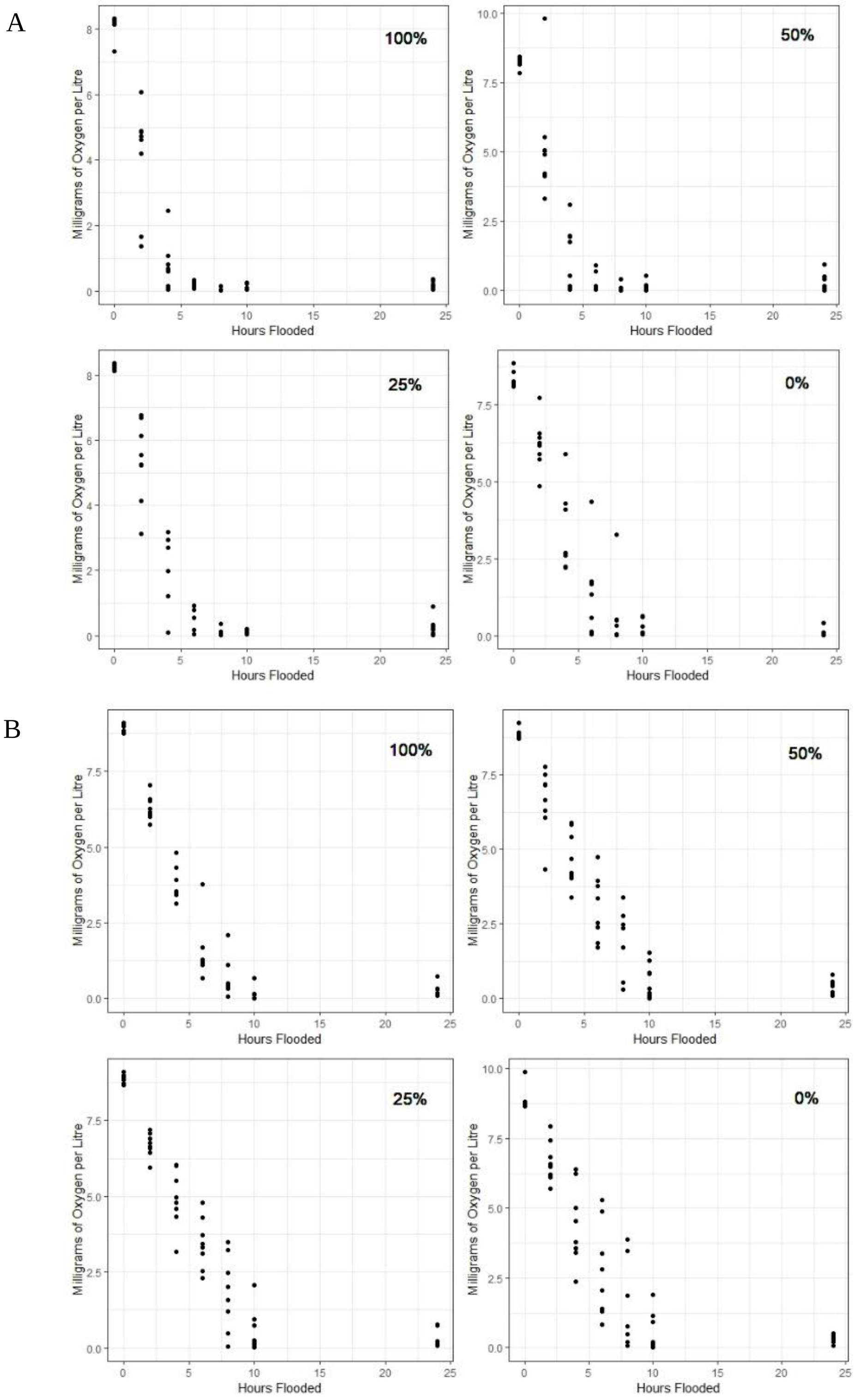

Figure 4-19. The oxygen depletion curves over unlogged time for the A. Heslington and B. Mansfield soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

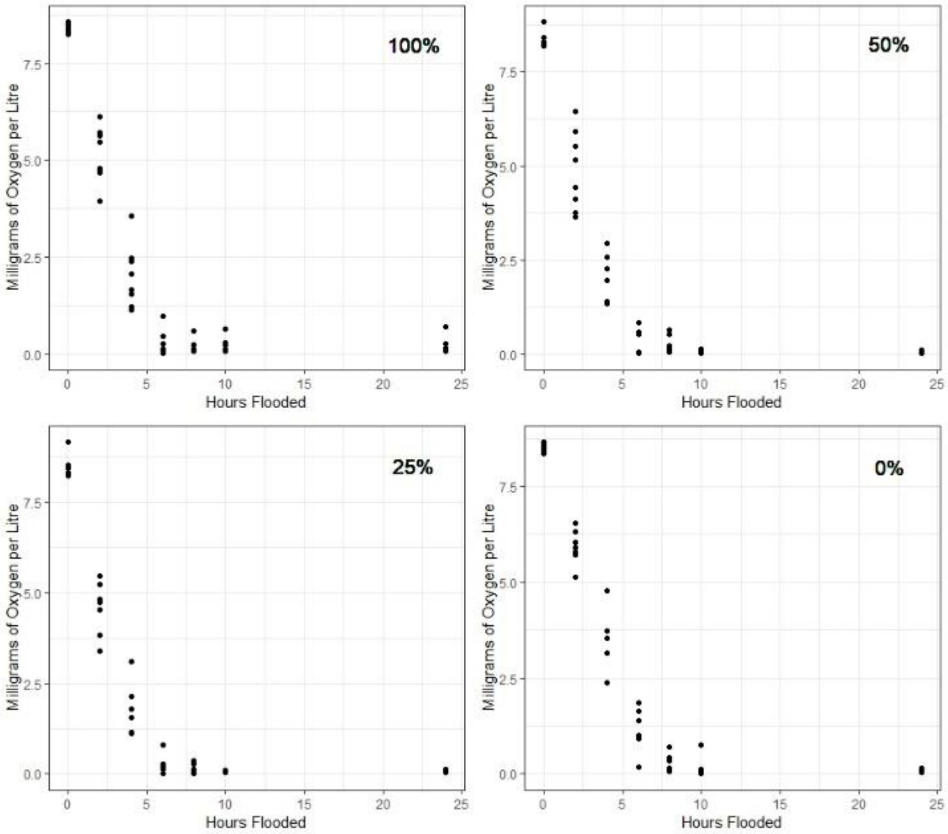

Figure 4-20. The oxygen depletion curves over unlogged time for the Newcastle soil origin site at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

4.8.2 Transformed oxygen depletion curves

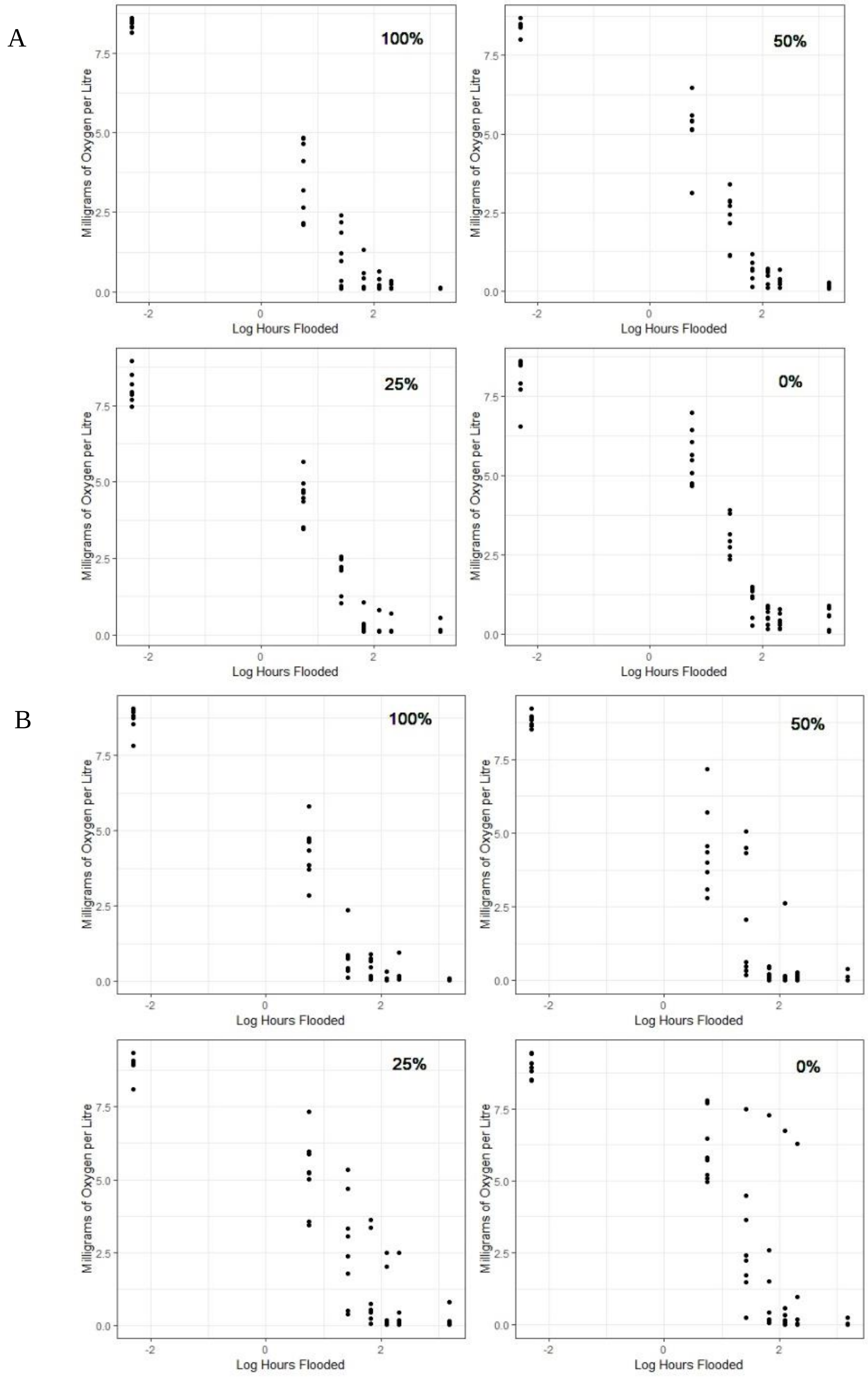

Figure 4-21. The oxygen depletion curves over logged time for the A. Loddington and B. Spen Farm soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

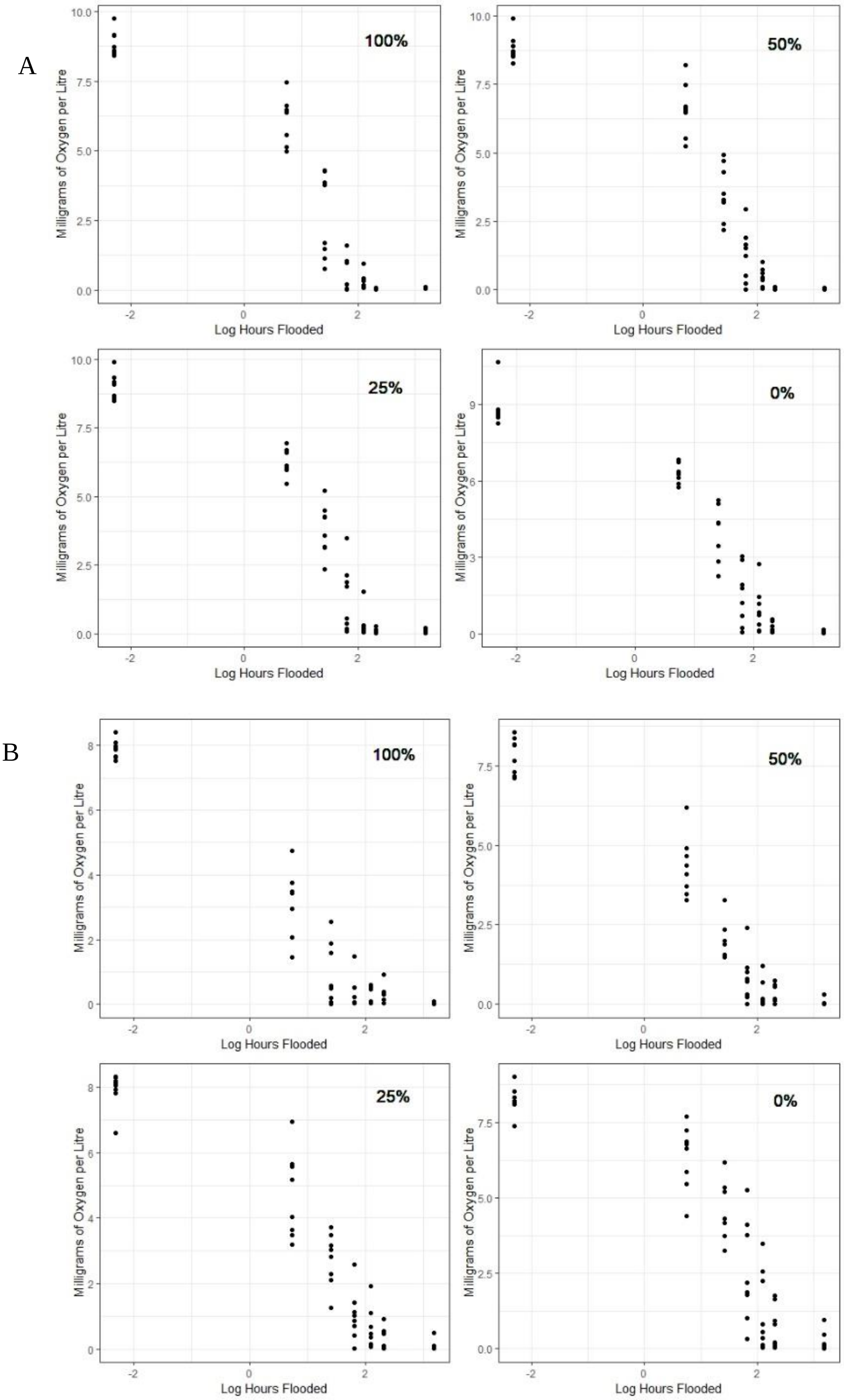

Figure 4-22. The oxygen depletion curves over logged time for the A. Cusgarne and B. Heslington East soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

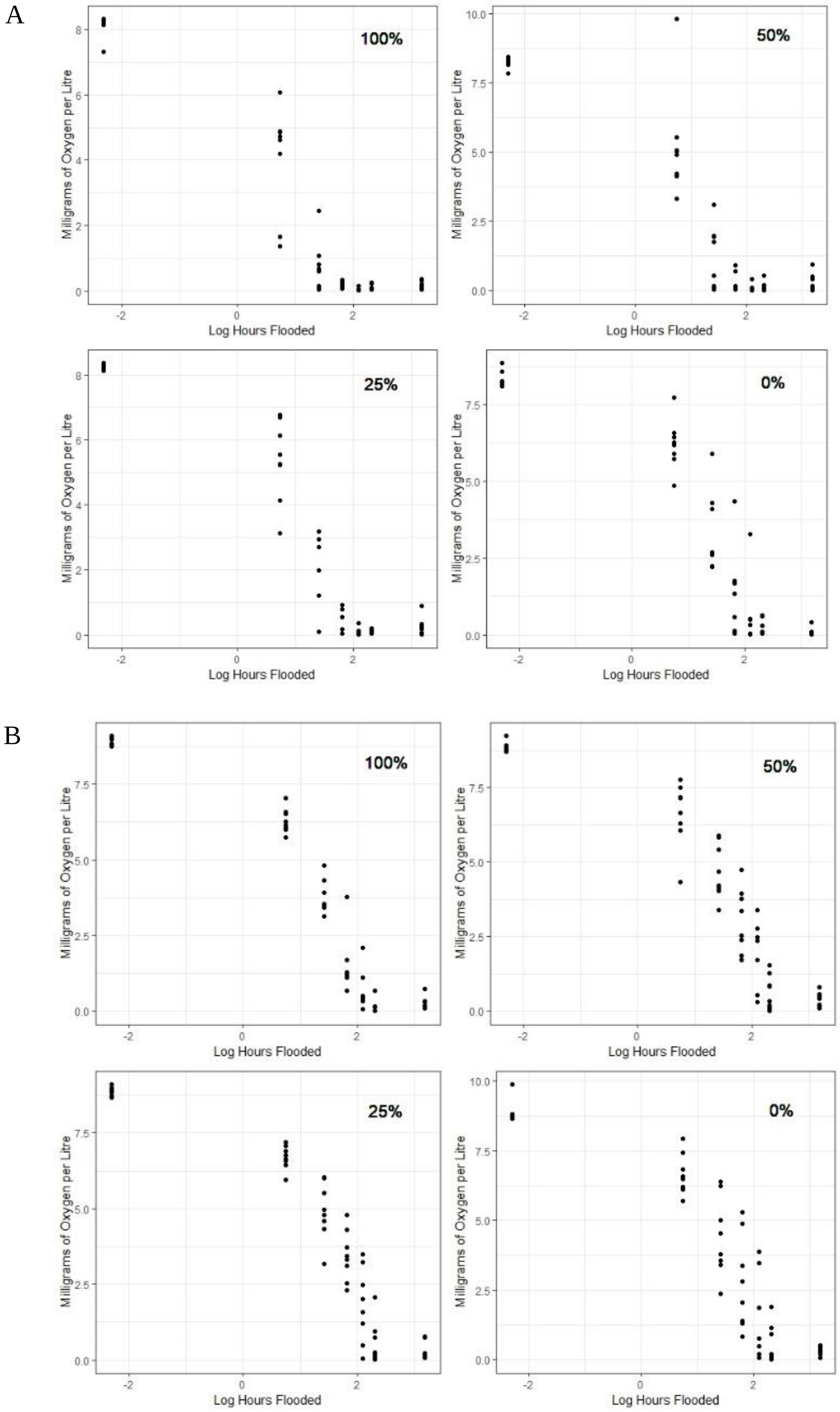

Figure 4-23. The oxygen depletion curves over logged time for the A. Heslington and B. Mansfield soil origin sites at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

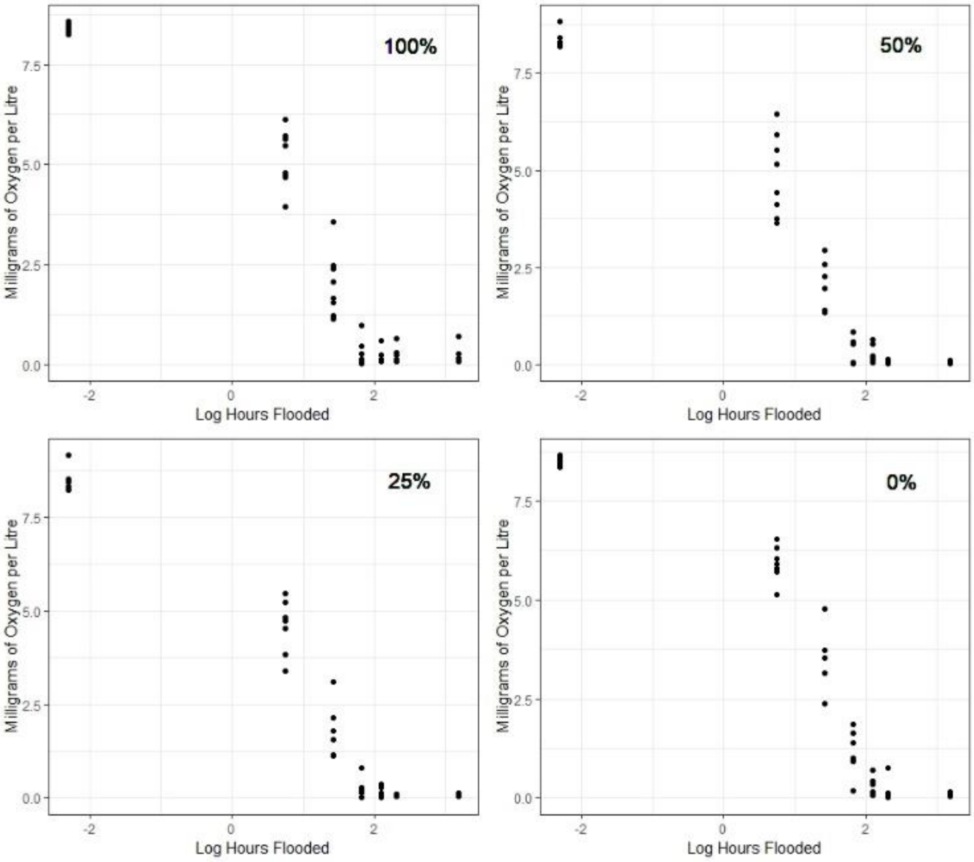

Figure 4-24. The oxygen depletion curves over logged time for the Newcastle soil origin site at different levels of maximum sewage sludge application (100%, 50%, 25% and 0% of maximum; n = 4 of each soil origin and sludge application). Each dot represents one of two measurements taken per beaker per timepoint.

5 Earthworm survival in water: the difference in oxygen requirements for an anecic (*Lumbricus terrestris*), an endogeic (*Allolobophora chlorotica*) and an epigeic (*Lumbricus castaneus*) species

5.1 Abstract

An earthworm's skin is its organ of oxygen exchange, allowing earthworms to survive in flooded environments provided that the water contains sufficient dissolved oxygen. However, we do not know the critical oxygen saturation level in solution below which earthworms will suffocate.

Three earthworms of contrasting ecotypes were investigated*:* *L. terrestris* (anecic), the green morph of *A. chlorotica* (endogeic), and *L. castaneus* (epigeic). Forty-eight tubes of reconstituted groundwater were prepared for weighed depurated earthworms: twenty-four control tubes, connected to an air pump, and twenty-four sealed treatment tubes. At specific time intervals, determined by pilot studies and varying between earthworm species, oxygen concentration and percentage oxygen saturation of the water, water temperature and pH were measured for three randomly selected tubes each from the control and treatment groups. The earthworms were removed from the selected tubes, weighed, and their length and diameter recorded before they were tested for life by prodding near the sensitive mouthparts with a sharp needle. *L. terrestris*, a large species which emerges at night to forage at the soil surface, consumed oxygen rapidly but only died when oxygen levels reached below approximately 1 milligram of oxygen per litre. *L. castaneus*, a smaller species which lives on the soil surface, consumed oxygen rapidly and died when oxygen levels reached below approximately 3 milligrams of oxygen per litre. *A. chlorotica* which is similar in size to *L. castaneus*, lives in the upper 20 cm of soil and is known to aestivate during the summer, consumed oxygen rapidly but did not die, even when oxygen levels reached approximately 1.5 milligrams of oxygen per litre after 280 hours.

The results suggest that earthworm respiration may be closely linked to both body size and to behavioural ecotype and that if flooding increases in frequency resulting in episodic reductions in soil oxygen levels, the species composition of earthworm communities may change.

5.2 Introduction

The soil environment is one in which both biotic and abiotic factors can be highly heterogeneous over scales ranging from hectares to millimetres (Ettema and Wardle, 2002). The spatial heterogeneity of distribution of soil aggregates and groupings of soil microorganisms (Stoyan et al., 2000), along with variation in factors such as soil moisture and soil organic matter content, lead to a highly spatially diverse distribution of the soil oxygen content (Parkin, 1993) not only through the soil pore space but also within aggregates (Sexstone et al., 1985). In this environment, which can rapidly change as an individual burrows through it, the respiratory system of the earthworm allows for passive diffusion of oxygen across the cuticle and epidermal tissues, as long as there is sufficient moisture to facilitate gas exchange and sufficient oxygen for respiration (Edwards and Lofty, 1977). However, in addition to conveying ecological niche benefits, the use of the skin as the organ of gas exchange allows earthworms to survive for some time in oxygenated water, with individuals of *A. chlorotica* surviving a mean average of 137 days, *L. terrestris* surviving 137, and *L. rubellus* surviving 78 days (Roots, 1956).

The differing lengths of survival in oxygenated water first noted by Roots (1956) may be linked to the different ecological niches that the earthworm species exploit. Known as earthworm ecotypes, different earthworm species display preferences for different zones of habitation in the soil, different food sources, and in some cases differences in behaviour. Earthworm species are broadly divided into three categories: anecic, epigeic and endogeic (Bouché, 1977), described in Section 2.6. These three distinct habitats may be subject to different levels of oxygenation, which may in turn mean that earthworms of different ecotypes are adapted to differing levels of oxygen availability. As soil moisture and organic matter disperses with increasing soil depth (Stoyan et al., 2000), transitions between oxic and anoxic zones becomes much smoother, meaning there are fewer distinctly anoxic and distinctly oxic zones, and more regions existing at partial oxygenation. This, along with the formation of deep burrows which may conduct oxygen down to deeper layers of soil (Lavelle, 1988) may mean that anecic earthworm species are less likely to be subject to the heterogeneity of soil anoxic zones potentially encountered by the endogeic species. Epigeic earthworms, meanwhile, live in and consume litter on the soil surface. In the soil surface litter environment, microbial activity is highly dependent on moisture and temperature, which may mean that the oxygen in the environment could be highly heterogeneous; however, the fact that the litter is on the soil surface, where oxygen can easily be replenished from the atmosphere, may mean that epigeic earthworms do not need to display any long term adaptations to cope with low oxygen conditions.

While earthworms of all ecotypes are likely adapted to some degree of oxygen stress, with *L. terrestris* and *L. rubellus* having been found to produce lactic acid (Davis and Slater, 1928) and other metabolites associated with anaerobic respiration (Gruner and Zebe, 1978), their ability to tolerate anoxic conditions is still unknown. As flooded soil can reach anoxic levels within as little as 24 hours (Ponnamperuma, 1984; Chapter 4), understanding how earthworms of different ecotypes respond to anoxia may inform understanding of earthworm population dynamics in regularly flooded regions, including how previously undisturbed populations may shift with the increased frequency and intensity of flooding predicted to occur with UK climate change.

In this study, a number of individuals of three UK species (*L. terrestris, A. chlorotica,* and *L. castaneus*) were maintained in sealed treatment tubes or in aerated control tubes filled with a reconstituted groundwater solution. These tubes were destructively sampled at set time points to determine how the dissolved oxygen concentration within the solution changed over time. Four broad hypotheses were considered:

1. The dissolved oxygen concentration (mg O_2 L^{-1}) of the solution surrounding the earthworm individuals would decrease in the treatment tubes but not in the control tubes, due to the aeration of the control tubes with a peristaltic pump.

2. The dissolved oxygen concentration at which individuals die would differ between species, depending on species characteristics such as the size of the individual or species behavioural patterns.

3. There would be no difference in the mass gained while maintained in solution between control and treatment individuals, and between living and dead individuals.

4. The solution pH in both the control and treatment tubes would decrease with time as respired CO_2 dissociates in solution. As the treatment tubes are sealed, more CO_2 may be expected to accumulate in the treatment tubes than in the control tubes.

5.3 Methods

5.3.1 Earthworm collection

At least fifty individuals of each earthworm species were obtained from different sources; individuals of *L. terrestris* were purchased from Wiggly Wrigglers (Blakemere, HR2 9PX, UK), while individuals of *A. chlorotica* and *L. castaneus* were collected from pasture fields at Spen Farm, near Leeds (SE 44300 41700). The experiments were run consecutively with each species – first *L. terrestris*, then *A. chlorotica*, and finally *L. castaneus*. The same methodology was used for each species, but changes were made to the time intervals for measurement taking based on preliminary studies. *L. terrestris* was selected as the anecic study species due to its prevalence in UK soils. *A. chlorotica* was selected as the endogeic study species as it was the most common species in the arable and pasture field sites (Section 3A and Section 3B), and easily collected from the Spen Farm location. *L. castaneus* was selected as the epigeic species as it was similar in size to *A. chlorotica*, and could be collected in sufficient numbers from the Spen Farm site.

5.3.2 Tube setup

For each species run, a number of individuals were depurated for forty-eight hours at 10°C on damp blue roll in order to empty the gut contents. Blue roll was changed approximately every 12 hours to prevent re-ingestion of soil matter. A greater number of earthworms than required were depurated in order to allow for any individuals that appeared to be in poor condition post depuration to be discarded and to account for loss of earthworms due to escape. Following depuration, forty-eight individuals that appeared to be in good condition were selected. The weight, length and diameter of these earthworms was recorded.

Twenty-four individuals were placed into the treatment tubes: sealed 50ml centrifuge tubes of reconstituted groundwater (Arnold et al., 2007) which had been maintained at 10°C in a controlled temperature cabinet. The remaining twenty-four individuals were placed into 50 ml centrifuge tubes of reconstituted groundwater maintained in a controlled temperature room at 15°C. The control tubes had been modified by drilling seven holes of approximately 2.5 mm width in the lid, and a length of flexible plastic tubing of approximately 2.5 mm internal diameter was inserted through one of these holes. Both the control and treatment tubes were weighed, filled to the brim with the reconstituted groundwater and weighed earthworms were added to the tubes. After the earthworms were placed in the tubes, the treatment tubes were returned to the 10°C controlled temperature cabinet. The control tubes were connected to a peristaltic pump set to rotate at 90 RPM to aerate the reconstituted groundwater solution. Due to the size and shape of the pumps, the setup could not be maintained in the 10°C controlled temperature chamber, so the control setup was maintained in a 15°C controlled temperature room. Despite the fact that the treatment and control tubes were maintained in different conditions, this was considered justified in the experimental setup as the purpose of the controls was to determine whether oxygen depletion was responsible for earthworm death, rather than being maintained in the centrifuge tubes. While temperatures of 15°C are optimal for high rates of earthworm cocoon production and growth under laboratory culture conditions (Lowe and Butt, 2005), earthworms are active in soil at 10°C

(Edwards and Lofty, 1977), and in the field surveys earthworms were found at soil temperatures below 5°C (Section 3A.4.1, Section 3B.4.1.

5.3.3 Measurements

The measurement intervals were determined using preliminary studies of the experimental species, when prior methodologies were being tested. The timepoints at which measurements were taken for *L. terrestris* were +0, +3, +6, +9, +24, +33, +48 and +72 hours following immersion. For *A. chlorotica*, the timepoints were +0, +9, +24, +48, +96, +144, +216 and +288 hours following immersion. For *L. castaneus*, the timepoints were +0, +9, +24, +48, +72, +120, +168, and +216 hours following immersion. At each interval, three tubes from both the treatment and control sets selected at random using a random number generator. The tubes were weighed before opening. After opening, the percent oxygen saturation, the concentration of oxygen in solution (mg O_2 L^{-1}), and the solution temperature were recorded by inserting a Thermo Scientific Orion Star A223 and Star A23 Portable Dissolved Oxygen Meter. The pH of the solution was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers. The earthworm from the tube was removed, blotted on blue roll, and again had its weight, length, and width recorded. The earthworms were tested to see if they were alive using a response test, in which they were prodded near the sensitive mouth parts using a sharp needle (OECD, 1984). If the earthworm did not respond to the prodding and did not show any signs of movement after two minutes on the bench surface, during which it was weighed and measured, it was recorded as dead.

5.3.4 Data analyses and statistics

Data were analysed using R version 3.4.2.

The oxygen concentration for each individual at each time point was normalised per gram biomass of the earthworm by dividing the oxygen concentration by the initial earthworm mass, and normalised per unit surface area by dividing the oxygen concentration by the earthworm surface area. Earthworm surface area was calculated by measuring the length and the width of the earthworms in millimetres, assuming that the earthworms were perfect cylinders. The initial measurements of earthworm biomass and surface area were used to control for any mass or dimension changes that may have occurred after the death of an individual. Datasets were statistically tested for normality, and non-parametric equivalents of statistical tests used where necessary.

The datasets were analysed to address the four broad hypotheses discussed in Section 5.2:

1. To determine how the oxygen concentration changed in the control and treatment tubes over time for each species, a generalised linear model (GLM) was performed as a non-parametric equivalent to a two way analysis of variance (ANOVA) comparing the effects of treatment and timepoint on the oxygen concentration for each earthworm species. This was performed for the absolute oxygen concentration (mg O_2 L^{-1}) in solution, the concentration per gram biomass (mg O_2 L^{-1} g^{-1}) and the concentration per mm2 surface area (mg O_2 L^{-1} mm^{-2}) of the earthworm individual.

2. To determine if the oxygen concentration at which individuals of *L. terrestris* and *L. castaneus* died differed, a two way t-test for the absolute concentration of oxygen, and a Wilcoxon signed ranks test for the concentration of oxygen normalised by gram biomass of the earthworm individual and by the surface area of the earthworm (mm^2). A further hypothesis, that the oxygen concentration at which individuals of *L. terrestris* and *L. castaneus* would significantly differ to the oxygen concentration at which individuals of *A. chlorotica* plateaued, was tested using a Kruskal-Wallis test, and pairwise Wilcox post hoc testing.

3. To determine if the mass gained by earthworms in solution differed between control and treatment, and between live and dead earthworms, a two way ANOVA and a non-parametric GLM for *L. terrestris* and *L. castaneus* were performed respectively. As no individuals of *A. chlorotica* died during the experiment, a one way Kruskal-Wallace test was performed comparing the mass gained between individuals in the treatment and control tubes.

4. To determine whether the pH of the solution changed with time and between control or treatment tubes, GLMs acting as a non-parametric two way ANOVA were performed between the pH of the solution and the measurement timepoint and treatment or control categorisation.

5.4 Results

5.4.1 Changes in oxygen concentration

A GLM, performed as a non-parametric two way ANOVA equivalent, found a significant effect of the sampling timepoint ($P < 0.01$), the treatment ($P < 0.01$), and the interaction term between the two ($P < 0.01$) for *L. terrestris* (Fig. 5-1), *A. chlorotica* (Fig. 5-2) and *L. castaneus* (Fig. 5-3)

No individuals of *L. terrestris* died in the control tubes over a 72 hour period. In the treatment tubes, 100% earthworm mortality was reached by 36 hours submerged. Tukey post hoc testing showed that, for individuals of *L. terrestris,* the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 6. From hours 9 to 72, the oxygen concentration was significantly lower in the treatment tubes than in the control tubes ($P < 0.05$). Across all sampling timepoints, there was no significant difference in the oxygen concentration in the control tubes. From hours 9 to 72, the oxygen concentrations in the treatment tubes did not significantly differ.

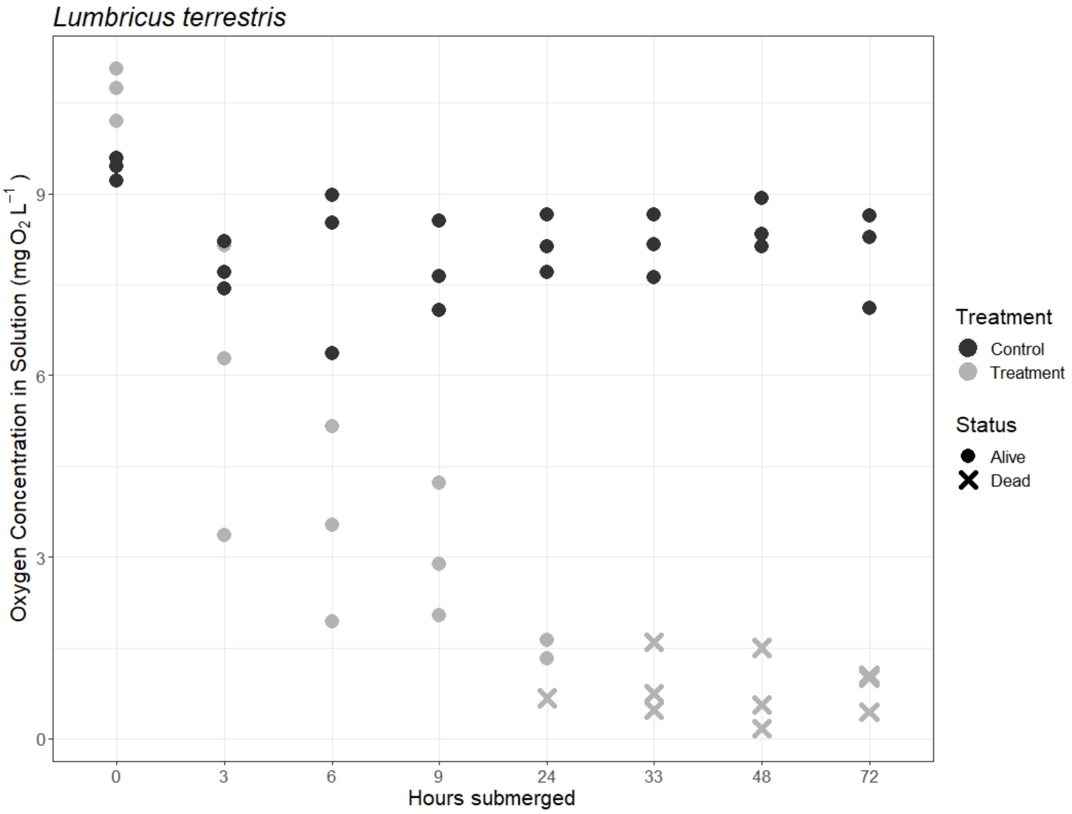

Figure 5-1. The changes in oxygen concentration in control and treatment tubes containing individuals of *L. terrestris* over time. 100% mortality in treatment individuals was reached by 36 hours, with no mortality observed in control individuals

No individuals of *A. chlorotica* died over the 288 hour sampling period. Tukey post hoc testing showed that, for individuals of *A. chlorotica*, the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 9. From hours 24 to 288, the oxygen concentration in the treatment tubes was significantly lower than in the control tubes ($P < 0.05$). Across all sampling timepoints, there was no significant difference in the oxygen concentration of the control tubes. Between hours 24 to 288, the oxygen concentrations within the treatment tubes did not significantly differ.

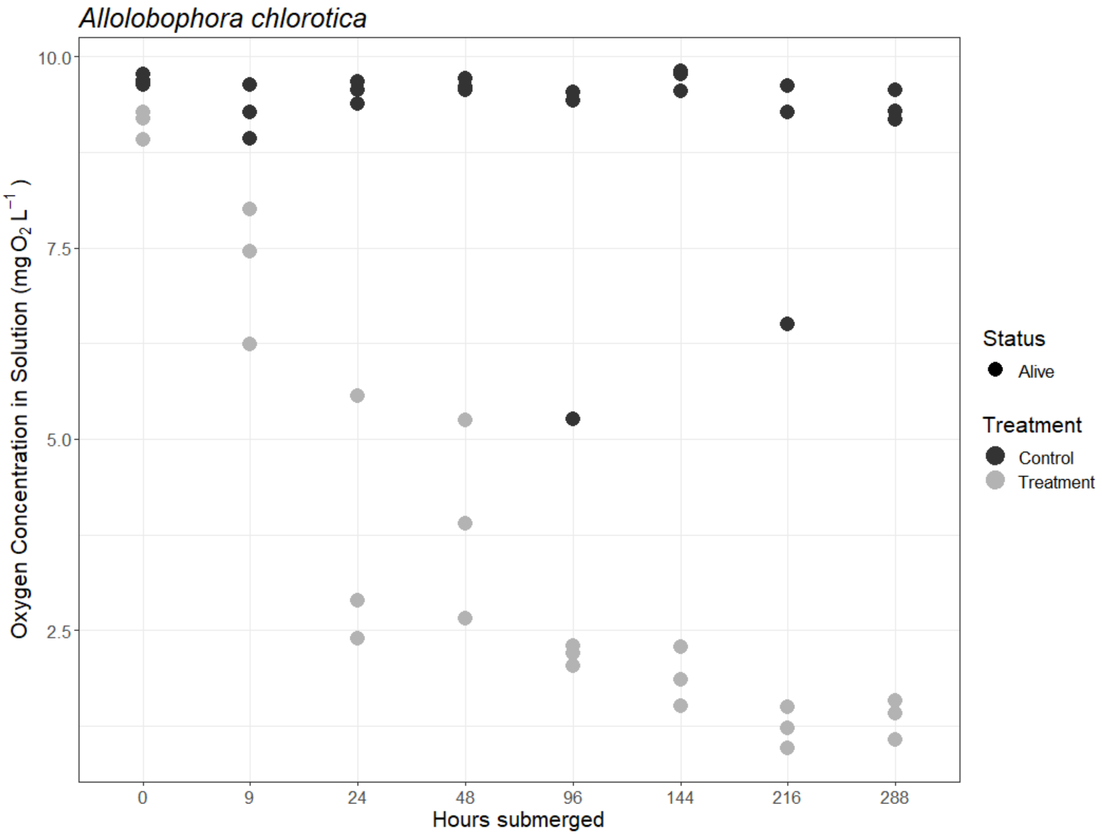

Figure 5-2. The changes in oxygen concentration in control and treatment tubes containing individuals of *A. chlorotica* over time. No mortality in either control or treatment individuals was observed in 288 hours.

One individual of *L. castaneus* was found dead in the control tubes, at hour 168. In the treatment tubes, 100% mortality was reached by 168 hours submerged, with 66% mortality reached at 28 hours. Tukey post hoc testing showed that, for individuals of *L. castaneus*, the oxygen concentration in the control and treatment tubes did not significantly differ from hours 0 to 9. From hours 24 to 216, the oxygen concentration in the treatments tubes was significantly lower than the oxygen concentration in the control tubes ($P < 0.05$). Across all sampling timepoints, there was no significant difference in the oxygen concentration of the control tubes. Within the treatment tubes, the oxygen concentration was significantly higher at hours 24 and 48 than the sampling timepoints between hours 72 and 216 ($P < 0.05$).

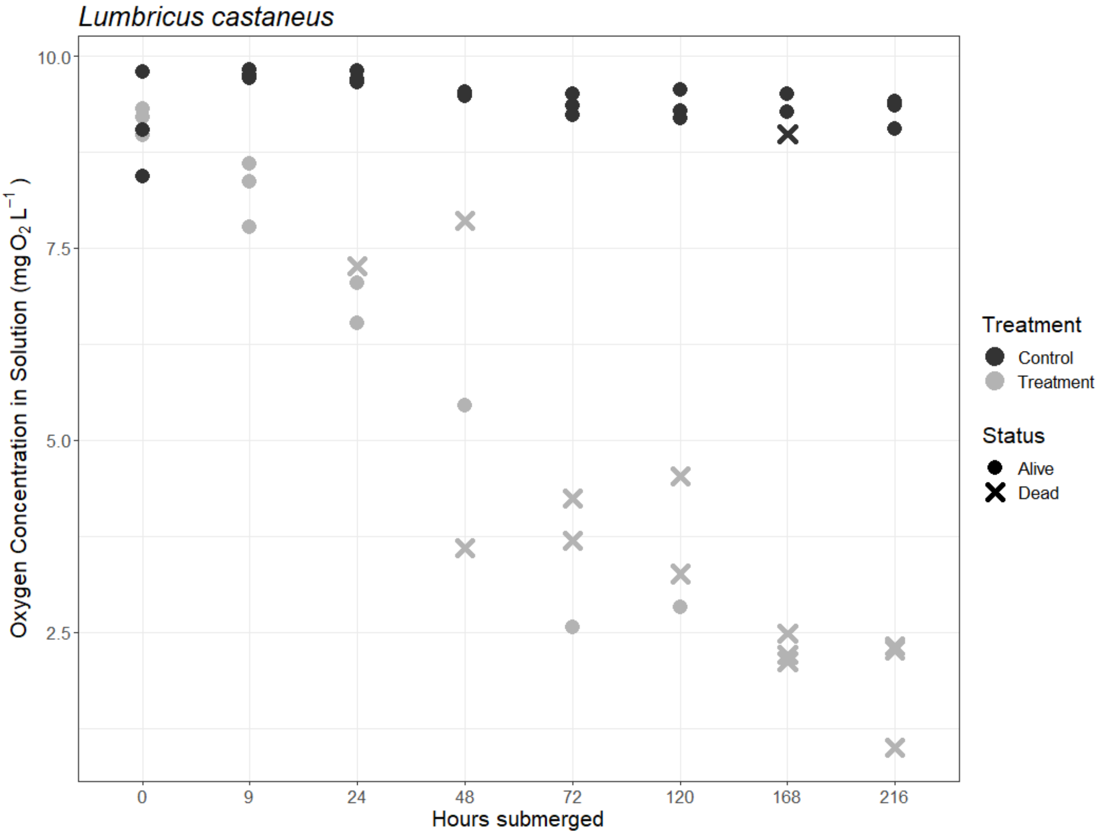

Figure 5-3. The changes in oxygen concentration in control and treatment tubes containing individuals of *L. castaneus* over time. 100% mortality in the treatment tubes was reached by 168 hours submerged, with 66% mortality reached at 48 hours. One control individual was found dead at the 168 hour sampling point.

The mean values of the normalised oxygen concentration per gram biomass of individuals of each earthworm species for each timepoint are presented in Table 5-1. GLMs examining the effect of timepoint and treatment on the normalised oxygen concentration per gram found a significant effect of timepoint ($P < 0.01$) treatment ($P < 0.01$) and the interaction term between the two ($P < 0.01$) for all three earthworm species.

Tukey post hoc testing showed that for all three earthworm species, there was no significant difference in the oxygen concentration normalised per gram at each timepoint for the control tubes ($P < 0.05$). For individuals of *L. terrestris*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 9 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 6 ($P < 0.05$).For individuals of *A. chlorotica*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 24 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 24 ($P < 0.05$). For individuals of *L. castaneus*, the oxygen concentration per gram biomass in the treatment tubes began to be significantly lower than the control tubes from hour 72 ($P < 0.05$). From hour 24, there was no significant difference in the oxygen concentration per gram biomass in the treatment tubes ($P < 0.05$).

Table 5-1. The mean and standard deviation oxygen concentration normalised by the gram biomass of the individual (mg O_2 L^{-1} g^{-1}) in control and treatment tubes containing individuals of *L. terrestris, A. chlorotica* and *L. castaneus* at each sampling time point. For each species, cells marked with the same letter within control and treatment columns are not significantly different (*P* > 0.05).

L. terrestris			***A. chlorotica***			***L. castaneus***		
Hours submerged	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**		**Hours submerged**	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**		**Hours submerged**	**Oxygen concentration normalised per unit biomass (mg O_2 L^{-1} g^{-1})**	
	Control	**Treatment**		**Control**	**Treatment**		**Control**	**Treatment**
0	2.58 (± 0.34) **EF**	3.16 (± 0.67) **F**	**0**	57.15 (± 12.15) **D**	59.54 (± 2.99) **D**	**0**	79.64 (± 13.06) **BC**	61.33 (± 7.03) **BC**
3	1.96 (± 0.20) **DE**	1.42 (± 0.78) **BCDE**	**9**	54.08 (± 19.31) **D**	47.75 (± 16.15) **BCD**	**9**	78.11 (± 24.06) **C**	77.88 (± 8.29) **C**
6	1.99 (± 0.50) **DEF**	0.84 (± 0.43) **ABCD**	**24**	54.97 (± 4.35) **D**	23.62 (± 13.84) **ABC**	**24**	76.26 (± 11.60) **C**	47.15 (± 1.95) **ABC**
9	1.48 (± 0.44) **DE**	0.74 (± 0.42) **ABC**	**48**	61.59 (± 7.79) **D**	22.15 (± 5.94) **AB**	**48**	61.67 (± 24.08) **C**	49.26 (± 27.09) **ABC**
24	1.98 (± 0.38) **EF**	0.28 (± 0.13) **AB**	**96**	53.89 (± 21.64) **D**	13.78 (± 1.47) **A**	**72**	75.17 (± 16.39) **BC**	25.93 (± 8.47) **AB**
33	2.21 (± 0.20) **CDE**	0.25 (± 0.19) **AB**	**144**	54.33 (± 6.80) **D**	10.93 (± 3.75) **A**	**120**	80.64 (± 14.46) **C**	28.83 (± 10.34) **AB**
48	1.76 (± 0.12) **EF**	0.19 (± 0.19) **A**	**216**	52.70 (± 12.93) **CD**	8.88 (± 1.40) **A**	**168**	81.64 (± 13.06) **C**	14.52 (± 1.67) **A**
72	2.38 (± 0.72) **EF**	0.21 (± 0.08) **A**	**288**	63.69 (± 4.18) **CD**	7.64 (± 0.68) **A**	**216**	79.64 (± 25.56) **C**	12.37 (± 2.88) **A**

The mean values of the oxygen concentration normalised per unit surface area (mm^{-2}) for each timepoint are presented in Table 5-2. GLMs examining the effects of timepoint and treatment on this value of oxygen concentration per unit surface area found a significant effect of timepoint ($P < 0.01$) and treatment ($P < 0.01$) for all earthworm species, with a significant interaction term ($P < 0.01$) present for *L. terrestris* and *A. chlorotica*.

Tukey post hoc testing showed that for all earthworm species, there was no significant difference in the oxygen concentration normalised per mm^2 surface area at each timepoint for the control tubes ($P < 0.05$). For individuals of *L. terrestris*, the oxygen concentration per mm^2 surface area in the treatment tubes began to be significantly lower than the control tubes from hour 24 ($P < 0.05$), with no significant difference in the oxygen concentration per gram biomass in the treatment tubes from hour 3 ($P < 0.05$).For individuals of *A. chlorotica*, the oxygen concentration per mm^2 surface area in the treatment tubes began to be significantly lower than the oxygen concentration per mm^2 at 9 hours from 48 hours ($P < 0.05$). There was no significant difference in the oxygen concentration per mm^2 in the treatment tubes from 24 hours ($P < 0.05$). For individuals of *L. castaneus*¸ Tukey post hoc testing showed that the oxygen concentration of treatment tubes was significantly lower than the oxygen concentration in the control tubes ($P < 0.05$). Statistical testing indicated a significant effect of timepoint, but post hoc testing showed that timepoints 9 and 168 were the closest to statistically significant difference ($P = 0.09$). Tukey post hoc testing of the interaction terms showed that the only statistically significant difference occurred between the treatment tubes at hours 216 and 168, which were significantly lower than the control tubes at hour 9 ($P < 0.05$).

Table 5-2. The mean and standard deviation oxygen concentration normalised by the mm^2 surface area of the individual ($\mu g\ O_2\ L^{-1}\ mm^{-2}$) in control and treatment tubes containing individuals of *L. terrestris, A. chlorotica* and *L. castaneus* at each sampling time point. For each species, cells marked with the same letter within control and treatment columns are not significantly different (*P* > 0.05).

	L. terrestris			***A. chlorotica***			***L. castaneus***	
Hours submerged	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**		**Hours submerged**	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**		**Hours submerged**	**Oxygen concentration normalised per unit surface area ($\mu g\ O_2\ L^{-1}\ mm^{-2}$)**	
	Control	**Treatment**		**Control**	**Treatment**		**Control**	**Treatment**
0	4.26 (± 1.63) **D**	4.38 (± 0.54) **D**	**0**	44.89 (± 11.65) **E**	46.53 (±3.99) **E**	**0**	63.14 (± 22.58) **AB**	63.69 (± 19.24) **AB**
3	2.88 (± 0.53) **CD**	2.53 (± 1.42) **ABCD**	**9**	37.78 (± 11.27) **CDE**	36.36 (± 11.02) **BCDE**	**9**	84.49 (± 12.05) **B**	76.18 (± 27.95) **AB**
6	3.17 (± 0.84) **CD**	1.27 (± 0.42) **ABC**	**24**	48.97 (± 1.78) **E**	18.82 (± 10.45) **ABCD**	**24**	64.84 (± 32.57) **AB**	47.34 (± 15.56) **AB**
9	3.24 (± 0.67) **CD**	1.44 (± 0.98) **ABC**	**48**	57.20 (± 12.67) **E**	15.89 (± 2.77) **ABC**	**48**	81.19 (± 38.19) **AB**	55.04 (± 33.70) **AB**
24	3.75 (± 1.05) **D**	0.48 (± 0.21) **AB**	**96**	43.50 (± 16.53) **DE**	12.24 (± 0.56) **AB**	**72**	60.02 (± 28.60) **AB**	21.69 (± 14.63) **AB**
33	2.71 (± 0.22) **BCD**	0.36 (± 0.28) **A**	**144**	33.51 (± 9.26) **CDE**	8.05 (± 3.18) **A**	**120**	59.44 (± 10.36) **AB**	21.07 (± 5.47) **AB**
48	2.86 (± 0.11) **CD**	0.28 (± 0.28) **A**	**216**	47.66 (±7.89) **DE**	6.00 (± 1.48) **A**	**168**	63.48 (± 13.18) **AB**	13.81 (± 4.05) **A**
72	3.16 (± 1.19) **CD**	0.36 (± 0.27) **A**	**288**	42.50 (± 3.97) **DE**	5.65 (± 0.88) **A**	**216**	74.46 (± 50.81) **AB**	13.44 (± 3.97) **A**

5.4.2 Differences in oxygen concentration at which earthworms died

As no individuals of *A. chlorotica* died, they were not included in this portion of the hypothesis testing. The absolute, normalised by biomass and normalised by surface area at which individuals of *L. terrestris* and *L. castaneus* were found dead was compared.

A two way t-test found that individuals of *L. terrestris* died at a significantly lower oxygen concentration (mg O_2 L^{-1}) than individuals of *L. castaneus* ($P = 0.003$; Fig. 5-4).

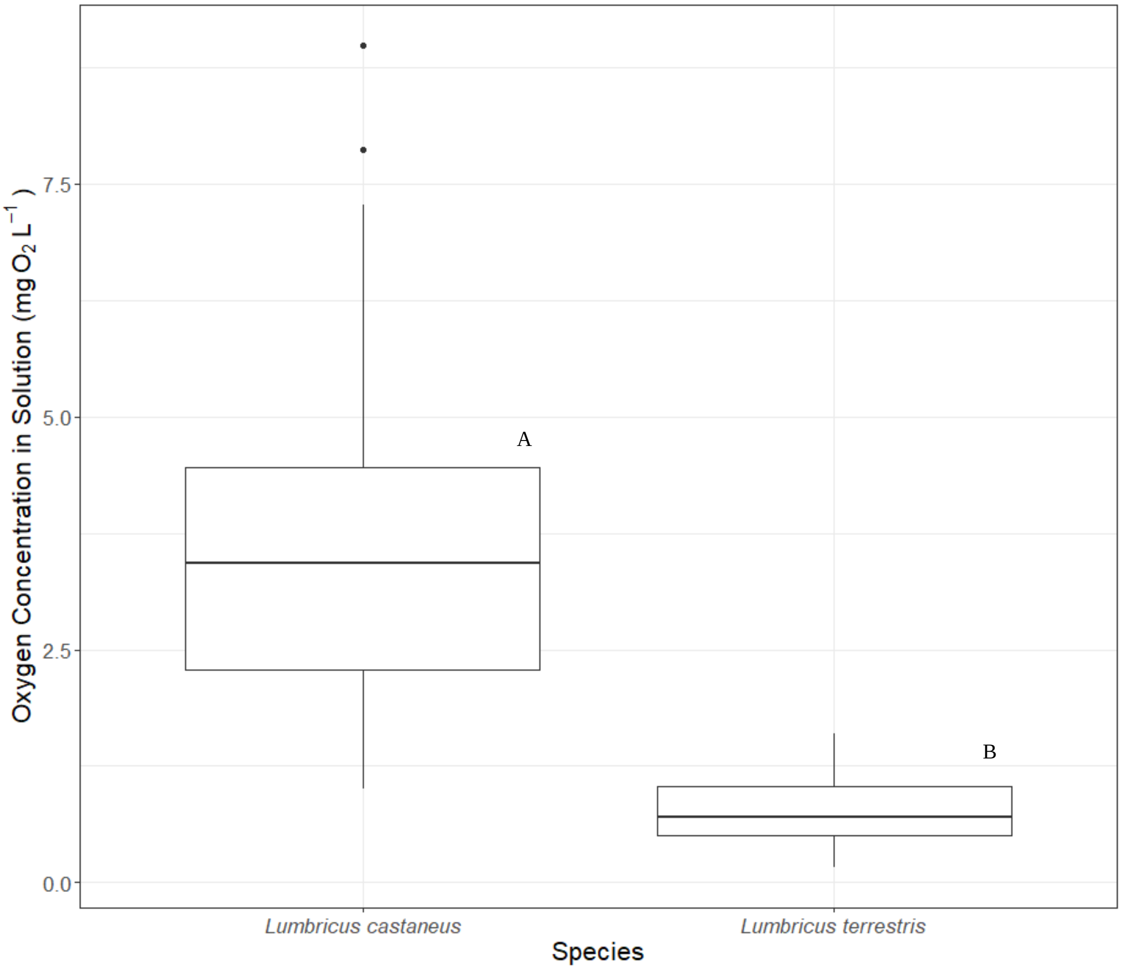

Figure 5-4. The differences in the absolute oxygen concentration in solution at which individuals of *L. castaneus* and *L. terrestris* were found dead, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Individuals of *L. castaneus* died at a significantly higher absolute oxygen concentration that individuals of *L. terrestris* ($P < 0.01$). Bars marked with the same letter are not significantly different ($P < 0.05$).

A Wilcox signed ranks test found that individuals of *L. terrestris* died at a significantly lower oxygen concentration per gram individual biomass than individuals of *L. castaneus* ($P < 0.0001$; Fig. 5-5)

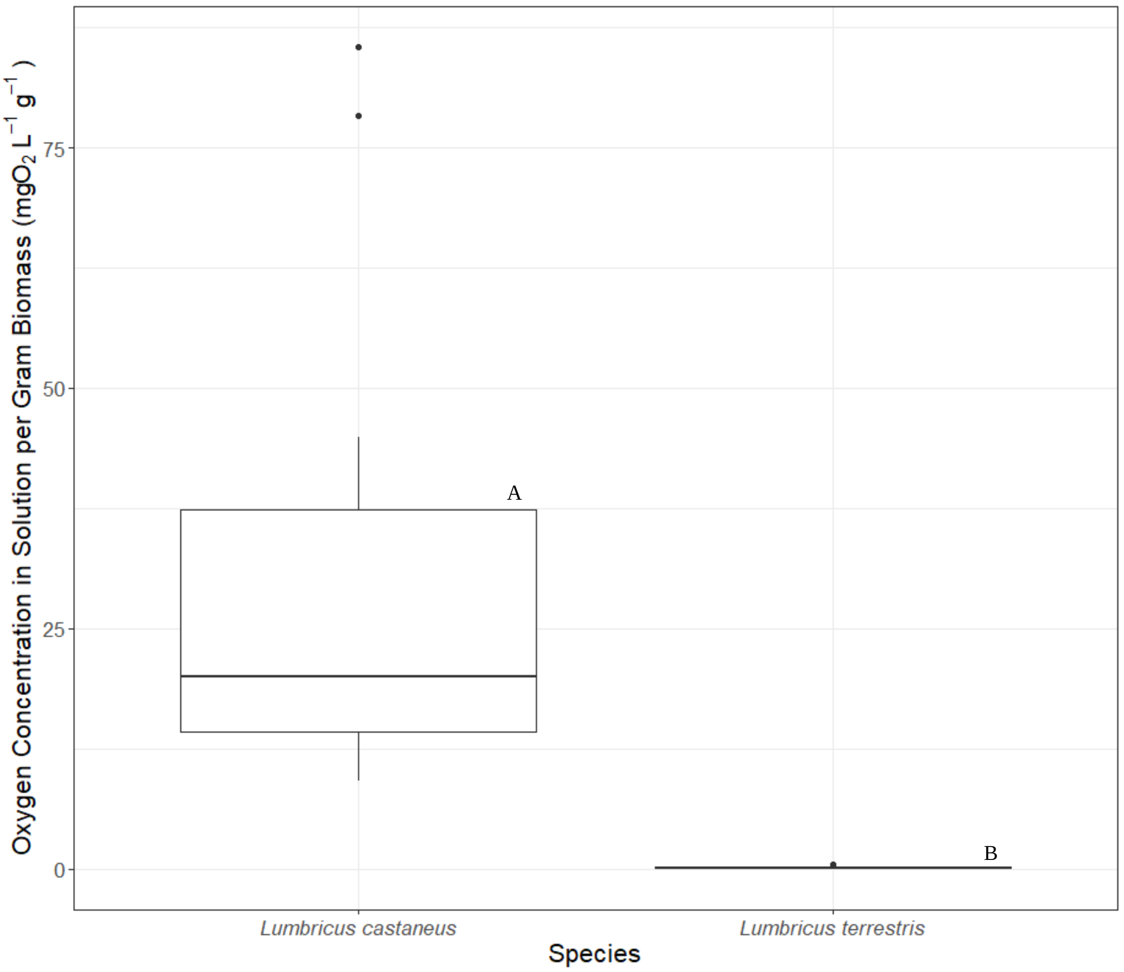

Figure 5-5. The oxygen concentration normalised per gram biomass at which individuals of *L. castaneus* and *L. terrestris* were found dead, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Individuals of *L. castaneus* died at a significantly higher mg O_2 L^{-1} g^{-1} than individuals of *L. terrestris* ($P < 0.01$). Bars marked with the same letter are not significantly different ($P < 0.05$).

A Wilcox signed rank test found that individuals of *L. terrestris* died at a significantly lower oxygen concentration mm^{-2} earthworm surface areas than individuals of *L. castaneus* ($P < 0.0001$; Fig. 5-6).

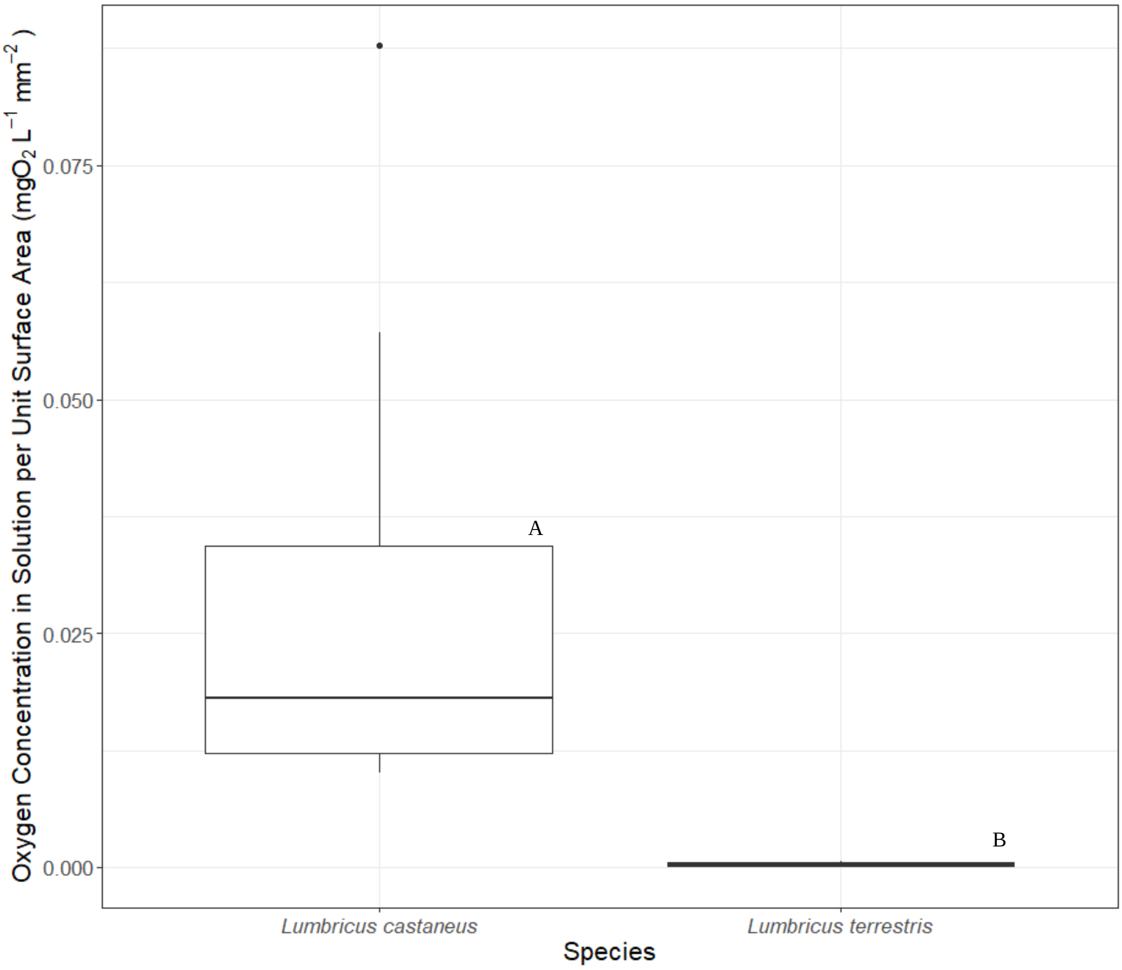

Figure 5-6. The oxygen concentration normalised mm^{-2} earthworm surface area at which individuals of *L. castaneus* and *L. terrestris* were found dead, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Individuals of *L. castaneus* died at a significantly higher mg O_2 L^{-1} mm^{-2} than individuals of *L. terrestris* ($P < 0.01$). Bars marked with the same letter are not significantly different ($P < 0.05$).

A Kruskal-Wallis test performed to determine if the oxygen concentration at which individuals of *L. terrestris* and *L. castaneus* died, and at which the oxygen concentration plateaued in *A. chlorotica* (hours 144 to 288) found a significant difference between the species ($P < 0.0001$; Fig. 5-7). Pairwise Mann-Whitney post hoc testing found a significant difference between the absolute oxygen concentration at which *L. terrestris* (n = 10) and *L. castaneus* (n = 13) died ($P = 0.002$) and at which *L. terrestris* died and *A. chlorotica* (n = 9) plateaued ($P = 0.042$).

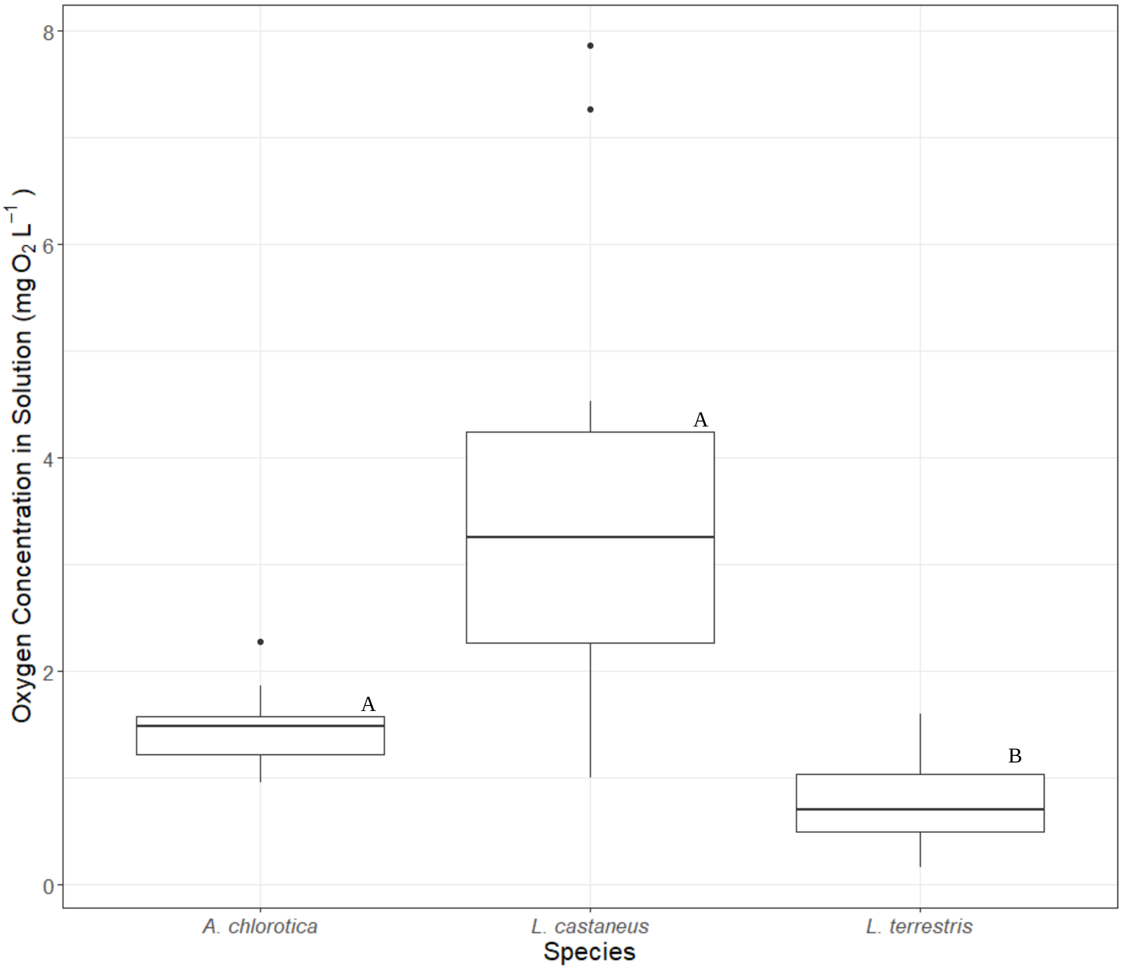

Figure 5-7. The absolute oxygen concentration in solution (mg O_2 L^{-1}) at which individuals of *L. terrestris* and *L. castaneus* died, and from hour 144 for *A. chlorotica*, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Bars marked with the same letter are not significantly different (*P* < 0.05)

5.4.3 Earthworm mass gain

A two way ANOVA found no significant difference in the mass gained (g) by individuals of *L. terrestris* between individuals in the control and treatment tubes and live and dead individuals.

As no individuals of *A. chlorotica* died, a Kruskal-Wallis test was performed between mass gained (g) between individuals in the control and treatment tubes. Individuals in the treatment tubes gained significantly more mass (*P* = 0.028) than individuals in the control tubes (Fig. 5-8).

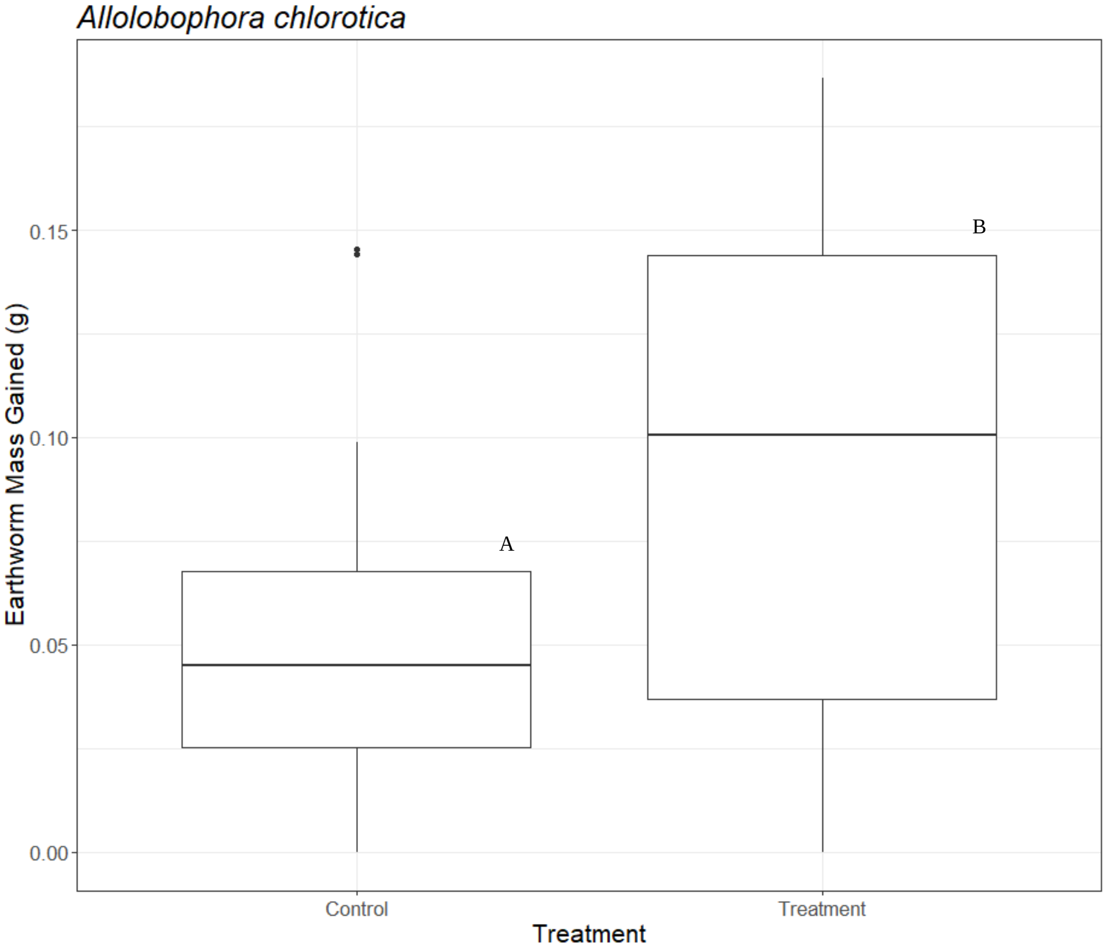

Figure 5-8. The mass gained by earthworms of *A. chlorotica* in the control and treatment tubes, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Individuals in the treatment tubes gained significantly more mass (P < 0.05) than individuals in the control tubes. Bars marked with the same letter are not significantly different (*P* < 0.05)

A GLM, performed as a non-parametric two way ANOVA, found no significant difference in the mass gained between the control and treatment tubes for individuals of *L. castaneus* (Fig. 5-9). There was a significant difference between live and dead earthworms ($P < 0.0001$) and a significant effect of the interaction term between treatment and control tubes and living and dead earthworms ($P = 0.002$). Tukey post hoc testing showed that dead treatment individuals gained significantly more mass than alive treatment, alive control, or dead control earthworms ($P < 0.05$).

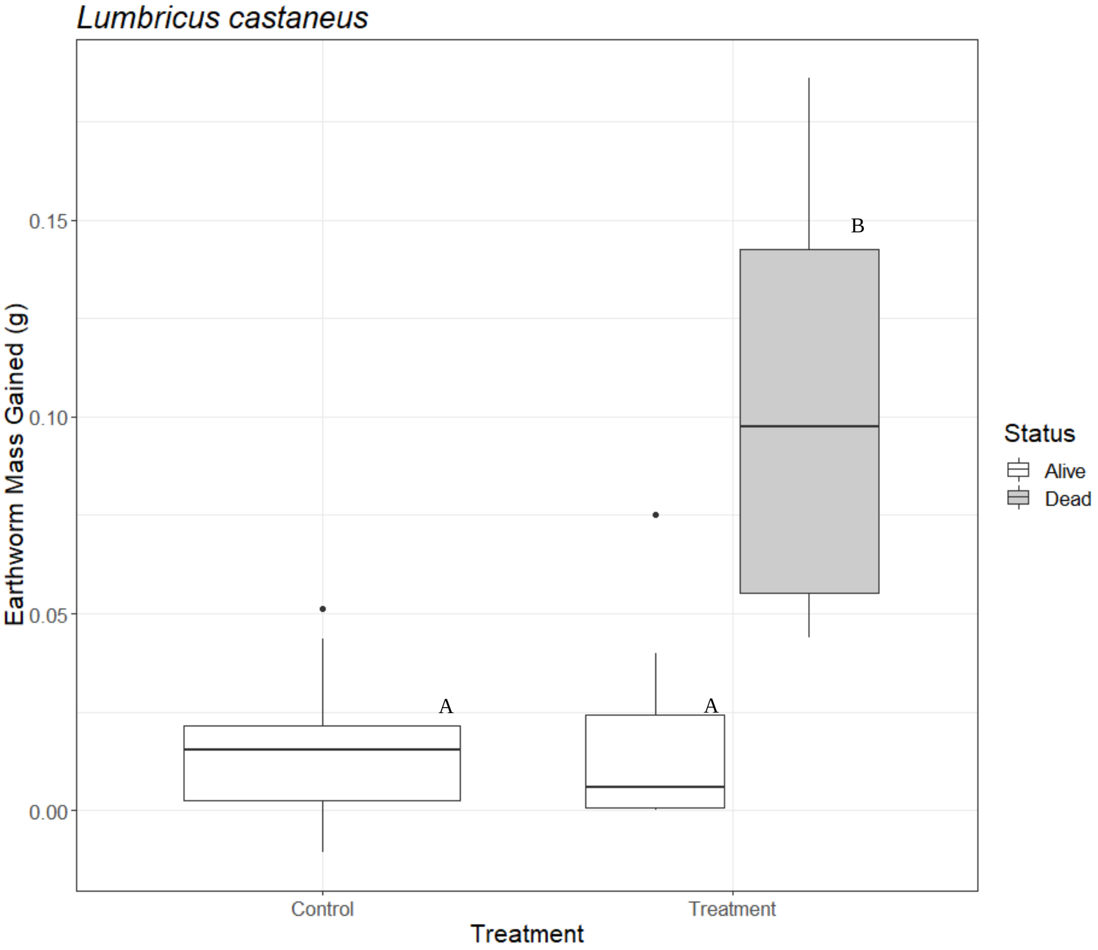

Figure 5-9. The mass gained by alive and dead earthworms of *L. castaneus* across the control and treatment tubes, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. No significant difference was observed between control and treatment tubes, but dead individuals gained significantly more mass than live earthworms. Bars marked with the same letter are not significantly different ($P < 0.05$)

5.4.4 Changes in solution pH

The pH of the ambient solution in which the earthworms were maintained changed over time. GLMs, performed as non-parametric two way ANOVAs, found that there was a significant effect of time ($P < 0.0001$), treatment ($P < 0.0001$) and the interaction term between the two ($P < 0.0001$) for *L. terrestris* (Fig. 5-10), *A. chlorotica* (Fig. 5-11), and *L. castaneus* (Fig. 5-12).

Tukey post hoc testing of the pH values over timepoints and treatments for individuals of *L. terrestris* found that the pH of the solution in the treatment tubes at timepoints 48 and 72 was significantly different to all other pH values throughout the testing ($P < 0.05$). The general trends showed that pH values were significantly lower in the treatment tubes than in the control tubes (P

< 0.05), and that pH values were significantly lower in hours 28 and 72 than in hours 0 to 24 (*P* < 0.05).

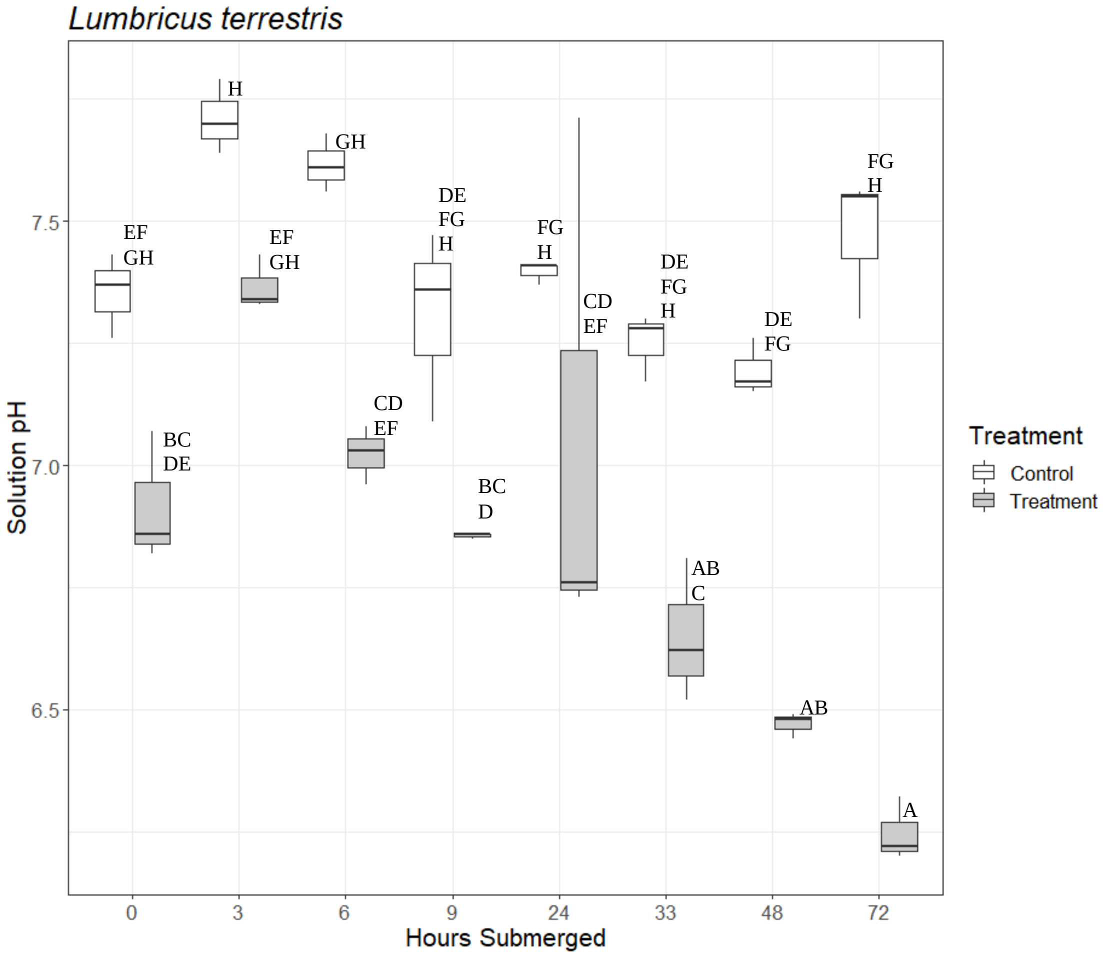

Figure 5-10. The pH values over the hours submerged in treatment and control tubes containing individuals of *L. terrestris,* with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. 100% mortality was reached by 36 hours in the treatment tubes, while no individuals died in the control tubes. Bars marked with the same letter are not significantly different (*P* < 0.05).

Tukey post hoc testing of the pH values over timepoints and treatments for individuals of *A chlorotica* found that the pH of the solution in the treatment tubes was significantly lower than the control tubes at timepoint 288 ($P < 0.05$). The pH values from 48 to 288 hours submerged were significantly lower than 0 to 9 hours submerged, with no significant difference in the pH between hours 48, 96, 144, and 288. The general trend shows decreasing pH with time in both the treatment and control tubes, but with the exception of timepoint 288 there is no significant difference between the pH in the control and treatment tubes at each timepoint.

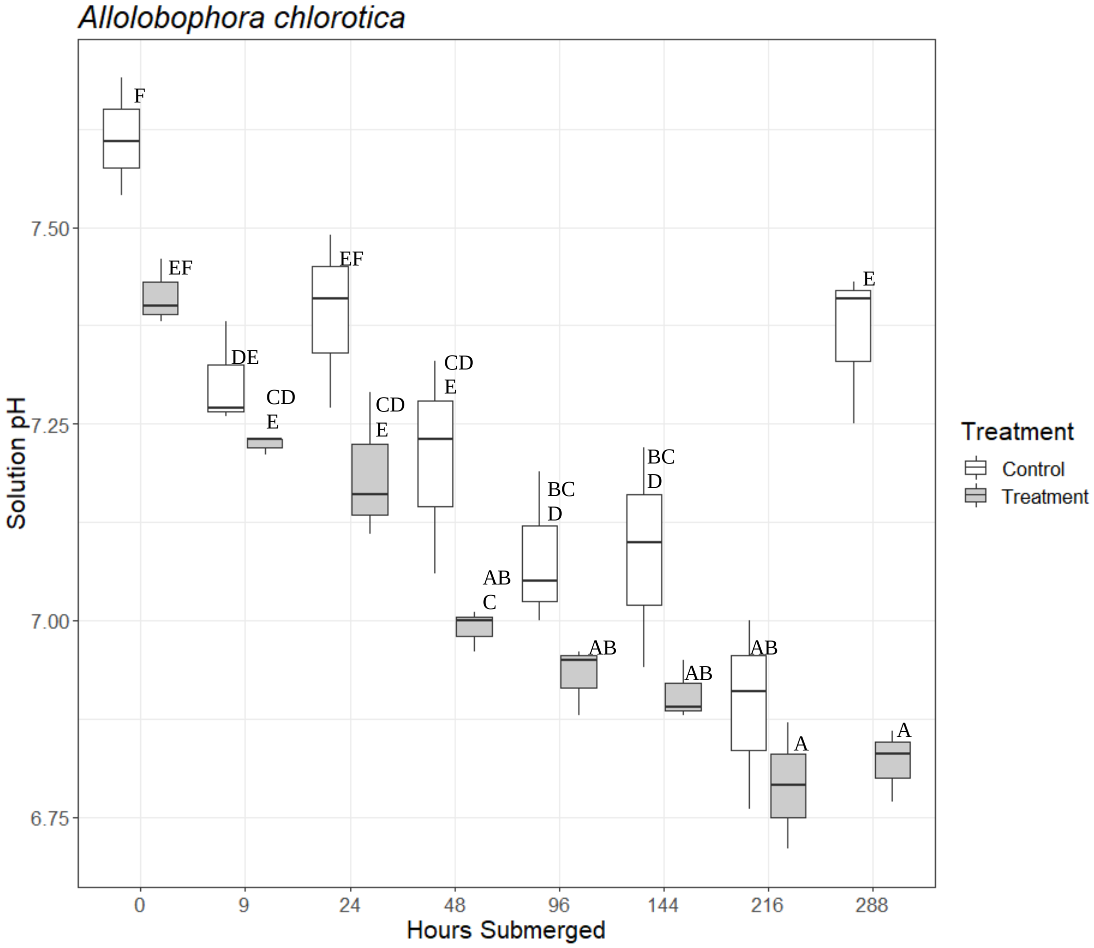

Figure 5-11. The pH values over the hours submerged in treatment and control tubes containing individuals of *A. chlorotica,* with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. No individuals died in either the treatment or control tubes. Bars marked with the same letter are not significantly different (*P* < 0.05).

Tukey post hoc testing of the pH values over timepoints and treatments for individuals of *L. castaneus* found that the pH of the solution in the treatment tubes was significantly lower than the control tubes ($P < 0.05$), with the solution pH decreasing over time. As the hours submerged increased, the difference between the tubes also increased, with treatment tubes significantly lower than control tubes at hours 168 and 216.

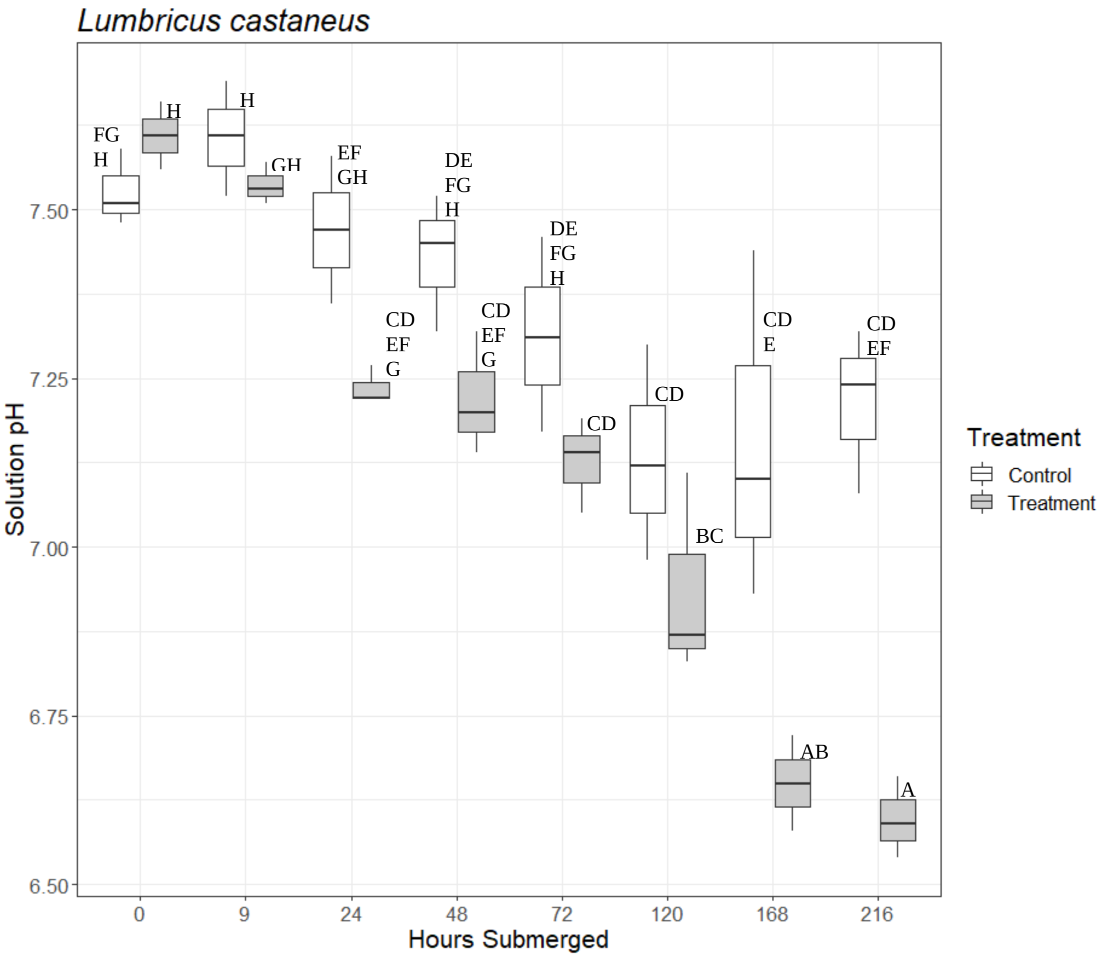

Figure 5-12. The pH values over the hours submerged in treatment and control tubes containing individuals of *L. castaneus,* with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. 100% mortality in treatment tubes was reached by 168 hours submerged, with 66% mortality reached at 48 hours. One control individual was found dead at the 168 hour sampling point. Bars marked with the same letter are not significantly different ($P < 0.05$).

5.5 Discussion

5.5.1 Control vs treatment deaths

The control and treatment individuals were maintained at different temperatures, with control individuals maintained at 15°C and treatment individuals at 10°C However, the deaths of treatment individuals is unlikely to be due to the temperature at which they were maintained.

100% mortality of individuals of *L. terrestris* was reached by 36 hours in the treatment tubes, and 66% mortality of *L. castaneus* was reached by 48 hours. However, individuals were depurated at 10°C for 48 hours before the start of the experiment. If the temperature caused earthworm deaths, then high levels of earthworm death would have been observed during the depuration process. As this did not occur, it is likely not the temperatures contributing to the deaths of individuals. As discussed in Section 5.3.2, the 10°C at which treatment individuals were maintained is above the temperatures at which individuals were still found active in the field (Section 3A.4.1, Section 3B.4.1), while earthworms of both *L. terrestris* and *A. chlorotica* both exhibit normal behaviours such as reproduction when maintained at 10°C (Butt, 1991; Butt, 1997).

Only one control earthworm death occurred: an individual of *L. castaneus* at hour 168. This indicates that the earthworm death is likely not linked to starvation or being maintained in the tubes. If these conditions lead to earthworm death there would have been an equal number of deaths in the control tubes as in the treatment tubes. Roots (1956) found that, in aerated water and not provided with food, *L. terrestris* and *A. chlorotica* were able to survive an average of 137 days, while *Lumbricus rubellus*, an epigeic species, was able to survive an average of 78 days. As the duration of the experiment was well within these limits, all individuals survived depuration at 10°C for 48 hours, the soil activity of earthworms below 10°C, and the death of only one individual in the control tubes, it suggests that earthworm deaths were not due to starvation, or temperature conditions but were instead due to other conditions such as the depletion of oxygen or changes in the pH.

For all three of the earthworm species, the pH of the treatment tubes was significantly lower than that of the control tubes. For *L. terrestris, A. chlorotica* and *L. castaneus*, there was no significant difference in solution pH between treatment and control at sampling time 0, but significant differences between the control and the treatment tubes were observed at hours 33 to 72 for tubes containing *L. terrestris*, 288 for tubes containing *A. chlorotica*, and hours 168 and 216 for tubes containing *L. castaneus*. In both the treatment and control tubes there is a general trend of decreasing pH across all timepoints. This acidification of the reconstituted groundwater is likely due to the production of CO_2 during earthworm respiration. CO_2 is highly soluble in water, where it dissolves to form carbonic acid (Equation 5-1) at a rate of 1.97 g L^{-1} at 15°C (Dean, 1972).

Equation 5-1. The formation of carbonic acid from CO_2 and H_2O

$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_{3(aq)}$$

The higher pH in the control tubes compared to the treatment tubes may be attributed to the control mechanism of continuous aeration with ambient air. Aeration with oxygen is a mechanism frequently used in aquaculture to strip carbon dioxide from solution (Summerfelt et al., 2000), which in turn leads to an increased pH. The pH in the treatment solutions was lower in the tubes containing individuals of *L. terrestris* and *L. castaneus* at the end of the experiment than the tubes containing *A. chlorotica*. This may be a result of reduced respiration rates in *A. chlorotica*, which could be a survival tactic used by the species in response to reduced oxygen or high stress conditions.

In culture, earthworms are able to tolerate a pH range of 4.5 to 7 (Lowe and Butt, 2005). In this study, pH values ranged from 7.8 to 6.2. While the highest pH is slightly above that which Lowe and Butt reported as preferable, other studies have found that the aversion to soil pH values of above 7.0 is slight, and weaker than the aversion earthworms show for very acidic soils, although the authors did not provide a reason for this observation (Baker and Whitby, 2003). In this study, therefore, although changes in pH were observed over time, and differences between control and treatment tubes were observed, these are unlikely to have contributed to the earthworm deaths.

5.5.2 Absolute oxygen concentration

One of the major findings of this study was that none of the individuals of *A. chlorotica* died during the experiment, despite being immersed in water for nearly 300 hours (12 days). However, the consumption of oxygen showed a similar pattern to that observed in both *L. terrestris* and *L. castaneus*, with the oxygen concentration reducing rapidly in the early stages of the experiment before plateauing. From the period of 144 hours to 288 hours, the mean oxygen concentration in the tubes containing *A. chlorotica* (1.49 mg O_2 L^{-1}) was significantly higher than the mean oxygen concentration at which individuals of *L. terrestris* died (0.82 mg O_2 L^{-1}), but did not significantly differ from the mean oxygen concentration at which individuals of *L. castaneus*, a similarly sized earthworm, died (3.60 mg O_2 L^{-1}). This suggests that individuals of *A. chlorotica* were able to survive within the oxygen range at which individuals of *L. castaneus* died. However, the oxygen concentration at which they were persisting was higher than that at which individuals of *L. terrestris* died. This may be an artefact of the different body sizes of the earthworms, with individuals of *L. terrestris* much larger than individuals of both *L. castaneus* and *A. chlorotica*, but the pattern of *L. terrestris* dying at a lower oxygen concentration continued when normalised by biomass and by surface area. This suggests that the difference in oxygen requirements may be a result of different lifestyles of the earthworm ecotypes requiring differing oxygen concentrations for survival, which will be explored further in Section 5.5.4.

5.5.3 Earthworm mass gain

There was no significant difference in the mass gained by individuals of *L. terrestris*, either between the live earthworms and the dead earthworms, or between the treatment and the control earthworms.

Individuals of *L. castaneus*, however, showed a significantly higher mass gain in the dead treatment earthworms relative to the dead control earthworms, in which only one individual died and was found to have not gained any mass. The difference in mass gain between the dead treatment earthworms and the live earthworms in both treatment and control tubes is likely because whilst 100% earthworm death in the randomly selected tubes occurred by 168 hours, 66% of earthworm death was reached by 48 hours. As tubes were randomly selected, it may be the case that many of the earthworms had been dead for some time when their tube was randomly selected between the 48 and 168 hour sampling times. Earthworms actively control their osmoregulation (Carley et al., 1983); in the period between death and sampling, the individuals of *L. castaneus* may have gained significant quantities of the ambient water via osmosis.

In contrast to the dead treatment earthworms of *L. castaneus*, individuals of *A. chlorotica* gained significantly more mass in the treatment tubes than in the control tubes. *A. chlorotica* is able to aestivate (Edwards and Lofty, 1977), meaning that the species is able to enter a period of dormancy in response to high temperatures and dry conditions. Studies performed on another endogeic aestivator, *Apporectodea caliginosa*, found that, when aestivating in soil, the earthworm water content increased in the early stages of aestivation. The earthworms increased their osmolarity, which resulted in the passive update of water from the soil; this strategy allowed increased chances of survival in hot and dry conditions (Bayley et al., 2010). It may be the case here that *A. chlorotica* is exhibiting a similar strategy and increased its osmolarity as it entered a dormant state, resulting in the passive uptake of water. As the earthworms were submerged in solution the mass gain was higher than may be observed in dry soil.

5.5.4 Earthworm lifestyle

It is not just the mass gain observed in *A. chlorotica* which indicates that the species may be aestivating in the treatment tubes. The aestivation process involves a number of behavioural characteristics, where the individuals roll themselves into a tight knot, with the head and the tail tucked into the centre, and excrete a mucus to help minimise water loss from the body (Edwards and Lofty, 1977). While any mucus excreted may have been washed away by the submergence in reconstituted groundwater, the behavioural response of aestivation was still observed (Fig. 5-13). After the tubes were emptied, individuals subject to the treatment tubes were found to have curled into tight balls, and took some time to begin behaving in the same manner as the control or the pre-treatment individuals.

Figure 5-13. The same individual of species *A. chlorotica* before (12) and after (12-2) submergence in a treatment tube for 144 hours. Before treatment the individual was relaxed and extended, moving across the workbench. After treatment the individual was curled into a tight ball, and it was only after a period of time on the workbench in ambient air that the individual uncurled and began moving again.

A. chlorotica consists of two colour morphs; a pink morph and a green morph. These species are known to display a strong preference for soil moisture, with the pink morph preferring drier areas than the green morph (Satchell, 1967) to the extent that lower soil moisture significantly restricts the growth of green morph juveniles (Lowe and Butt, 2007), with some studies arguing for their classification as two separate species (Lowe and Butt, 2008). The precise reasons for the strong preference of high soil moisture content is still not known, but there is evidence that the colouration differences are due to a differing haem pigment found in the green morph than in the pink morph (Kalmus et al., 1955). In this study, the green morph of *A. chlorotica* was used, although this does not show up well in Fig. 5-13 due to poor lightning. It may be the case that this haem pigment allows for the uptake of oxygen from solution at lower oxygen concentrations, thereby making oxygen available for respiration at low concentrations in solution.

The key driver in the differences in the oxygen requirements between *L. terrestris* and *L. castaneus* are likely a result of differences between the characteristics of the two ecotypes, related to the organism's lifestyle and strategy rather than characteristics of the preferred soil habitat. As an anecic species, *L. terrestris* leads what could be described as a mostly sedentary lifestyle, living in deep vertical burrows in the soil. Laboratory experiments have shown that the oxygen consumption of other anecic species is greatest during night time periods (Chuang and

Chen, 2008) when the earthworms typically emerge to forage on the soil surface, which suggests that the anecic earthworms may experience more extremes in their physical activity rates, and therefore oxygen consumption, than epigeic or endogeic species. The lifestyle of epigeic species, on the other hand, is dramatically different to that of anecic species. Living in, and consuming, the soil-litter layer, the ecotype displays many of the characteristics of rapid colonisers (Eijsackers, 2011), rapidly coming to dominate in regularly disturbed areas (Pižl, 2001; Klok et al., 2006) The hypothesis that epigeic earthworm species are *r* strategy organisms, and that their strategy differs to that of anecic earthworms, was first posited by Satchell (1980). *r* strategy organisms favour large productivity (MacArthur and Wilson, 1967), typically displaying a shorter lifespan, greater reproductive output, and a smaller size of individuals, while *K* species are characterised by a longer lifespan, reduced reproductive output, and a larger individual size. Butt and Lowe (2011) summarised studies regarding a number of characteristics between earthworm species, including the number of days required to grow to maturity, the number of days required to incubate cocoons, and the number of hatchlings per cocoon. Their findings for an anecic species (*L. terrestris*) and an epigeic species (*Eisenea fetida*) are summarised in Table 5-3. *E. fetida* has been included as one of the most broadly studied epigeic earthworm species. However as a non-native species to the UK it is not necessarily representative of temperate epigeic species. Available information about a UK epigeic species, *L. rubellus*, has also been included in Table 5-3; however, the species is not as well studied as *E. fetida*.

Table 5-3. A summary of key life cycle characteristics of an anecic (*L. terrestris*), two epigeic (*E. fetida, L. rubellus),* and an endogeic (*A. chlorotica)* species, summarised from a number of studies

Characteristics	*L. terrestris*	*E. fetida*	*L. rubellus*	*A. chlorotica*
Ecotype	Anecic	Epigeic	Epigeic	Endogeic
Growth to maturity (days)	112 (Svendsen et al., 2002)	53 – 76 (Edwards, 1988)	74 (Elvira et al., 1997)	70 – 98 (Lowe and Butt, 2005)
Cocoon incubation (days)	90 (Butt, 1991)	32 – 73 (Edwards, 1988)	36.5 (Elvira et al., 1997)	63 – 79 (Lowe and Butt, 2007)
Hatchlings per cocoon	1 (Butt, 1991)	3.3 (Edwards, 1988)	1 (Elvira et al., 1997)	1 (Butt, 1997)

Butt and Lowe's summary of earthworm reproductive characteristics corresponds with a number of the expected characteristics of *r* species, with a shorter time to maturity, shorter incubation period and greater number of hatchlings per cocoon. Other characteristics such as lifespan differences have been harder to determine outside of laboratory conditions. Experiments performed on *L. terrestris* have led to a lifespan estimate of 7 to 8 years (Lakhani and Satchell, 1970), while laboratory studies found that individuals of the epigeic species *Eisenia andrei* had a median lifespan of 5 to 6 years, with longevity directly correlating with the adult body mass (Mulder et al., 2007). At the time of writing, no laboratory studies have been performed to determine the potential lifespan of earthworms of *L. castaneus*, and it must be remembered that the practical lifespan of the organism in the field is likely much shorter than under laboratory conditions, due to factors such as predation and food availability.

The lifestyle of an *r* species organism, prioritising reproduction and growth to sexual maturity at the cost of lifespan of the individual, likely comes at higher metabolic cost of that of a *k* species. If we apply these *r* and *K* strategies respectively to *L. castaneus* and *L. terrestris,* it may explain why *L. castaneus* individuals died at a much higher oxygen concentration than *L. terrestris*, both absolutely and when normalised for body mass and surface area. With *L. castaneus* representing an *r* species, the survival of the individual in a flooding event is less important, as the high reproductive output of cocoons, each with a high number of individuals per cocoon, means that the species is likely to rapidly recolonise after a disturbance event, despite lower survival rates of individuals in low oxygen, flooded conditions.

With its small body size, and relatively fast growth to maturity and cocoon incubation period, *A. chlorotica* is more similar in lifestyle to the *r* strategy, epigeic species than to the *K* strategy, anecic species. However, this study suggests that for this species, the aestivation response overrides any expected oxygen requirements associated with either the *r* or *K* strategy.

5.5.5 Field site context

This study does not represent field conditions. While this study has focused on the absolute oxygen concentrations required for survival, some experiments have found that earthworms are able to survive for 120 days in flooded soil samples (Ausden et al., 2001). This suggests that earthworm behavioural responses in flooded soil may be equally as influential for a species surviving a flooding event as their absolute oxygen requirement. This is also reflected in previous iterations of the experiment described in Section A1.2. While developing the alternative methods for this experiment, individuals of *L. terrestris* were observed exhibiting a ‘snorkelling’ behaviour, where a segment of the body was maintained out of the water, allowing the earthworms to respire.

This experiment also did not emulate the field environment because the tubes were sealed. As described in Section A1.2, previous iterations of the experiment investigated the oxygen consumption of earthworms in open beakers, where oxygen was able to diffuse across the air-water interface. This may also explain why the results obtained in this experiment are so different to studies such as those performed by Ausden et al. (2001).

This study does partially explain some of the patterns of earthworm distribution within the flooded sites surveyed in Chapter 3. The regularly flooded, slow-draining region of the pasture field (Chapter 3A) and the regularly flooded crop sites of the arable field (Chapter 3B) were dominated by *A. chlorotica*, with few individuals of other species or ecotypes present. While *A. chlorotica* is one of the most common species of earthworm in the UK, representing 34% of all UK earthworms (Natural England, 2014), their presence in regularly flooded sites has been recorded in a number of studies (Plum, 2005; Plum and Filser, 2005), with little variance in their abundance regardless of recent flooding events (Zorn et al., 2005). The persistence of *A. chlorotica* populations in soils may in part be due to the ability of the species to resist extremes in soil environmental conditions, such as both drought and flooding conditions.

5.5.6 Further work

Further work from this chapter could involve repeating the experiment with different earthworm species. Endogeic species known to aestivate, such as the alternative colour morph of *A. chlorotica*, *A. caliginosa* (Bayley et al., 2010) and *A. rosea* (Gerard, 1967) could be used to confirm whether the aestivation behavioural response to reduced oxygen concentration occurs across multiple earthworm species. A native UK anecic species, *A. longa*, is also known to exhibit obligatory diapause during the summer months (Morgan and Winters, 1990). The combination of anecic earthworm *k* life strategy and aestivation behaviour would be beneficial to aid understanding of aestivation behaviour as a stress response.

Further work could also allow for earthworm choice tests between flooded and unflooded soils. Performed with a variety of earthworm species, this may provide insight into species specific earthworm responses to flooding soils when escape would be available.

As UK epigeic species are relatively under-represented in the literature, further work examining not only their responses to flooding but also their life cycles, cocoon production, and time to maturity would be beneficial for UK species such as *L. castaneus*, *D. octaedra* and *S. mammalis*, all of which were found at very low occurrences during the field sampling in Chapter 3.

This work was not performed with any compensations for diurnal cycles of earthworm behaviour. Anecic earthworm behaviour in particular is strongly influenced by diurnal rhythms, and their oxygen consumption has been shown to vary based on the time of day (Chuang and Chen, 2008). Future work could examine in finer detail the influence of light and diurnal patterns on earthworm oxygen consumption, and examine whether there is a different behavioural response to flooding if the earthworms are already on the soil surface, as anecic earthworms at night or epigeic earthworms are, or if they are within the soil, as endogeic and anecic earthworms are in the day.

5.6 Conclusion

The three earthworm species of *L. terrestris*, *A. chlorotica* and *L. castaneus* each illustrated different oxygen requirements when placed in unaerated solution. Individuals of *L. terrestris* needed comparatively low concentrations of oxygen to survive, particularly when accounting for the body mass or size of the individual, while individuals of *L. castaneus* needed comparatively higher oxygen concentrations. Individuals of *A. chlorotica* did not die after 288 hours submerged, and instead appeared to exhibit an aestivation response. Previously thought to be a response to very hot and dry soil conditions, this research suggests that the aestivation response may be a more general stress response than previously thought, and highlights the need for more research into the aestivation response of multiple earthworm species.

The relative difference in oxygen concentration requirements of *L. terrestris* and *L. castaneus* earthworms may be attributed to the oxygen demands of their ecotypes. As largely sedentary earthworms that live in potentially lower oxygen conditions, emerging onto the soil surface at night to feed, the oxygen demands of anecic earthworms may be relatively low. In contrast, the consistent foraging behaviour on the soil surface of epigeic earthworms, in a potentially higher oxygen environment, may lead to higher oxygen requirements. Anecic and epigeic earthworms can broadly be grouped into slower reproducing, longer lived *K* species, and faster reproducing and shorter lived *r* species respectively. The metabolic requirements of these two different species persistence strategies may also be reflected in the oxygen needs of the individual.

This study highlights the need for more research into the oxygen requirements of UK species, including anecic species which are known to exhibit aestivation-like behaviour, and the need for more understanding of the life cycles of relatively understudied UK epigeic earthworm species.

5.7 References

Arnold, R.E., Hodson, M.E., Comber, S. 2007. Effect of organic complexation on the toxicity of Cu to the earthworm *Eisenia fetida. Applied Geochemistry* 22: 2397-2405.

Ausden, M., Sutherland W.J., James, R. 2001. The Effects of Flooding Lowland Wet Grassland on Soil Macroinvertebrate Prey of Breeding Wading Birds. *Journal of Applied Ecology* 38(2): 320 – 338

Baker, G.H., Whitby, W.A. 2003. Soil pH Preferences and the Influences of Soil Type and Temperature on the Survival and Growth of Aporrectodea longa (Lumbricidae): The 7th International Symposium on Earthworm Ecology. Cardiff. Wales. 2002. *Pedobiologia* 47(5 – 6): 745 – 753

Bayley, M., Overgaard, J., Høj, A.S., Malmendal, A., Nielsen, N.C., Holmstrup, M., Wang, T. 2010. Metabolic Changes during Estivation in the Common Earthworm Apporectodea caliginosa. *Physiologica and Biochemical Zoology* 83(3): 541-550.

Bouché, M. B., 1977. Strategies Lombriciennes. *Ecological Bulletins* 25: 122-132.

Butt. K.R. 1991. The Effects of Temperature on the Intensive Production of *Lumbricus terrestris* (Oligochaeta: Lumbricidae). *Pedobiologia* 35(4): 257-264

Butt, K.R. 1997. Reproduction and Growth of the Earthworm *Allolobophora chlorotica* (Savigny, 1826) in Controlled Environments. *Pedobiologia* 41: 369 – 374

Butt, K.R., Lowe, C.N. 2011. Controlled Cultivation of Endogeic and Anecic Earthworms. In: Karaca A. (eds) Biology of Earthworms. Soil Biology, vol 24. Springer, Berlin, Heidelberg.

Carley, W.W., Caracciolo, E.A., Mason, R.T. 1983. Cell and Coelomic Fluid Volume Regulation in the Earthworm *Lumbricus terrestris*. *General and Comparative Endocrinology* 27(4): 509-516.

Chuang, S.C., Chen, J.H. 2008. Role of Diurnal Rhythm of Oxygen Consumption in Emergence from Soil at Night after Heavy Rain by Earthworms. *Invertebrate Biology* 127(1): 80-86.

Davis, J.G., Slater, W.K. 1928. The Anaerobic Metabolism of the Earthworm (Lumbricus terrestris). *Biochemical Journal* 22(2): 338-343.

Dean, J.A. 1972. Lange’s Handbook of Chemistry 14th Edition. New York: McGraw-Hill, Inc

Edwards, C.A., Lofty, J.R. 1977. Biology of Earthworms (2nd Edition). London: Chapman and Hall.

Edwards, C.A., 1988. Breakdown of animal, vegetable and industrial organic wastes by earthworms. Earthworms in waste and environmental management. Edited by Clive A. Edwards and Edward F. Neuhauser.

Eijsackers, H. 2011. Earthworms as Colonisers of Natural and Cultivated Soil Environments. *Applied Soil Ecology* 50: 1-13.

Elvira, C., Domínguez, J., Mato, S. 1997. The Growth and Reproduction of *Lumbricus rubellus* and *Dendrobaena rubida* in Cow Manure Mixed Cultures with *Eisenia Andrei*. *Applied Soil Ecology* 5(1): 97 – 103

Ettema, C.H., Wardle, D.A. 2002. Spatial Soil Ecology. *Trends in Ecology and Evolution*. 17(4): 177-183.

Gerard, B. 1967. Factors Affecting Earthworms in Pastures. *Journal of Animal Ecology* 36(1): 235 – 252

Gruner, B., Zebe, E. 1978. Studies on the Anaerobic Metabolism of Earthworms. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*. 60(4): 441-445.

Kalmus, H., Satchell, J.E., Bowen, J.C. 1955. XCIV - On the Colour Forms of *Allolobophora chlorotica* Sav. (Lumbricidae). *Annals and Magazine of Natural History* 8(94): 795-800.

Klok, C., Zorn, M., Koolhaas, J.E., Eijsackers, H.J.P., van Gestel, C.A.M. 2006. Does Reproductive Plasticity in *Lumbricus rubellus* Improve the Recovery of Populations in Frequently Inundated River Floodplains? *Soil Biology and Biochemistry* 38(3): 611-618.

Lavelle, P. 1988. Earthworm Activities and the Soil System. *Biology and Fertility of Soils* 6(3): 237 – 251

Lakhani, K.H., Satchell, J.E. 1970. Production by *Lumbricus terrestris*. *Journal of Animal Ecology* 39(2): 473-492.

Lowe, C.N., Butt, K.R. 2005. Culture Techniques for Soil Dwelling Earthworms: A Review. *Pedobiologia* 49(5): 401 – 413

Lowe, C.N., Butt. K.R. 2007. Life-Cycle Traits of the Dimorphic Earthworm Species *Allolobophora chlorotica* (Savigny, 1826) Under Controlled Laboratory Conditions. *Biology and Fertility of Soils* 43: 495-499.

Lowe, C.N., Butt, K.R. 2008. *Allolobophora chlorotica* (Savigny, 1826): Evidence for Classification as Two Separate Species. *Pedobiologia* 52(2): 81 – 84

MacArthur, R.H., Wilson, E.O. 1967. The Theory of Island Biogeography. Princeton University Press: Princeton.

Morgan, A.J., Winters, C. 1990. Diapause in the Earthworm, *Aporrectodea longa*: Morphological and Quantitative X-Ray Microanalysis of Cryosectioned Chloragogenous Tissue. *Scanning Microscopy* 5(1): 219 – 227

Mulder, C., Baerselman, R., Posthuma, L. 2007. Empirical Maximum Lifespan of Earthworms is Twice that of Mice. *Age* 29(4): 229-231.

Natural England, 2014. Earthworms in England: Distribution, Abundance and Habitats (NECR145)

OECD, 1984. Test No. 207: Earthworm, acute toxicity tests. OECD Publishing, Paris.

Parkin, T.B. 1993. Spatial Variability of Microbial Processes in Soil – A Review. *Journal of Environmental Quality* 22(3): 409-417.

Pižl, V. 2001. Earthworm Succession in Afforested Colliery Spoil Heaps in the Sokolov Region, Czech Republic. *Restoration Ecology* 9(4): 359-364.

Plum, N.M. 2005. Terrestrial Invertebrate in Flooded Grassland: A Literature Review. *Wetlands* 25(3): 721-737

Plum, N.M., Filser, J. 2005. Floods and Drought: Response of Earthworms and Potworms (Oligochaeta: Lumbricidae, Enchytraeidae) to Hydrological Extremes in Wet Grassland. *Pedobiologia* 49(5): 443-453

Ponnamperuma, F.N. 1984. Effects of Flooding on Soils. In: Flooding and Plant Growth. London; Academic Press, Inc.

R Core Team 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Roots, B. I. 1956. The Water Relations of Earthworms II. Resistance to Desiccation and Immersion, and Behaviour when Submerged and when Allowed a Choice of Environments. *Journal of Experimental Biology* 33: 29-44.

Satchell, J.E. 1967. Colour Dimorphism in *Allolobophora chlorotica* Sav. (Lumbricidae). *Journal of Animal Ecology* 36(3): 623-630.

Satchell, J.E. 1980. *r* worms and *K* worms: A basis for classifying lumbricid earthworm strategies. In: Soil Biology as related to Land Use Practicies, (ed D.L. Dindal), Prc. 7th Int. Coll. Soil Zool. EPA, Washington DC: 848 – 854

Sexstone, A.J., Revsbech, N.P., Parkin, T.B., Tiedje, J.M. 1985. Direct Measurement of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society of America Journal* 49: 645-651.

Stoyan, H., De-Polli, H., Böhm, S., Robertson, G.P., Paul, E.A. 2000. Spatial Heterogeneity of Soil Respiration and Related Properties at the Plant Scale. *Plant and Soil* 222(1-2): 203-214.

Summerfelt, S.T., Vinci, B.J., Piedrahita, R.H. 2000. Oxygenation and Carbon Dioxide Control in Water Reuse Systems. *Aquacultural Engineering* 22(1-2): 87 – 108

Svendsen, T.S., Sommer, C., Holter, P., Grønvold, J. 2002. Survival and Growth of *Lumbricus terrestris* (Lumbricidae) Fed on Dung from Cattle Given Sustained-Release Boluses of Ivermectin or Fenbendazole. *European Journal of Soil Biology* 38(3-4): 319-211

Zorn, M.I., Van Gestel, C.A.M., Eijsackers, H. 2005. Species-Specific Earthworm Population Responses in Relation to Flooding Dynamics in a Dutch Floodplain Soil. *Pedobiologia* 49(3): 189 – 198

6 The effect of flooding on greenhouse gas emissions from sewage sludge amended, earthworm populated mesocosms

6.1 Abstract

Flooding, addition of organic soil amendments, and earthworm burrowing are all known to change soil emissions of greenhouse gases. With increased flooding in the UK, and over 70% of sewage sludge disposed of through application to agricultural land, it is important to understand how the interaction of these factors may affect greenhouse gas emissions.

Mesocosms were prepared with a number of soil treatments: control soils (C), added earthworms (*Lumbricus terrestris*) (E), added sewage sludge (S), and earthworms and sewage sludge in combination (ES). Fluxes of methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O) were measured weekly for eight weeks. Half of the mesocosms were flooded after two weeks (F and UF), and the surface water drained from these columns after four weeks of flooding. Measurements continued for another two weeks after draining. Following the experiment, live earthworms were weighed, the mass of cast material and the number of burrow openings on the soil surface recorded, and soil pH determined.

Flooding or sewage sludge addition had no impact on earthworm biomass, burrowing or casting activity. Soil pH was significantly lower in unflooded soils, and lowest within unflooded soils amended with both earthworms and sewage sludge. Fluxes of all greenhouse gases varied with the flooding treatment, the soil treatment, and the time spent submerged. CH_4 fluxes were negative for both F and UF mesocosms, and only increased to emissions in the F mesocosms once surface water was drained off. CO_2 fluxes were variable, but higher in the unflooded soils than in the flooded soils, and highest in the soil treatments of earthworms and earthworms and sewage sludge. Across all soil treatments, N_2O fluxes were below detectable levels after flooding. In the unflooded mesocosms, fluxes of N_2O were highest in ES mesocosms.

Earthworm burrowing stimulates microbial populations, which can lead to increased N_2O fluxes, particularly following the application of low C:N ratio organic amendments, such as sewage sludge. Increased disposal of sewage sludge through its application to soil, particularly in arable soils with healthy earthworm populations, may lead to high N_2O emissions from UK agricultural land. Further work is needed to understand the effect of more complex flooding and drying cycles on greenhouse gas emissions.

6.2 Introduction

When soil is flooded, oxygen levels rapidly drop to anoxic conditions. Ponnamperuma (1984) stated that this occurs within 24 hours; the experiments performed in Chapter 4 found that on average, and across a number of soil types of different organic matter contents, this occurred within eight to ten hours. The oxygen levels within the soil reduce as microorganisms use the available oxygen for the aerobic respiration of soil organic matter. However, once the soil has reached anoxic conditions, bacterial breakdown of organic matter does not stop. Instead anaerobic respiration begins to dominate, with bacterial activity moving down the REDOX chain (Bell, 1969; Tanji et al., 2003).

The REDOX chain is a series of reduction reactions that occurs in anoxic soils. Once the oxygen available in aerated soil has been consumed, the next most readily available electron donor is used for the breakdown of organic matter. This sequence continues for as long as there is sufficient organic matter to break down and a lack of oxygen in the soil (Patrick and Delaune, 1977). This sequential use of electron donors results in the production of various molecules such as N_2, Mn^{2+}, Fe^{2+}, S^{2-}, and CH_4 (Takia and Kamura, 1966). Two of the products of these reduction sequences are then released as greenhouse gases; nitrous oxide (N_2O) and methane (CH_4) (Equations 2-1 and 2-4). Studies performed on soils that remain flooded for long periods of time, such as rice paddies, which can remain flooded for weeks at a time, find high levels of CH_4 production in these soils. However, the rates of CH_4 emissions by diffusions through floodwater are typically low, with diffusion through water representing less than 1% of emissions from these waterlogged soils (Schütz et al., 1989a).

As discussed in Chapter 4, the addition of sewage sludge to soil has a limited effect on the rate of oxygen depletion in soil. This was hypothesised to be because while there was an increase in the total organic matter content of the soil with the addition of sludge, the available organic matter content of these added sludges was very low. Previous studies suggest that, as the sludge had already been through a process of anaerobic digestion, as little as 20% of the organic matter present is readily available for bacterial breakdown (Terry et al., 1979). However, although the material may be recalcitrant the overall organic matter content of the soil will still increase with the addition of the sludge, and this increased pool of organic matter may be sufficient to drive an increased rate of bacterial respiration under anoxic conditions in soils amended with sewage sludge. A study performed in 2000 found that under anaerobic, flooded conditions there was increased mineralisation of carbon from recalcitrant pools of lignin and cellulose (Devêvre and

Horwáth, 2000). While Devêvre and Horwáth examined the breakdown of rice straw, their findings on the rate of decomposition of recalcitrant matter suggest that similar effects on the breakdown of sewage sludge may be observed under flooded, anoxic conditions, and could in turn lead to increased emissions of greenhouse gases. However, other studies have found variable results, with some studies reporting no effect of rice straw amendment on CH_4 emissions from rice paddies (Schütz et al., 1989b), while others have found a non-linear relationship (Ye et al., 2015). However, these studies have been performed on the recalcitrant organic matter of rice straw, rather than sewage sludge. Studies examining the greenhouse gas emissions from stockpiles of sewage sludge found high emissions of CH_4, N_2O and carbon dioxide (CO_2), despite the relatively recalcitrant nature of the organic matter (Majumder et al., 2014). In turn, soils amended with sewage sludge show higher emissions of CO_2 (Wijesekara et al., 2017), and N_2O, which is strongly linked to the soil water content (Willén et al., 2016). With this strong effect of soil moisture, and the potentially higher rates of recalcitrant organic matter breakdown under anaerobic conditions, the greenhouse gas emissions of flooded soil amended with sewage sludge may be higher still than those already observed in amended, unflooded soils.

The presence of earthworms also alters the emissions of greenhouse gases from soil. Through their burrowing activity, anecic earthworms facilitate gas exchange with the surface (Kretzschmar, 1987). This allows for increased flow of oxygen from the atmosphere into the soil and increased soil aeration (Lavelle, 1988), but also allows the emissions of gases that are accumulating in the soil. This increased aeration of the soil, along with the increased microbial activity and biodiversity that earthworms promote in the soil (Scheu, 1987; Wolters, 2000), leads to increased emissions of CO_2 due to increased soil respiration (Binet et al., 1998), but has also been found to lead to increased emissions of CH_4 under aerobic soil conditions (Singer et al., 2001) and increased emissions of nitrous oxide (N_2O) due to increased N mineralisation (Lubbers et al., 2011, Rizhiya et al., 2007). As discussed in Section 2.9.3, earthworms are known to feed on sewage sludge (Bouldin et al., 2016) when no other food sources are available, with changes in the enzymatic profile of casts indicating the shift in the soil C:N ratio caused by biosolid addition (Kızılkaya and Hepşen, 2004), and the presence of sewage sludge appears to increase earthworm populations (Hamilton and Dindal, 1989). Through their feeding and burrowing activity, the presence of earthworms may lead to increased rates of breakdown of sewage sludge in soil, which may in turn lead to increased rates of emissions of greenhouse gases.

In isolation, the effects of flooding, sewage sludge amendments, and earthworms are each known to alter the rates of emissions of greenhouse gases from soil. However their interaction is not currently understood. This study aims to understand the interplay between these three factors using mesocosm experiments and individuals of *L. terrestris*, considering the following four broad hypotheses:

1. Fluxes of CH_4, CO_2, N_2O, and levels of soil oxygen will differ under flooded conditions from unflooded conditions, and based on the presence or absence of earthworms and sewage sludge. As discussed in Section 6.2, anaerobic conditions produced in flooded soil lead to increased emissions of CH_4 and N_2O, as bacterial breakdown of organic matter moves down the REDOX chain. Under flooded conditions, therefore, higher fluxes of CH_4 and N_2O are expected. As CO_2 is a measure of soil respiration, it may be greater under unflooded conditions than flooded conditions. Greenhouse gas fluxes may be greater with the presence of earthworms, as earthworms lead to increased rates of nitrogen mineralisation within soil. Finally, there may be higher CH_4 fluxes with the presence of sewage sludge, due to the increased quantities of recalcitrant organic matter.

2. Soil oxygen levels in the flooded treatments would deplete to zero in the flooded treatments. Chapter 4 found that there was significant effect of the application of sewage sludge on rates of dissolved oxygen depletion in flooded soil in the very short term, but that overall the dissolved oxygen levels in all flooded soils rapidly depleted to zero, with long term depletion rates more depending on the organic matter content of the soil itself than the addition of sewage sludge. Oxygen in flooded soils or sediments typically follows a gradient (Brune et al., 2000). In flooded rice paddy soils, oxygen levels can be depleted to below detectable levels as little as 2mm from the soil surface (Lüdemann et al., 2000; Noll et al., 2005). In unflooded soils, soil oxygen content is dependent on factors such as inter-aggregate pore size and complexity (Sexstone et al., 1985; Refsgaard et al., 1991; Horn and Smucker, 2005) rather than soil depth. This suggests that no difference in the oxygen concentration at different depths is expected in either flooded or unflooded soil treatments.

3. Soil pH would differ based on the soil treatments. As discussed in Section 2.5.2, under flooded conditions soil pH of both acid and alkaline soils may be expected to move back to neutral conditions. As the Spen Farm soil initially had an average pH of 7.69, flooded soil may be lower than this value. The presence of earthworms can directly lead to increases in soil pH, due to

secretion of mucus (Schrader, 1994 in Sizmur and Hodson, 2009), while the promotion of microbial activity caused by earthworm activity may indirectly lead to increases in soil pH (Haimi and Huhta, 1990). The effect of sewage sludge on soil pH can be more variable. A comparison of three different sewage sludges compiled in Singh and Agrawal, 2008, found that the pH of sewage sludge can be variable, ranging from 6.8 to 7.1 (Singh and Agrawal, 2008). It is not surprising, therefore, that some studies report an increase in soil pH following sewage sludge application (Tsadilas et al., 1995), others a decrease (Epstein et al., 1977), and others still high variability in the effect observed (Neilsen et al., 1998). The effect of biosolid application on the pH of the soils in this experiment, therefore, is not something than can be easily predicted.

4. Earthworm condition and behaviour is altered under flooded conditions, and with the application of sludge. The earthworms under flooded conditions may be expected to have gained less, or even reduced in, mass compared to the earthworms under unflooded conditions. Flooding may disrupt burrowing and foraging behaviour of L. terrestris; the species prefers to avoid waterlogged soil and flee to drier conditions when available (Roots, 1956), however very few studies exist examining how burrowing and foraging behaviour may change under flooded conditions.

6.3 Method

6.3.1 Earthworms

L. terrestris was chosen for the experiment due to its prevalence in UK soils. As an anecic species, their deep, vertical burrows were considered potentially influential on gas exchange between the soil and ambient air, as earthworms increase soil aeration (Lavelle, 1988). *L. terrestris* were purchased online from Wiggly Wrigglers (Blakemere, HR2 9PX, UK) and depurated in a controlled temperature chamber at 10°C for twenty-four hours before the start of the experiment. Before the individuals were placed into the mesocosms, the weight of the individuals was recorded.

6.3.2 Sewage sludge

The sewage sludge was collected from the Esholt water treatment works in West Yorkshire. The soil surface area in the 1800 ml ‘Lock and Lock’ plastic containers used in the experiments (see below) and internal diameter of the containers was 12 cm; sewage sludge additions of 20.9 g wet sludge per container were calculated to give additions equivalent to the maximum application of 250 kg N Ha^{-1} (Section 4.3.1).

In Chapter 4, the sewage sludge was dried and ground before addition to the dried soil. In this study, the decision was made to add the field-wet sewage sludge to the rewetted soil. While this meant that the sewage sludge was not homogenously mixed through the soil, it allowed for conditions to more accurately mimic field applications of sewage sludge, where the field wet sludge would be surface applied and mixed into the top soil through ploughing activity.

6.3.3 Soil

The arable soil used in this experiment was collected from Spen Farm, near Leeds (SE 44300 41700; Table 4-2). The soil was air dried over a number of days, ground by hand in a large pestle and mortar to break up larger aggregates, and sieved to < 2 mm. Any large stones, plant matter, or litter fragments were removed and discarded. Larger soil aggregates that could not be sieved were returned to the pestle and mortar for further breakdown before being sieved again.

The dried soil was rehydrated in bulk at a rate of 100 ml of water per 400 g of dried soil; the same rewetting rates used in Chapter 4. As the experimental measurements were to be staggered over two days, half of the soil to be used was rehydrated on the first day, and half on the second.

6.3.4 Oxygen sensor spots

Eighty clear plastic tubes were prepared. Forty were of length 15.5 cm, and forty of length 10.5 cm. These lengths represented the bottom of the mesocosm and the half-filled depth of the mesocosm. The centre of the base of the clear plastic tubes was prepared with a Presens Oxygen Sensitive Spot, of the same type used in Chapter 4. These tubes were prepared to allow measurement of the oxygen concentration of the soil at different depths without disturbing the soil. Measurements were taken with a Presens fibre optic sensor (Chapter 4). The fibre optic cable was stiffened with a piece of wire, to allow the sensor to be placed on the sensor spots accurately.

6.3.5 Experimental design

The experiment was set up as a factorial design of eight treatments (Figure 6-1). The forty mesocosms were divided first into two treatments: twenty flooded (F) and twenty unflooded (UF). Within each of these treatments there were five replicate mesocosms for each of the following sub-treatments: no added treatment (C), added earthworms (E), added sewage sludge (S), and added earthworms and sewage sludge (ES). In subsequent sections, C, E, S, and ES will be used to refer to treatments that were both flooded and unflooded. When the flooded or unflooded treatments are being described they will be referred to as e.g. UF-ES for the unflooded, earthworm and sewage sludge treatment.

Figure 6-1. Experimental factorial design of eight treatments, with flooded and unflooded mesocosms subdivided into no added treatment, added earthworms, added sewage sludge, and added earthworms and sewage sludge. Each soil treatment within each flooding treatment contained five replicates, to give a total of forty mesocosms.

Flooded (F)	
No added treatment (C)	Added earthworms (E)
Added sewage sludge (S)	Added earthworms and sewage sludge (ES)

Unflooded (F)	
No added treatment (C)	Added earthworms (E)
Added sewage sludge (S)	Added earthworms and sewage sludge (ES)

Seven days after the initial rewetting of the bulk soil, 1300 g of the rewetted soil (equivalent to 1050 g of air-dried soil) was weighed for each mesocosm, and for the S treatments, the sludge was added and mixed into the soil manually. This was done in a separate container before transfer to the mesocosm, as the presence of the oxygen sensors in the mesocosm would prevent mixing later on. A 1 cm layer of soil was placed into the mesocosm before the 15.5 cm long plastic tube was inserted and soil packed around it. Once soil had been filled to a depth of 7 cm, the second plastic tube of length 10.5 cm was inserted. The soil was then packed around the two plastic tubes to a total depth of 12 cm. After insertion of the plastic tubes, cork stoppers were placed into their tops to ensure that no soil would enter the tubes during filling with soil, and to ensure that once the earthworms were added they would not be able to get into the tubes. Figure 6-2 is a representative diagram and a photograph of the tubes with the sensors in position on the tube base.

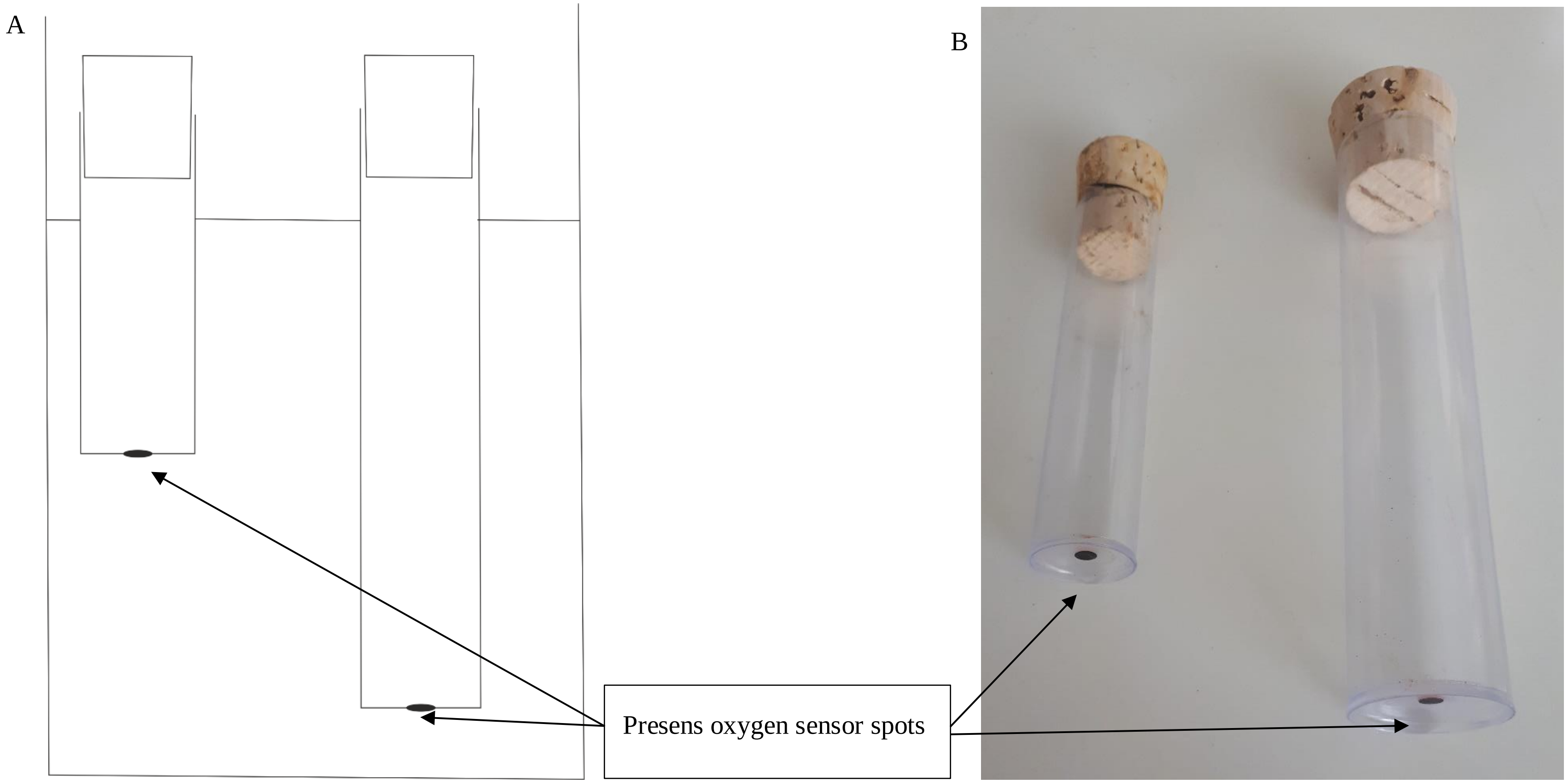

Figure 6-2. A. Representative diagram of the placement of sensor spot tubes in the soil filled mesocosms and B. Photograph of the tubes of length 10cm (L) and 15 cm (R). Tubes were stoppered with corks to prevent earthworm intrusion. Sensor spots were placed on the outside of the tube bases.

24 hours after the mesocosms were prepared, two earthworms were added to the surface of each earthworm treatment mesocosm, following a 24 hour depuration period, and a further 50 ml of water added to the surface of the mesocosm to facilitate earthworm movement on the surface. All mesocosms had 5 g of ground, air-dried horse manure added to the soil surface, intended for food for the mesocosms containing earthworms. Sewage sludge was not used as a food source for the earthworms, as the quantities of sludge mixed through the soil represented the maximum rate of sludge application that would be observed in the field. Following these additions, and before being placed *in situ* in the controlled temperature room the completed mesocosms were weighed. Nylon mesh was placed across the surface of each mesocosm and secured with an elastic band to prevent earthworms escaping E treatments. For consistency of treatment, all mesocosms were covered with nylon mesh. Before measurements began, the mesocosms were left in the controlled temperature room for seven days, to allow the earthworms to acclimatise. Gas flux measurements (see Section 6.3.6) were made weekly for two weeks, after which F mesocosms were flooded. 250 ml of deionised water was added to the mesocosms slowly, to allow the water to saturate the soil. The flood level was intended to simulate groundwater flooding, and so left very little standing water on the soil surface. This was also to allow a higher chance of survival for the earthworms. Previous experiments into earthworm survival in water had found that individuals of *L. terrestris* were able to survive in submerged conditions by maintaining a segment of their body outside of water, allowing them to effectively 'snorkel' (see Sections 5.5.2 and A1.2), a flooding behavioural response that has been noted by other researchers (Roots, 1956). This behaviour was observed in the flooded treatments as the experiment continued, as was the presence of earthworms on the soil surface (Figure 6-3). Following the application of the flooding treatment, no further food was added to the mesocosms, in either UF or F treatments.

Figure 6-3. Individual of *L. terrestris* on the surface of a flooded mesocosm at 2 weeks post flooding.

Measurements continued weekly until the flooded mesocosms had been saturated for four weeks. As the soil had settled during these four weeks, some standing water had formed on the soil surface. This surface water was siphoned off and the mesocosms left to dry, with no additional water added to the flooding treatment mesocosms. Measurements continued for another two weeks, until the soil had been under saturated conditions for a total of six weeks.

6.3.6 Measurements

Before flux measurements were taken, the oxygen concentration at depths 11 cm and 5 cm was recorded for each mesocosm. The mesocosms were weighed to establish the quantity of water lost while *in situ*, and rehydrated to the same value as their initial weight. From weeks -1, the second week of the experiment but before flooding, to 6, unflooded columns lost an average of 9.73 g mass (n = 180, sd = 4.74) each week. From weeks 0 to 4, post flooding but before water siphoning, flooded columns lost an average of 10.14 g mass (n = 100, sd = 7.08) each week.

Flux measurements were taken using the ‘White Dwarf’ EGA61 Multi Channel Gas Exchange System connected to a Los Gatos Research (LGR) CO_2 and CH_4 analyser and an LGR NO_x analyser using a through flow system. Ambient air was drawn into a large barrel, which mixed the air before it was distributed to the White Dwarf distribution unit. The White Dwarf distributed the ambient air through a total of 24 channels sequentially by passing air over the surface of the contents of a column at a flow rate of 350 µmol s^{-1} for two minutes, then drawing the displaced air through to the LGR analysers (Fig. 6-3) The sequence of each of the 24 pots having the air drawn through using the through flow system for two minutes apiece was performed for approximately 5 hours on each experimental run, allowing LGR readings to stabilise.

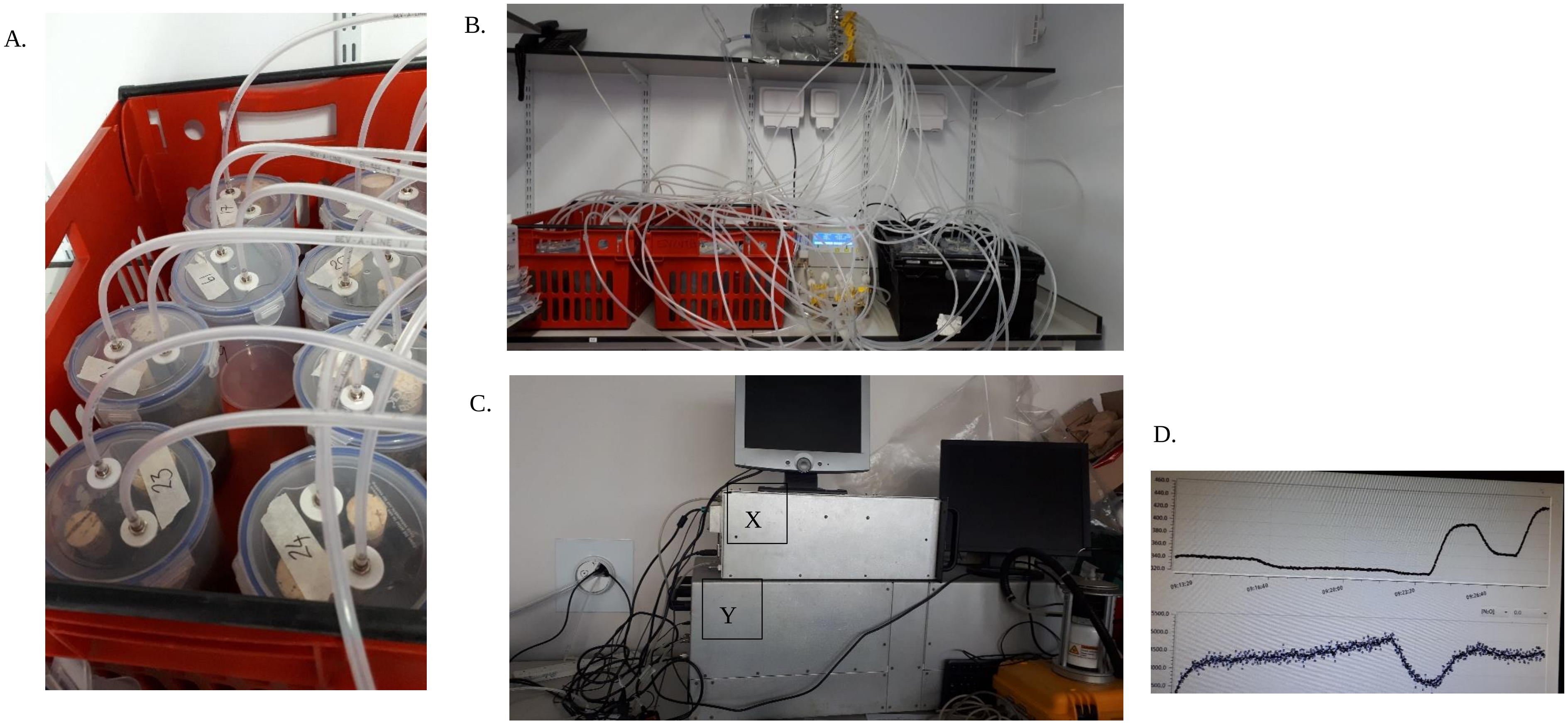

Figure 6-4. Experimental setup for gas flux measurements. A. Sealed 'Lock and Lock' containers. B. Containers were connected by flexible tubing to the White Dwarf EGA61 Multi Channel Gas Exchange System. Following samples being drawn by the White Dwarf, samples passed through tubing through a hole in the wall to be analysed by the LGR CH4 & CO_2 and N_2O analysers. C. LGR CH_4 (X) and CO_2 and N_2O (Y) analysers in the next laboratory. The hole through which tubing passed can be seen on the left of the image. Real-time outputs out gas fluxes were visible on the monitors. D. An example of the output for N_2O fluxes.

Measurements were first taken (week -2) seven days after the mesocosms had been placed in the controlled temperature room. The start dates of the mesocosms were staggered to ensure sufficient time for all measurements, with the F treatments being started 24 hours after the UF treatments. Timings reported below account for this staggered start. Measurements continued every seven days, with the measurements of the designated unflooded and flooded mesocosms occurring on sequential days.

6.3.7 Mesocosm deconstruction

After the six week period of flooding, and the eight weeks total period of measurements, the mesocosms were deconstructed.

For mesocosms without earthworms, the layer of ground, air-dried horse manure was removed from the soil surface and disposed of. For mesocosms with earthworms, cast material was removed from the surface and weighed moist. The number of earthworm burrow openings visible on the soil surface were counted.

The mesocosms containing earthworms were emptied out into a tray, and any live earthworms removed and weighed. For all mesocosms, triplicate soil samples were removed for pH analysis, dried, and mixed with deionised water at a ratio of 1:5 g. The pH was measured using a Thermo Orion 420A plus pH/ISE Meter, calibrated with pH 4, pH 7 and pH 10 buffers. The remaining mesocosm soil was left to air dry for storage and potential future analysis.

6.3.8 Data analysis and statistics

Data were analysed using R version 3.4.2.

The LGR produced a curve of the gas concentrations in the displaced air from each mesocosm in ppm. To convert these values to unit surface areas, the final sampling run of the 24 mesocosms was used. From this final run, the mean ppm value from between 1:00 and 1:30 of the two minute run was calculated for each treatment mesocosm and for the empty air pots. The difference between the measured ppm in the experimental mesocosm and the air pot was calculated, and converted to the flux value in mol l^{-1} by multiplying the ppm by the molar volume of the gas sample at 15°C. This value was multiplied by the flow rate in l min^{-1} and by the molecular weight of the greenhouse gas being analysed to get the flux value of the gas in mg min^{-1}. Finally, this value was divided by the surface area of the soil (m^2) and multiplied by 60 to get the flux of the greenhouse gas in mg m^2 h^{-1}. As flux values of CH_4 and N_2O were smaller than those of CO_2, these values were further multiplied by 1000 to get the flux values in µg m^2 h^{-1}.

To address the four hypotheses, the data were analysed in the following ways:

1. To determine whether fluxes of CH_4, CO_2, N_2O, and levels of soil oxygen will differ under flooded conditions from unflooded conditions, and based on the presence or absence of earthworms and sewage sludge, repeated measures ANOVAs were performed.

2. To determine whether soil oxygen levels in the flooded treatments would deplete to zero in the flooded treatments over time, repeated measures ANOVAs were performed for both the 11 cm and 5 cm sampling depths.. In order to determine if the oxygen concentration at the different soil depths of 5 cm and 11 cm was significantly different, a generalised linear model (GLM) was performed comparing the oxygen concentration against the measurement depth and soil treatment factors.

3. A two way ANOVA was used to determine whether soil pH differed based on the soil treatments

4. To determine the differences in earthworm condition across flooding and sludge application treatments, the total biomass of earthworms placed into the mesocosm was summed, as was the total biomass of earthworms removed. The difference in earthworm biomass between the beginning and the end of the experiment was then compared based on the flooding treatment and the inclusion of sewage sludge in the mesocosm using a GLM. A two-way ANOVA was performed between the mass of cast material, and the flooding and soil treatments, while a linear model with a Poisson link was performed between the number of earthworm burrows and the flooding and soil treatments to determine whether the flooding and sludge application treatments had an effect on earthworm burrowing behaviour.

6.4 Results

When deconstructing the mesocosms flooded container 12, a treatment of just earthworm addition, was found to have no casting material, no evidence of incorporation of the dried manure into the soil, and, when broken apart, very little evidence of burrowing and no earthworm presence. Due to all these factors, this replicate was excluded from the data analysis.

6.4.1 Earthworm activity

All earthworms were recovered from all but two of the mesocosms. As discussed at the beginning of Section 6.4, no earthworms or casting activity were recorded in F-E container 12. UF-ES container 22 had only one earthworm retrieved. During the experiment, one earthworm was found to have escaped into the controlled temperature room; it is likely that this individual escaped from UF-ES container 22. Unlike container 12, container 22 was not excluded from the data analysis, as there was evidence of earthworm burrowing and casting activity.

GLMs, or a two way ANOVA performed on the mass of casting material between flooding and sludge addition, found no effect of flooding or of the addition of sludge was found on earthworm mass changes, the mass of casting material removed from the surface of the mesocosms, or the number of burrows in the mesocosms (Table 6-1).

Table 6-1. The mean and range of changes in earthworm biomass, the wet mass of cast material, and the number of burrow openings counted on the soil surface across UF and F flooding treatments and E and ES soil treatments.

Treatment	Earthworm biomass change (g)	Mass of casting material (g)	Number of burrows
UF – E	0.79 (-0.74 – 1.88)	85.86 (58.49 – 100.53)	2.2 (1 – 3)
UF – ES	0.41 (-4.96 – 2.49)	94.27 (3.75 – 149.93)	3.6 (1 – 7)
F – E	2.22 (1.02 – 3.13)	98.71 (61.29 – 149.39)	0.75 (0 – 2)
F - ES	2.30 (1.83 – 3.21)	91.08 (65.12 – 110.03)	1.8 (1 – 3)

6.4.2 Gas Fluxes

Repeated measure three way ANOVAs found that fluxes of CH_4, CO_2 and N_2O varied with the number of weeks flooded, the C, E, S or ES treatments, and the flooding treatment (UF, F) ($P < 0.01$). All repeated measure ANOVAs also showed significant interaction terms for gas flux between the number of weeks flooded and flooding treatment, the number of weeks flooded and the soil treatment, and the number of weeks flooded, flooding and soil treatments ($P < 0.01$).

Post hoc testing revealed complex interaction terms for all the greenhouse gases. General trends are described here, and key significant differences described. All significant differences reported in post hoc testing differed at the level of $P < 0.05$.

6.4.2.1 CH_4 fluxes

In both F and UF treatments (Figure 6-5), there were few statistical differences between the flooding and soil treatments between the start of the experiment (week -2) and week 4, with all soils acting as a methane sink for the majority of the experiment. Although there was a large amount of overlap, in weeks 5 and 6 a general trend began of CH_4 fluxes from the F treatments becoming CH_4 sources, with CH_4 fluxes at week 6 being significantly lower in the F-C treatments than the F-E, F-S, and F-ES treatments. At week 6, the only F treatments still acting as a CH_4 sink rather than a source were the F-C treatment soils.

Throughout the experiment, UF-ES treatments were significantly different to the majority of other soils, and particularly at week 6. In general, UF-ES treatments acted as the greatest CH_4 sink.

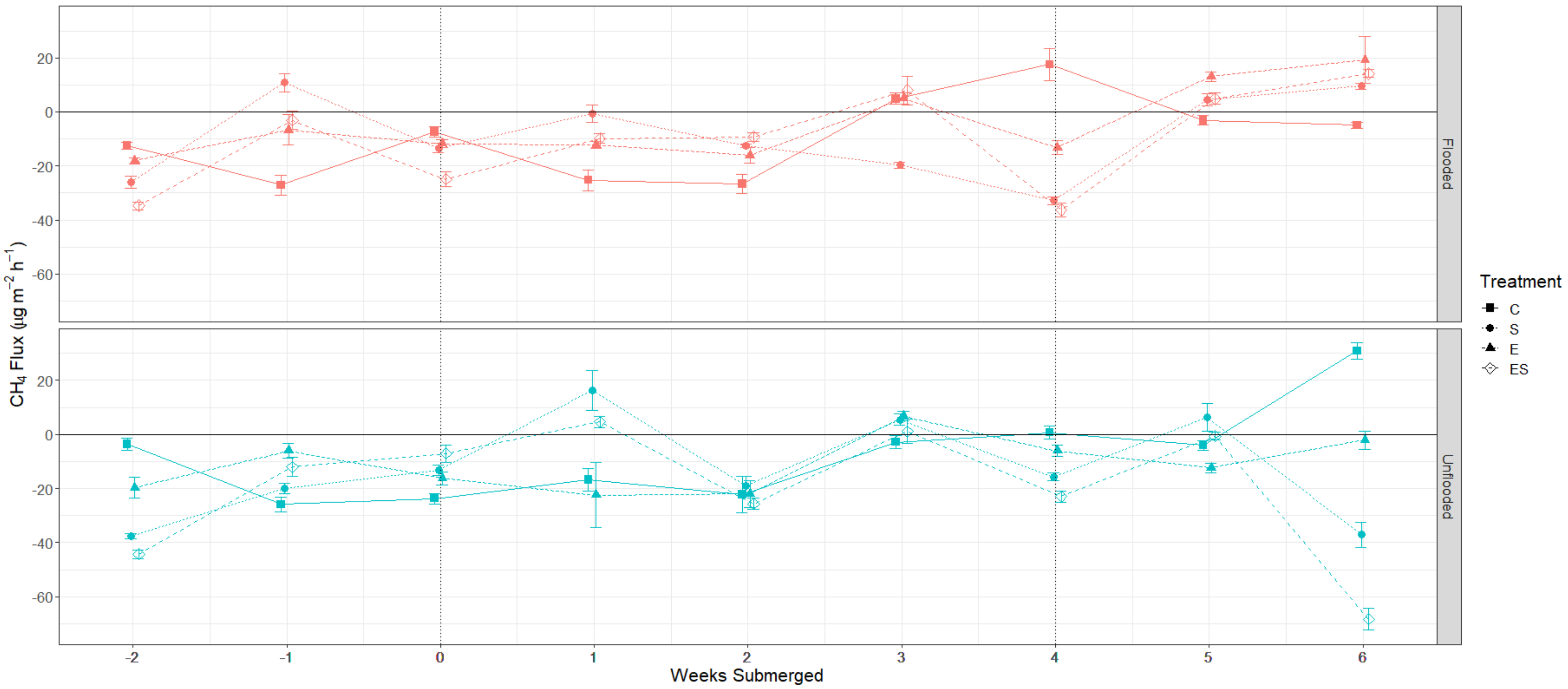

Figure 6-5. Mean fluxes of CH_4 (µg m^{-2} h^{-1}) for each soil treatment of no treatment (C), sewage sludge addition (S), earthworm addition (E), and earthworms and sewage sludge addition (ES) under flooded and unflooded conditions over the duration of the experiment. The vertical Dotted lines represent the time of flooding (0 weeks submerged) and the sampling point after which surface water was drained (4 weeks submerged). Error bars represent standard error (n=5).

6.4.2.2 CO_2 fluxes

CO_2 fluxes (Figure 6-6) were highly variable throughout the experiment in both F and UF treatments. In week -2, before the flooding event, CO_2 fluxes were highest from F-ES soils, but by week -1 there was no significant differences between any of the soils. From week 2, fluxes from the UF-E and UF-ES treatments were significantly higher than other fluxes at that timepoint, with no significant difference between F treatments and UF-C and UF-S treatments. From week 4, there were no significant differences between UF-C, UF-S, and F treatments across weeks 4, 5 and 6, with fluxes remaining highest in the UF-E and UF-ES treatments.

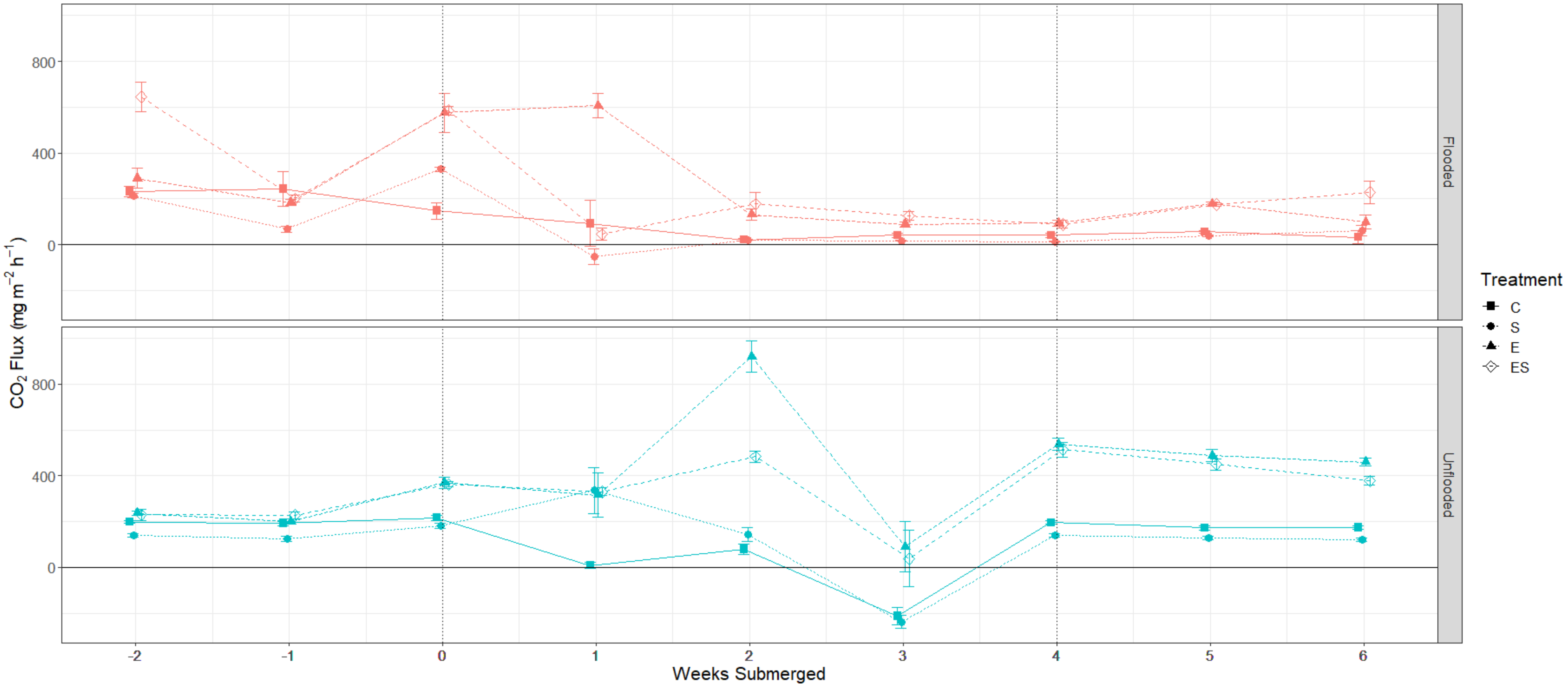

Figure 6-6. Mean fluxes of CO$_2$ (mg m^{-2} h^{-1}) for each soil treatment of no treatment (C), sewage sludge addition (S), earthworm addition (E), and earthworms and sewage sludge addition (ES) under flooded and unflooded conditions over the duration of the experiment. Vertical dotted lines represent the time of flooding (0 weeks submerged) and the sampling point after which surface water was drained (4 weeks submerged). Error bars represent standard error (n=5).

6.4.2.3 N_2O fluxes

At week -2, there was no significant difference in N_2O fluxes (Figure 6-7) between UF-ES and F-ES treatments. At week -1, N_2O fluxes in both F- and UF-ES treatments decreased, and were not significantly different to E treatments. From week 1, following the flooding event at week 0, only UF-ES treatments showed a significant difference to the other soil treatments, but did not significantly differ from UF-ES treatment fluxes at other time points. Following application of floodwater, fluxes from F treatments fell below detectable levels in all soil treatments, and these values did not significantly differ to those observed in UF-C, UF-S and UF-E treatments.

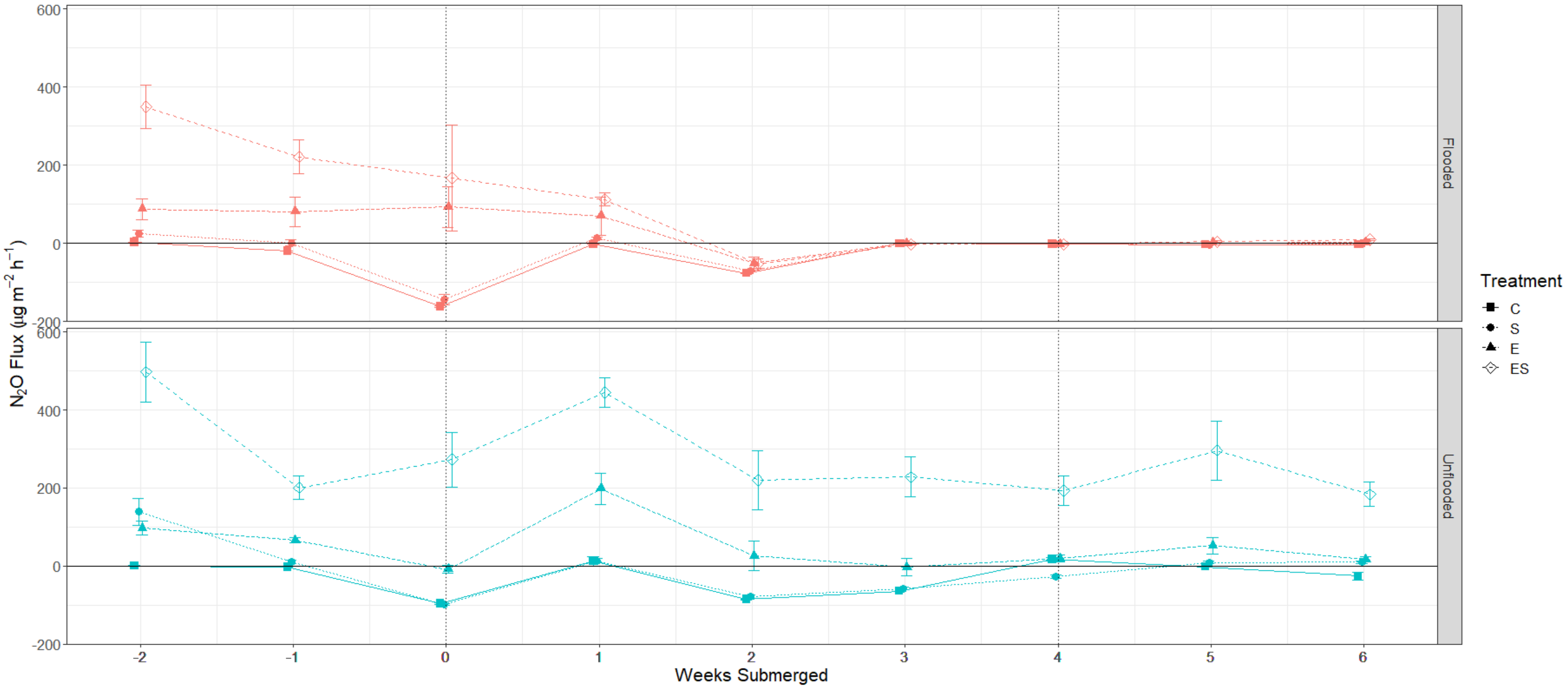

Figure 6-7. Mean fluxes of N_2O ($\mu g\ m^{-2}\ h^{-1}$) for each soil treatment of no treatment (C), sewage sludge addition (S), earthworm addition (E), and earthworms and sewage sludge addition (ES) under flooded and unflooded conditions over the duration of the experiment. Vertical dotted lines represent the time of flooding (0 weeks submerged) and the sampling point after which surface water was drained (4 weeks submerged). Error bars represent standard error (n=5).

6.4.3 Soil oxygen depletion

A repeated measures three way ANOVA examining the changes in oxygen concentration at a depth of 11 cm (Figure 6-8) across the number of weeks flooded, the flooding treatment and soil treatment found a significant effect of the duration of the experiment ($P < 0.0001$), the flooding treatment ($P = 0.031$), and the interaction between the duration of the experiment and the floodint treatment ($P < 0.0001$)

In week -2, oxygen concentrations were significantly higher in F treatments than in UF treatments. In week -1, oxygen concentrations began to decrease, with no significant difference between F-ES and UF-C and –S treatments. Within F treatments, oxygen concentration was significantly higher in F-ES than in F-S. From week 0 to week 6, there was no significant difference between soils or the week submerged, with high variability and large error bars in UF soils, and oxygen concentration below detectable levels in F soils.

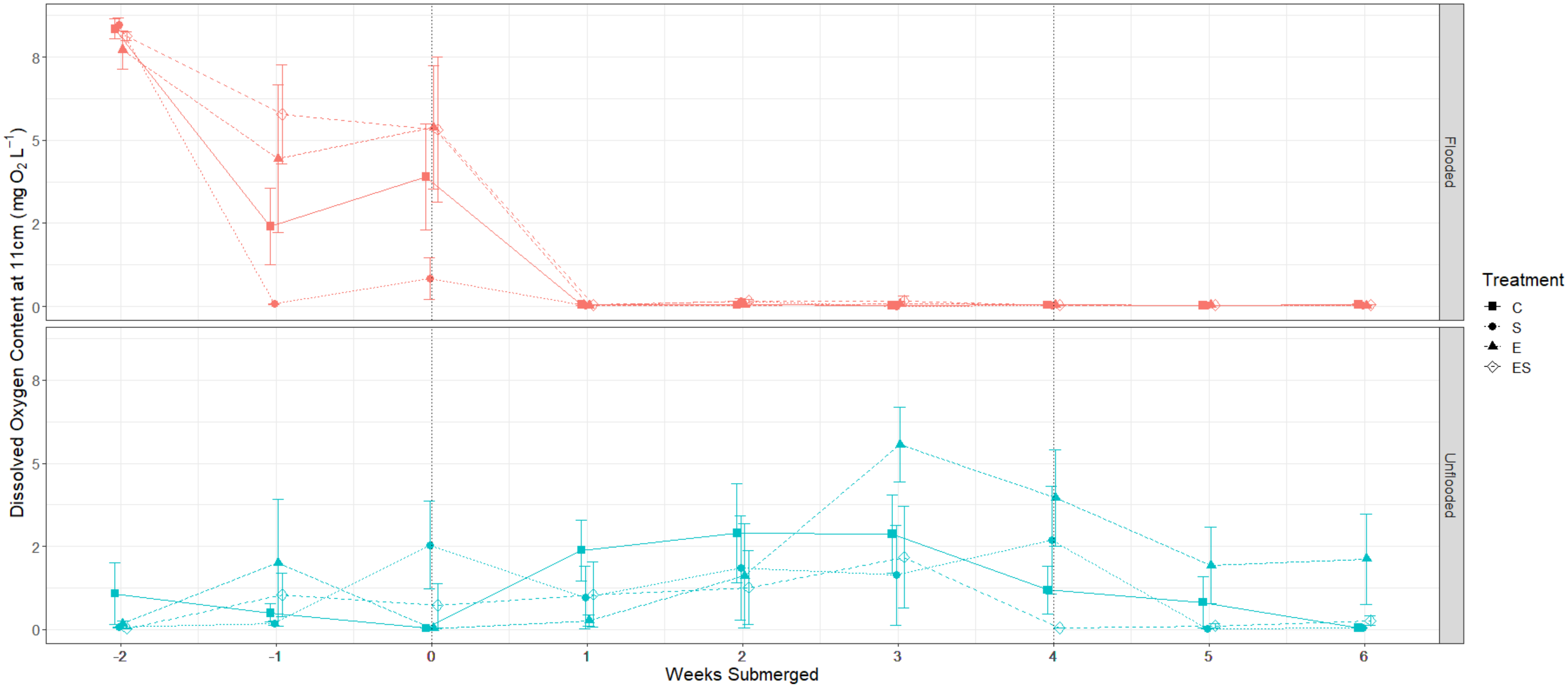

Figure 6-8. Mean dissolved oxygen content (mg O_2 L^{-1}) at a depth of 11 cm for each soil treatment of no treatment (C), sewage sludge addition (S), earthworm addition (E), and earthworms and sewage sludge addition (ES) under flooded and unflooded conditions over the duration of the experiment. Vertical dotted lines represent the time of flooding (0 weeks submerged) and the sampling point after which surface water was drained (4 weeks submerged). Error bars represent standard error (n=5).

A repeated measures ANOVA of the same factors at a soil depth of 5 cm (Figure 6-9) found a significant effect of the number of weeks flooded ($P < 0.001$), flooding treatment ($P = 0.002$), and an interaction term between the time submerged and flooding ($P < 0.0001$).

As with the 11 cm depth, once the flooding event occurred in the F treatment, the dissolved oxygen content of the soil dropped rapidly to below detectable levels across all soil treatments. In F treatments, the dissolved oxygen concentration in weeks -2 to 0 was significantly higher than the UF columns of the same time period. From weeks 1 to 6, the oxygen concentration in F treatment mesocosms was significantly lower than UF treatments across all weeks..

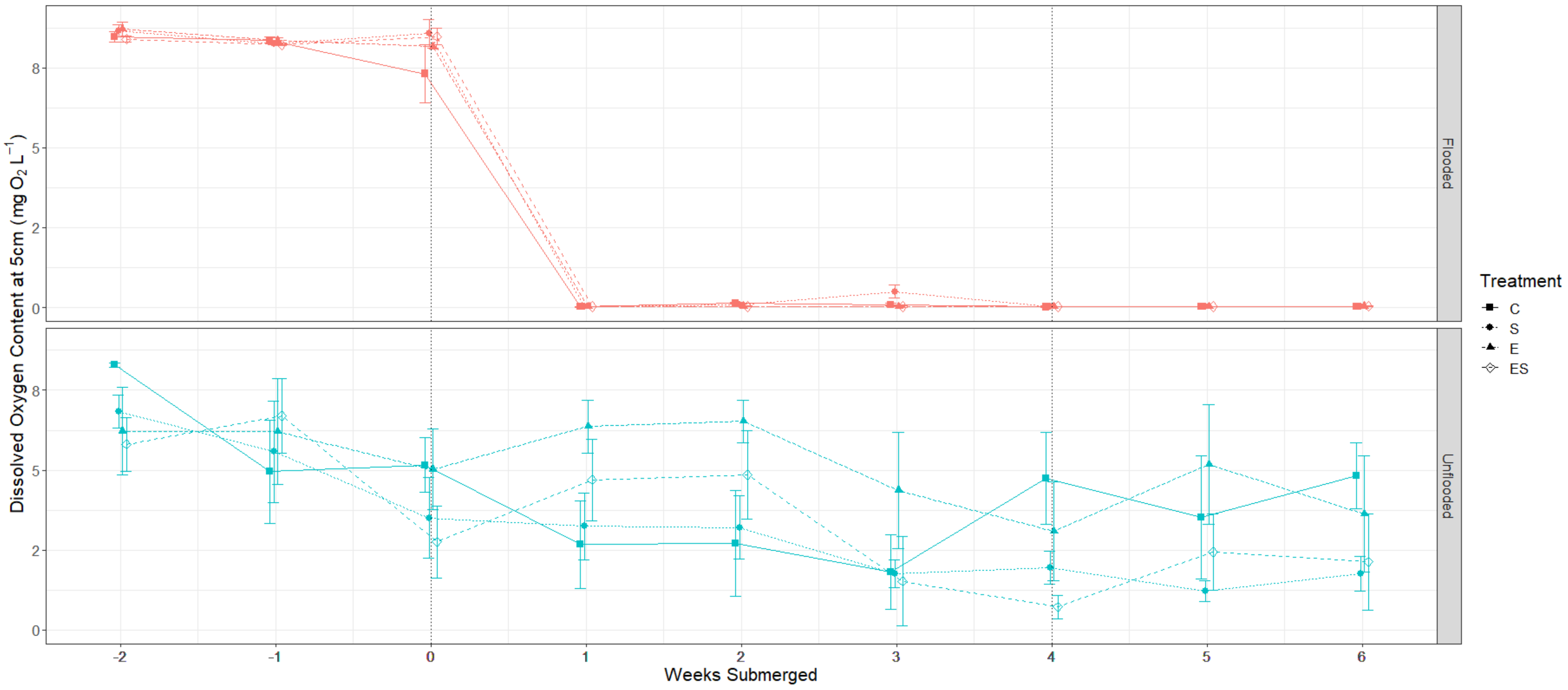

Figure 6-9. Mean dissolved oxygen content (mg O_2 L^{-1}) at a depth of 5 cm for each soil treatment of no treatment (C), sewage sludge addition (S), earthworm addition (E), and earthworms and sewage sludge addition (ES) under flooded and unflooded conditions over the duration of the experiment. Vertical dotted lines represent the time of flooding (0 weeks submerged) and the sampling point after which surface water was drained (4 weeks submerged). Error bars represent standard error (n=5).

A GLM comparing the oxygen concentration at the measurement depths and a combination of flooding and soil treatment found a significant effect of depth (*P* < 0.0001) and a significant interaction term between depth and treatment (*P* = 0.011).

Oxygen concentrations were significantly higher at depth 5 cm than at depth 11 cm (*P* < 0.0001). Post hoc testing examining the interaction term found that the oxygen concentration of UF-ES treatment at depth 11 cm was significantly lower than the oxygen concentration in UF-E and UF-C at 5 cm, but did not significantly differ to any other soil treatments at either depth.

6.4.4 Soil pH

A two way ANOVA showed a significant effect of sludge or earthworm treatment (*P* < 0.0001), flooding (*P* < 0.0001) and the interaction term (*P* < 0.0001) on the soil pH (Figure 6-10).

Post hoc testing showed the pH of the unflooded treatments was significantly lower than the flooded treatments (*P* < 0.05), with the unflooded treatments containing earthworms significantly lower in pH than the control or sludge only treatments. In the flooded treatments, there was much less variability in the pH values.

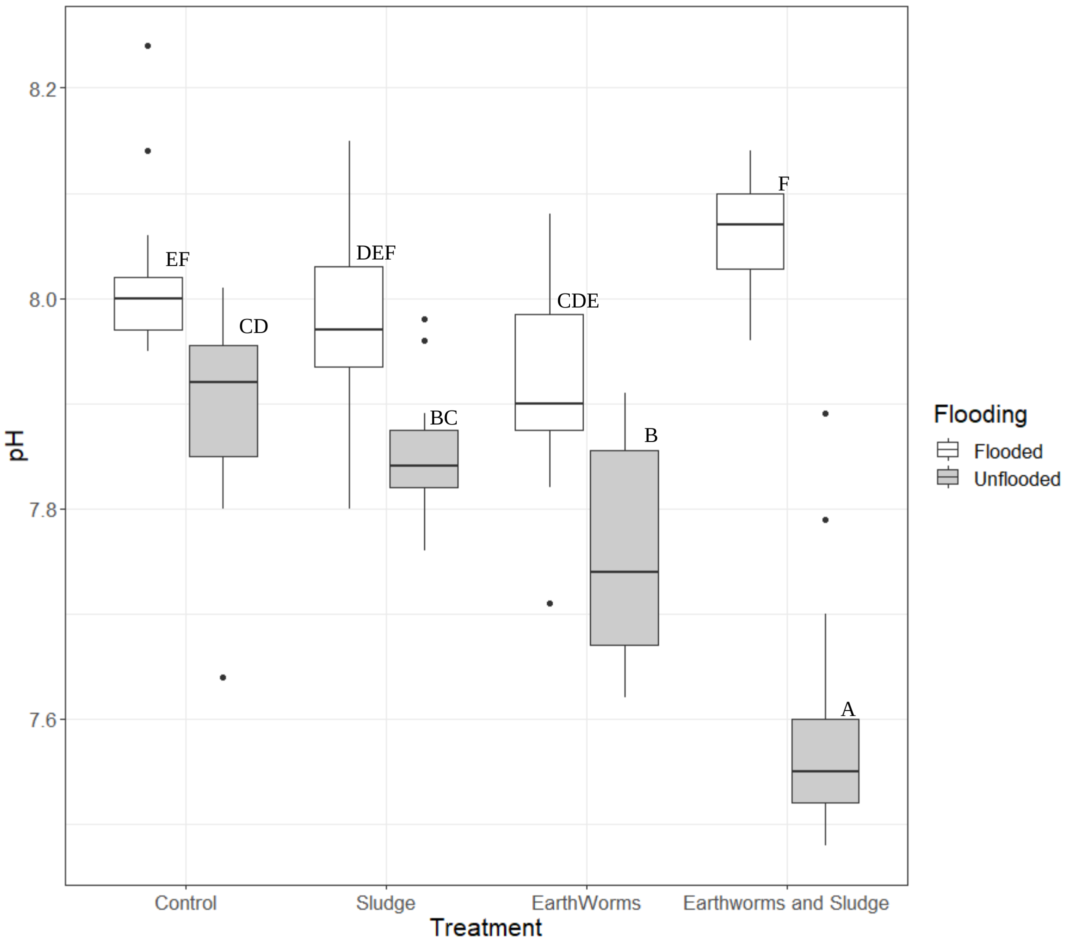

Figure 6-10. The pH of the flooded and unflooded mesocosm soils in the different soil treatment categories, with 25th, 50th, and 75th percentile, data ranges, and outliers illustrated in boxplots. Bars marked with the same letter are not significantly different ($P < 0.05$).

One sample t-tests between the observed pH values after treatment and the expected pH value of 7.69, determined during soil characterisation in Chapter 4, found a significant difference for all combinations of flooding and soil treatment ($P < 0.01$). All flooding and soil treatment combinations had a significantly higher pH than the expected value of 7.69, with the exception of UF-ES, which had an average pH of 7.59, and was significantly below the expected value (Figure 6-10).

6.5 Discussion

6.5.1 Earthworms

There was no significant effect of flooding or of sewage sludge addition on the mass gain of earthworms in the experiment, on the mass of cast material produced, or on the number of burrows counted on the surface of the soil. The lack of statistical significance in the mass gain of earthworms can likely be attributed to the wide range of changes in earthworm biomass. However, it is important to consider that the number of burrows and mass of casting material does not necessarily accurately illustrate the earthworm activity occurring within the mesocosms after flooding had occurred. The mesocosms were left *in situ* for twenty-one days before the flooding event, with seven days before sampling began and two weeks of unflooded measurements occurring. Although casts were observed after flooding, it is likely that much of the initial casting and burrowing activity occurred during these twenty-one days. Overall, it is likely that there were differences in earthworm behaviour once flooding occurred. Throughout the experiment, there were several instances of earthworms in the flooded treatments being observed on the soil surface (Fig. 6-2), or exposing segments of their bodies to the air; a behaviour observed during previous studies (Roots, 1956). This behaviour may be a result of the earthworm response of evacuating flooded soils, potentially due to the reduced oxygen conditions of the flooded soil. As discussed in Chapter 5, individuals of *L. terrestris* typically died at oxygen concentrations of > 1 mg O_2 L^{-1} (Section 5.4.2; Figure 5-4). As conditions within the flooded beakers reached below these levels within 7 days of flooding, the earthworm presence on the soil may be a behavioural response to low oxygen conditions within the flooded mesocosm itself.

6.5.2 Gas fluxes and soil oxygen

6.5.2.1 Soil oxygen concentration

The soil oxygen concentration at depths 5 cm and 11 cm were as expected given the results reported in Chapter 4. Seven days after flooding, the oxygen in all F treatments had reduced to levels below detection. In the UF treatments, there was no significant difference in oxygen concentration over the duration of the experiment or across soil treatments.

However, there was a difference observed between the oxygen concentrations at the two depths, with higher oxygen concentrations at 5 cm than 11 cm. Post hoc testing showed that this difference between the depths was driven by UF-E and UF-ES treatments, which had significantly lower oxygen concentrations at 11 cm than 5 cm. This result was unexpected, as earthworm burrowing may be expected to increase the aeration of soils (Lavelle, 1988). However, there were high levels of variability in the UF treatment at both depths, with overlapping error bars. It may be the case that the burrowing activity lead to increases in the soil microbial content, or increased the porosity and aeration in the soil (Lavelle, 1988). These changes may then lead to higher spatial variability of oxygen distribution within the soils at depth, causing the differences between 5 cm and 11 cm in the UF – E and –ES treatments.

6.5.2.2 Greenhouse gas fluxes

The ranges of reported fluxes for the three greenhouse gases from the literature are varied (Table 6-2). The CH_4 fluxes observed in my dataset are consistent with emissions from wetland, temperate soil (Oertel et al., 2016; Kasimir-Klemedtsson et al., 1997).

CO_2 fluxes from soil appear to be highly variable, reaching as high as 21×10^5 µg m^{-2} h^{-1} (Muñoz et al., 2010). However, it must be considered that soil production of CO_2 occurs as a result of microbial and root respiration, both of which are highly influenced by soil conditions such as temperature and moisture (Davidson and Janssens, 2006). As the review papers gathered data from across the globe, it is not surprising that such high ranges of CO_2 production are present. The CO_2 fluxes observed in this study in both the UF and F treatments fall towards the lower end of the scale reported in Muñoz et al., but within the range reported in Oertel et al..

N_2O fluxes are also highly variable. The majority of N_2O fluxes reported in the literature are typically lower than those observed in the UF dataset in this study. When the UF-ES treatments were removed, the UF fluxes of N_2O were more in line with the averages reported. The exception was Muñoz et al., which reported a maximum emission of 209 µg N_2O m^{-2} h^{-1}, with a maximum emission of 178 µg m^{-2} h^{-1} in soils amended with animal waste (Saggar et al., 2009, in Muñoz et al., 2010). This suggests that the very high flux values observed in the UF-ES treatments may still be within an expected emission range for organically amended soil.

Table 6-2. The ranges of gas fluxes of CH_4, CO_2, and N_2O reported in review papers collating sources from a number of soil types and regions compared to the ranges observed in this study

	Greenhouse gas		
Flooding treatment	**CH_4 range** **µg m^{-2} h^{-1}**	**CO_2 range** **mg m^{-2} h^{-1}**	**N_2O range** **µg m^{-2} h^{-1}**
Flooded	-44.6 – 35.7	-310.0 – 814.3	-169.9 – 585.4
Unflooded	-77.9 – 38.3	-451.5 – 1088.7	-105.0 – 1133.8
Unflooded (excluding UF-ES)	-	-	-105.0 – 337.7
Paper			
Oertel et al., 2016	-0.1 – 1.7^{-1}	0 – 1.1 x 10^{-2}	0 – 0.1
Kasimir-Klemedtsson et al., 1997	2.3 – 228.3	-61.6 – 4794.5	0 – 43.4
Muñoz et al., 2010	*Not reported*	0 – 21 x 10^{5}	0 – 208.9

6.5.2.3 *CH_4 fluxes*

The CH_4 fluxes observed are different to those expected. As discussed in Section 6.3.8, the expected pattern of CH_4 emission was that, as the flooded soils moved into an anaerobic state, the CH_4 emissions would increase accordingly (Le Mer and Roger, 2001). However, this was not observed. Although the flooded soil oxygen reduced to anoxic conditions after seven days, there was no increase in CH_4 fluxes until the standing water was drained from the mesocosms after 4 weeks submerged. This is very similar to the observed emissions of CH_4 in saturated soil environments such as rice paddies, where emissions of CH_4 greatly increase after floodwater recedes (Neue, 1993), due to the slow rates of diffusion through the floodwater. However, other studies found that this effect only continued for 2 to 4 days following the removal of floodwater (Denier van der Gon et al., 1996), while in this study high CH_4 emissions were still observed two weeks after the removal of floodwater. These observed results may have occurred because, although the floodwater was removed, the soil remained saturated and anaerobic. Studies investigating the link between CH_4 emission and water table depth found that, as the distance between the water table and the soil surface increased, soil emissions of CH_4 decreased (Moore and Roulet, 1993), likely due to the presence of methanotrophs in aerobic conditions, which oxidise CH_4 to carbon dioxide (Amaral and Knowles, 1995; Topp and Pattey, 1997). In this study, following the draining of the surface water, the soils remained anaerobic at both 5 cm and 11 cm in the flooded treatments, meaning no aerobic breakdown of CH_4 would be occurring, resulting in higher rates of CH_4 emissions than the unflooded treatments. The high flux values following removal of surface water also suggests that the surface water may have had a cushioning effect on CH_4 emissions. In flooded rice paddies, one study suggested that < 1% of CH_4 emissions came from diffusion through surface water, with the majority caused by active transport through plants (Schütz et al., 1989a). The low emissions of CH_4 from floodwater is unlikely to be due to the dissolution of CH_4 in floodwater; as a non-polar molecule, at low temperature and ambient pressure the solubility of methane in water is very low (Frolich et al., 1931), at 2.59×10^{-2} g L^{-1} (Dean, 1972), which explains why studies into rice paddy soils typically find very little methane in surface water (Mariko et al., 1991).

The patterns of CH_4 uptake observed may be due to the aerobic conditions of the surface floodwater. Studies examining unflooded soil mesocosms found that methane emissions drop to below detectable levels as oxygenation increased at the soil surface, despite low oxygenation at depths of below 10 cm (Sheppard and Lloyd, 2002). In this study, oxygen concentrations in the floodwater were not recorded; however, as a relatively thin layer of water on the soil surface, the larger surface area may have allowed for a relatively fast rate of oxygen reabsorption into the water, meaning it maintained aerobic conditions allowing for methanotrophic activity. Studies examining floodwater in rice paddies find diverse communities of methanotrophic bacteria (Escoffier et al., 1997), which can oxidise over 60% of CH_4 produced in the soils (Holzapfel-Pschorn and Seiler, 1986). These aerobic conditions leading to methanotrophic conditions may also explain why, generally, both the flooded and unflooded soils act as CH_4 sinks until the removal of floodwater, along with the movement of atmospheric CH_4 down concentration gradients (Koschorreck and Conrad, 1993). The balance between CH_4 sink or source status, therefore, depends on the relative rates of atmospheric uptakes, methanotrophic, and methanogenic activity (Topp and Pattey, 1997).

There was an observed effect of soil treatment on the emissions of CH_4 from the flooded soils once the water was removed. The F - E treatments have the highest rates of CH_4 emissions, followed by the F - ES treatments, and F - S treatments. The F - C soils did not emit CH_4, and the soils remained CH_4 sinks. This suggests that the addition of organic material, and the burrowing actions of earthworms, also influence the rates of CH_4 production within the flooded soils. The presence of organic amendments leads to higher emissions of CH_4 in rice paddy soils (Denier van der Gon and Neue, 1995; Neue et al., 1997), which may explain why CH_4 fluxes are higher from the F-S treatments than the F-C treatments. Meanwhile, the effect of earthworms on soil CH_4 emission is not well understood (Lubbers et al., 2013). One study found higher gross production of CH_4 in intensively farmed pasture soils when the soil was incubated with epigeic earthworms, with higher concentrations of methanogenic genes present (Bradley et al., 2012), while another study attributed the increased emissions of CH_4 with earthworms to their influence on bacterial growth (Héry et al., 2008; Singer et al., 2001). Under anaerobic conditions, the presence of these methanogenic bacteria may be more evident, with no methanotrophic activity reducing the CH_4 to CO_2. In both the E and ES treatments, the promotion effect of earthworms on bacterial activity may have led to the higher rates of CH_4 emissions once the surface water was removed, although the reason for higher fluxes in E treatments than ES treatments is not currently understood.

6.5.2.4 CO_2 fluxes

The CO_2 fluxes are higher overall in the UF treatments than the F treatments, with emissions of CO_2 suppressed following the addition of floodwater. However, there are higher fluxes of CO_2 in the ES and E treatments than in the S or C treatments, which may be an effect of higher rates of CH_4 emissions being converted to CO_2 by methanotrophic bacteria in the soil water in the F treatments. If the F - E and – ES soils are producing higher levels of CH_4, methanotrophic activity in the floodwater may then in turn lead to higher CO_2 fluxes under flooded conditions. However, the higher rates of CO_2 emissions in E and ES treatments in both F and UF conditions are more likely the effect of earthworm respiration (Binet et al., 1998), or of the stimulation of microbial population that earthworms can cause in soil (Scheu, 1987; Wolters, 2000). Overall, the presence of the surface water may lead to reduced rates of gas emission due to the dissolution of CO_2 into the floodwater. As discussed in Section 2.5.2, CO_2 dissolves in water to form carbonic acid, at a rate of 1.97 g L^{-1} at 15°C (Dean, 1972). This relatively high rate of solubility in water may explain the low flux values of CO_2 in F treatments from weeks 0 to 4, with increases in CO_2 fluxes in weeks 5 and 6, after the removal of surface water.

6.5.2.5 N_2O fluxes

Fluxes of N_2O differed greatly between UF and F treatments. In the F treatments, following flooding, the emissions of N_2O rapidly dropped to levels below detection. This reduction may be partly due to dissolution of the gas into floodwater (Terry et al., 1981) or N_2O consumption by microbial activity (Payne, 1973; Majumdar, 2013). In the unflooded soils, the control and sewage sludge only treatments frequently showed negative fluxes of N_2O. This may be due to passive uptake, with the N_2O moving according to a concentration gradient (Chapuis-Lardy et al., 2006), or, as in the floodwater, bacterial activity and consumption.

Unlike the fluxes of CH_4, there was no statistically significant increase in the fluxes of N_2O following the removal of floodwater. Emissions of N_2O are highly associated with soil water content, with waterlogged soil emissions of N_2O remaining low even after removal of floodwater (Zou et al., 2005), likely due to the microbial consumption of N_2O under anaerobic conditions in waterlogged soils. However, as flooded soils drain, N_2O emissions typically increase (Zou et al., 2005; Towprayoon et al., 2005). This may be a result of high N_2O solubility in water; at 10°C, 88 ml volume of N_2O dissolves in 100 ml of water (Dean, 1972). As the water content of soil decreases, N_2O emissions likely correspondingly increases. Had the experiment continued for a longer period of time, there may have been an increase in soil N_2O emissions, which would have coincided with a reduction in CH_4 emissions as the soil began to return to aerated conditions.

In the UF treatments and in the F treatment before flooding, fluxes of N_2O were highest in the ES mesocosms. The E treatments, while showing higher fluxes, did not differ significantly from the control and sludge only treatments. The increases earthworms cause on soil N_2O emissions have long been known (Borken et al., 2000; Lubbers et al., 2011, Rizhiya et al., 2007), as their guts act as a reservoir for denitrifying microbes, which are subsequently incorporated into soil through their burrowing and casting activity (Drake and Horn, 2006). However, in this study it is the interaction between earthworms and sewage sludge which had the greatest effect on N_2O fluxes. This is not wholly unexpected; a previous meta-analysis found that the influence of earthworms on N_2O emissions from soil was highly associated with the addition of organic residues to soil (Lubbers et al., 2013). Lubbers et al. suggested that this may be due to decomposition of organic material leading to relatively anaerobic conditions, in turn contributing to higher denitrification activity of bacteria. This study found no effect of soil treatment on the oxygen concentration of the soil, but an overall trend of soil oxygen depletion occurring in UF soils, likely due to the breakdown of organic matter, although they did not reach anaerobic conditions. This in turn may have led to small, localised anaerobic regions within the soil, leading to increased N_2O emissions from the mesocosms with added organic residues and earthworms. There is also evidence that bacterial reduction of NO_3^- to N_2O still occurs under aerobic conditions (Müller et al., 2004). Although the study by Müller et al. (2004) found this occurring at soil depths of 20 to 50cm depths, the combination of earthworm activity and aerobic reduction of NO_3^- suggests that even in the aerobic, but reduced oxygen, conditions, the observations made in Lubbers et al. (2013) are in agreement with the observed results in this study.

The carbon to nitrogen (C:N) ratio of the sludge addition to earthworm-containing soil may explain the higher N_2O emissions in the UF-ES soils. There is a strong negative association between soil emissions of N_2O and the C:N ratio of organic amendments (Baggs et al., 2000; Huang et al., 2004) and the C:N ratio of unamended soil (Klemedtsson et al., 2005), likely due to the high nitrogen content of these low C:N ratio amendments. The sewage sludge used in Chapter 4 had a C:N ratio of 7.75. In comparison, the soil used in this study had a C:N ratio of 11.57, with a high proportion of carbon and low nitrogen content. Although, as discussed in Chapter 4, the majority of the organic matter content of sewage sludge is likely locked into recalcitrant matter, the promotion of the activity of denitrifying bacteria by earthworms combined with the low C:N ratio of the sewage sludge organic amendment may have led to the high rates of N_2O emission observed in this study.

6.5.3 Soil pH

The differences between the expected pH value and the observed pH values in the soils after the experiment may simply be a result of changes in the soil over the course of the experiment; while in Chapter 4 the experimental soil was dried and rewetted before pH was determined, in this chapter the soil has been through a process of drying, rewetting, and long term incubation under a variety of conditions. Given that the increase from the expected pH to the observed pH in both F and UF treatments is relatively small, from 7.69 to approximately 8.0, the differences observed between the treatments is overall of more interest in the context of the study.

Variation in pH between the different UF treatments was far greater than between F treatments. In the F treatments, the pH was significantly higher than the unflooded soils, and there was little variation in the pH of the different soil treatments. It may be the case that the differences between F and UF treatments were driven by changing soil conditions in the UF treatments, rather than the effect of flooding on the flooded columns. The pH of the UF treatments was affected by soil treatment, with the pH of ES treatments statistically lower than the C, S and E treatments, and significantly lower than the expected pH value. This low pH value of the UF-ES may be a result of increased breakdown of sewage sludge promoted by earthworm activity. As suggested by the CO_2 and N_2O emissions, the combination of earthworms and sewage sludge leads to higher rates of bacterial decomposition of sewage sludge than sewage sludge alone. This breakdown of organic matter in turn leads to the release of H^+ ions (Porter et al., 1980), increasing the acidity of the soil over time. The process of the aerobic breakdown of carbohydrates into carbon dioxide and H^+ ions is summarised in Equation 6-1. In all UF treatments, there was likely some breakdown of organic matter occurring, although the rates of breakdown vary based on the soil treatment; only the ES treatments had sufficient organic matter breakdown to reach pH levels statistically more acidic than the base pH level. In the F treatments, breakdown of organic matter was considerably slower under the anaerobic conditions caused by flooding, meaning that flooding suppressed any potential effects of soil treatment on pH. This is supported by the lower CO_2 fluxes in the F treatments than the UF treatments, suggesting slower turnover of organic matter under anaerobic conditions (Kristensen et al., 1995).

Equation 6-1. The aerobic breakdown of carbohydrates

$$\boldsymbol{CH_2O + H_2O = CO_2 + 4H^+ + 4e^-}$$

However, this lack of change in the F treatments runs counter to the expectation expressed in Section 6.3.8 and Section 2.5.2, where it was suggested that the pH of flooded acid or alkaline soils will shift towards neutrality. In alkaline soils, this is believed to be due to the accumulation of CO_2 in waterlogged soils (Greenway et al., 2006) forming carbonic acid with water (Sahrawat, 2005). The increase in H^+ ion concentration this causes drives the pH of flooded, alkaline soils back towards neutrality. When characterised in Chapter 4, the soil was found to have a pH of 7.69: mildly alkaline. However, the average pH for the soils in the F treatments ranged from 7.92 in F - E to 8.02 in F - C. This suggests that not only did the pH not begin to shift towards neutrality, but that the pH of the flooded soils increased slightly, becoming more alkaline. Other studies have found that the decrease in soil pH on submergence was temporary, with soil pH increasing again after approximately 5 weeks, when soil Redox potential values had reached negative conditions

(Islam and Islam, 1973). It may be the case that it is the soil Redox conditions driving these increases in soil pH in the flooded conditions. As the soils had been saturated for a period of six weeks, and soil oxygen depleted to levels below detection by week 1, it is likely that soil conditions in the flooded treatments were highly reducing. Under anaerobic conditions, the production of CH_4, observed to increase in emissions during weeks 5 and 6 following the removal of surface water, consumes H^+ ions (Equation 6-2). Although not significant, there was an increasing trend in emissions of CH_4 from mesocosms following the removal of floodwater, suggesting that CH_4 producing processes were occurring.

Equation 6-2. The anaerobic formation of CH_4

$$CO_2 + 8H^+ + 8e^- = CH_4 + 2H_2O$$

In the flooded soils, the rate of H^+ consumption for methane production may have outstripped the rates of H^+ caused by the accumulation of carbonic acid, leading to an overall increase in the alkalinity of flooded soils, rather than a shift towards neutrality.

Despite the unexpected changes to pH observed in the flooded soils, the flooding treatment appears to have suppressed the effects of soil treatment observed in the unflooded soils. Flooding has been referred to as a “great equalizer” of soil chemistry (Sahrawat, 1998), and the differences in pH of the different soil treatments, along with the similar patterns of soil gas fluxes under flooded conditions, support this assessment.

6.5.4 Further work

This experiment has provided a number of questions for potential future investigation.

Were this experiment to be repeated, further work could extend the drying period of the previously flooded soils. Initially, the experimental design was to drill a hole into the base of the flooded containers and allow water to drain from the soil over the last two weeks of the experiment. However, when this was tested on a spare container set up in the same manner as the mesocosms, the water did not successfully drain due to the compaction of the soil, and so the method was not used on the experimental containers. Perhaps moving the mesocosms to a warmer controlled temperature room would encourage the drying of the soil. As discussed in Section 6.5.1, draining of previously flooded soils can lead to an increase in N_2O production; as the data shows a strong effect of the combination of earthworms and sewage sludge on N_2O production in the UF treatments, it may be the case that this pattern would also be observed as the soil dried.

As discussed in 6.5.1, the effect of flooding on earthworm behaviour in the mesocosms is not well quantified. Examination of the burrow network through the use of CT scanners, or close observation of changes in earthworm burrow use with flooding, may provide valuable understanding of survival strategy and decision making during flooding events. The earthworms in the flooded mesocosms showed different survival mechanisms, such as exposing segments of the body or remaining on the soil surface. Further experimentation could also examine a hierarchy of decision making in employing different survival strategies in stressful circumstances, such as flooding or drought.

Other further work could explore some of the knowledge gaps in the literature. The majority of work that has been performed on flooded soils have focused on regularly inundated soils, such as wetlands and rice paddies. However, relatively little work has been done on arable and pasture floodplain soil. An understanding of greenhouse gas emissions from these soils with flooding, and how gas transport through grasses and arable crops differs to gas transport in crops such as rice or to wetland plants, may be of use for understanding how greenhouse gas emissions from arable soil will change with the predicted increases in UK flooding.

Finally, this study has shown the strong interaction between earthworms, sewage sludge amendment, and N_2O emissions. Further work needs to examine the effect of earthworms belonging to the endogeic and epigeic ecotypes on greenhouse gas emissions from sewage sludge amended soil. As discussed in Chapter 3, arable soils typically have low abundances of anecic earthworms (Chan, 2001), and are dominated by endogeic earthworm species (Pelosi et al., 2009; Schmidt et al., 2001). This study was performed using *L. terrestris*, as the earthworm species is easily available for purchase in large quantities from online suppliers, and we thought that the impact of permanent vertical burrows to the surface may have an impact on gas transport and greenhouse gas fluxes. However, as this study has only been performed with anecic earthworms, it is not necessarily representative of how greenhouse gas emissions may change with soil amendment and flooding. Understanding differences in the microbial community, or differences in gene expression, may aid understanding of what causes the interaction between earthworms and sewage sludge observed in this study. As this study was based on a single application of sewage sludge to previously unamended soils, further studies examining soils that have had multiple applications of sewage sludge over time, and the effects of earthworms and the microbial community on greenhouse gas emissions from these soils, could also improve our understanding of how fluxes may change in the field.

6.6 Conclusion

The gas fluxes of CH_4, CO_2 and N_2O all changed with flooding and soil treatments. Earthworms greatly influenced the fluxes of CO_2 and N_2O from the unflooded soils, but overall did not appear to greatly impact soil fluxes of CH_4. The addition of sewage sludge alone to unflooded soils did not lead to any changes in greenhouse gas fluxes from soils. When both earthworms and sewage sludge were added to soils, soil respiration, and therefore emissions of CO_2, increased, as did soil fluxes of N_2O.

Fluxes of greenhouse gases from flooded soils were overall very low, with little to no effect of soil treatment on greenhouse gas emissions. Fluxes of CO_2 and CH_4 increased with the removal of surface water, although the soil remained saturated. Fluxes of N_2O, however, remained below detectable levels while the soil was saturated. Further work is needed to see how N_2O fluxes may increase as soil dries.

There were no adverse effects of flooding on a number of indicators of earthworm activity. This is in contrast to the results observed in Chapter 5, which suggested that the individuals of *L. terrestris* were likely to drown when oxygen concentrations dropped below 1 mg O_2 L^{-1}. However, this result was not observed during this experiment, as all individuals were recovered from the all but one of the flooded mesocosms. This demonstrates the effect that earthworm behaviours have on their survival in flooded conditions, and helps explain some of the results observed in Chapter 3, where anecic earthworm populations were still present, albeit reduced, in regularly flooded regions.

Overall, these results suggest that, while flooding may lead to increased CH_4 emissions from soil once surface water drains, the effect of flooding soil that contains both earthworms and sewage sludge amendments does not lead to greater greenhouse gas emissions. However, in unflooded soil, the addition of sewage sludge as an organic amendment to soils containing earthworms led to greatly increased emissions of N_2O. While waterlogged, N_2O emissions from soil typically remain low. However, the drying of soil is typically associated with pulses of N_2O emissions. Further work is needed to determine if, with increased flooding and drying cycles, and high levels of soil amendment with sewage sludge in the UK, there is the potential for UK emissions of N_2O to increase.

6.7 References

Amaral, J.A., Knowles, R. 1995. Growth of Methanotrophs in Methane and Oxygen Counter Gradients. *FEMS Microbiology Letters* 126(3): 215 – 220

Baggs, E.M., Rees, R.M., Smith, K.A., Vintent, A.J.A. 2006. Nitrous Oxide Emission from Soils after Incorporating Crop Residues. Soil Use and Management 16(2): 82 – 87

Bell, R.G. 1969. Studies on the Decomposition of Organic Matter in Flooded Soil. *Soil Biology and Biochemistry* 1(2): 105 – 116.

Binet, F., Fayolle, L., Pussard, M., Crawford, J.J., Traina, S.J., Tuovinen, O.H. 1998. Significance of Earthworms in Stimulating Soil Microbial Activity. *Biology and Fertility of Soils* 27(1): 79 – 84

Bouldin, J.L., Klasky, J.W.P., Green, V.S. 2016. Earthworm Preference Bioassays to Evaluate Land Management Practices. *Bulletin of Environmental Contamination and Toxicology*, 96: 767-772.

Borken, W., Gründen, S., Beese, F. 2000. Potential Contribution of *Lumbricus terrestris L.* to Carbon Dioxide, Methane, and Nitrous Oxide Fluxes from a Forest Soil. *Biology and Fertility of Soils* 32(2): 142 – 148

Bradley, R.L., Chroňáková, A., Elhottová, D., Šimek, M. 2012. Interactions between Land-Use History and Earthworms Control Gross Rates of Soil Methane Production in an Overwintering Pasture. *Soil Biology and Biochemistry* 53: 64 – 71

Brune, A., Frenzel, P., Cypionka, H. 2000. Life at the Oxic-Anoxic Interface: Microbial Activities and Adaptations. *FEMS Microbiology Reviews* 24(5): 691 – 710

Chapuis-Lardy, L., Wrage, N., Metay, A., Chotte, J-L., Bernoux, M. 2006. Soils, a Sink for N_2O? A Review. *Global Change Biology* 13(1): 1 – 7

Chan, K.Y. 2001. An Overview of Some Tillage Impacts on Earthworm Population Abundance and Diversity – Implications for Functioning in Soils. *Soil and Tillage Research* 57(4): 179 – 191.

Davidson, E.A., Janssens, I.A. 2006. Temperature Sensitivity of Soil Carbon Decomposition and Feedbacks to Climate Change. *Nature* 440(7081): 165

Dean, J.A. 1972. Lange's Handbook of Chemistry 14th Edition. New York: McGraw-Hill, Inc

Denier van der Gon, H.A.C., Neue, H.U. 1995. Influence of Organic Matter Incorporation on the Methane Emission from a Wetland Rice Field. *Global Biogeochemical Cycles* 9(1): 11 – 22

Denier van der Gon, H.A.C., van Breemen, N., Neue, H.U., Lantin, R.S., Aduna, J.B., Alberto, M.C.R., Wassman, R. 1996. Release of Entrapped Methane from Wetland Rice Fields upon Soil Drying. *Global Biogeochemical Cycles* 10(1): 1 – 7

Devêvre, O.C., Horwáth, W.R. 2000. Decomposition of Rice Straw and Microbial Carbon Use Efficiency under Different Soil Temperatures and Moistures. *Soil Biology and Biochemistry* 32(11-12): 1773 – 1785.

Drake, H.L., Horn, M.A. 2006. Earthworms as a Transient Heaven for Terrestrial Denitrifying Microbes: A Review. *Engineering in Life Sciences* 6(3): 261 – 165

Epstein, E., Taylor, J.M., Chaney, R.L. 1977. Effects of Sewage Sludge and Sludge Compost Applied to Soil on some Soil Physical and Chemical Properties. *Journal of Environmental Quality* 5(4): 422 – 426

Escoffier, S., Le Mer, J., Roger, P.A. 1997. Enumeration of Methanotrophic Bacteria in Rice Field Soils by Plating and MPN Techniques: A Critical Approach. *European Journal of Soil Biology* 33(1): 41 – 51

Frolich, P.K., Tauch, E.J., Hogan, J.J., Peer, A.A. 1931. Solubilities of Gases in Liquids at High Pressure. *Industrial & Engineering Chemistry* 25(3): 548 – 550

Greenway, H., Armstrong, W., Colmer, T.D. 2006. Conditions Leading to High CO2 (>5Kpa) in Waterlogged-Flooded Soils and Possible Effects on Root Growth and Metabolism. *Annals of Botany*, 98: 9-32.

Haimi, J., Huhta, V. 1990. Effect of Earthworms on Decomposition Processes in Raw Humus Forest Soil: A Microcosm Study. *Biology and Fertility of Soils* 10(3): 178 – 183

Hamilton, W. E., Dindal, D. L. 1989. Impact of Land-Spread Sewage-Sludge and Earthworm Introduction on Established Earthworms and Soil Structure. *Biology and Fertility of Soils*, 8: 160 – 165

Héry, M., Singer, A.C., Kumaresan, D., Bodrossy, L., Stralis-Pavese, N., Prosser, J.I., Thompson, I.P., Murrel, J.C. 2008. Effect of Earthworms on the Community Structure of Active Methanotrophic Bacteria in a Landfill Cover Soil. *The ISME* 2(1): 92

Holzapfel-Pschorn, A., Seiler, W. 1986. Methane Emission during a Cultivation Period from an Italian Rice Paddy. *Journal of Geophysical Research: Atmospheres* 91(D11): 11809 – 11814

Horn, R., Smucker, A. 2005. Structure Formation and its Consequences for Gas and Water Transport in Unsaturated Arable and Forest Soils. *Soil and Tillage Research* 82(1): 5 - 14

Huang, Y., Zou, J., Zheng, X., Wang, Y., Xu, X. 2004. Nitrous Oxide Emissions as Influenced by Amendment of Plant Residues with Different C:N Ratios. *Soil Biology and Biochemistry* 36(6): 973 – 981

Islam, A., Islam, W. 1973. Chemistry of Submerged Soils and Growth and Yield of Rice I. Benefits from Submergence. *Plant and Soil* 39(3): 555 – 565

Kasimir-Klemedtsson, Å., Klemedtsson, L., Berglund, K., Martikainen, P., Silvola, J., Oenema, O. 1991. Greenhouse Gas Emissions from Farmed Organic Soils: A Review. *Soil Use and Management* 13: 245 – 250

Kızılkaya, R. and Hepşen, S., 2004. Effect of biosolid amendment on enzyme activities in earthworm (Lumbricus terrestris) casts. *Journal of Plant Nutrition and Soil Science* 167(2): 202 – 208

Klemedtsson, L., Von Arnold, K., Weslien, P., Gundersen, P. 2005. Soil CN Ratio as a Scalar Parameter to Predict Nitrous Oxide Emissions. *Global Change Biology* 11(7): 1142 - 1147

Koschorreck, M., Conrad, R. 1993. Oxidation of Atmospheric Methane in Soil: Measurements in the Field, in Soil Cores and in Soil Samples. *Global Biogeochemical Cycles* 7(1): 109 – 121

Kristensen, E, Ahmed, S.I., Devol, A.H. 1995. Aerobic and Anaerobic Decomposition of Organic Matter in Marine Sediment: Which is Fastest? *Limnology and Oceanography* 40(8): 1430 – 1437

Kretzschmar, A. 1987. Soil Partitioning Effect of an Earthworm Burrow System. *Biology and Fertility of Soils* 3(1 – 2): 121 – 124

Lavelle, P. 1988. Earthworm Activities and the Soil System. *Biology and Fertility of Soils* 6(3): 237 – 251

Le Mer, J., Roger, P. 2001. Production, Oxidation, Emission and Consumption of Methane by Soils: A Review. *European Journal of Soil Biology* 37(1): 25 – 50

Lubbers, I.M., Brussaard, L., Otten, W., van Groenigen, J.W. 2011. Earthworm-Induced N Mineralisation in Fertilised Grassland Increases both N_2O Emission and Crop-N Uptake. *European Journal of Soil Science* 62(1): 152 – 161

Lubbers, I.M., van Groenigen, K.J., Fonte, S.J., Six, J., Brussaard, L., van Groenigen, J.W. 2013. Greenhouse-Gas Emissions from Soils Increased by Earthworms. *Nature Climate Change* 3(3): 187

Lüdemann, H., Arth, I., Liesack, W. 2000. Spatial Changes in the Bacterial Community Structure along a Vertical Oxygen Gradient in Flooded Paddy Soil Cores. *Applied and Environmental Microbiology* 66(2): 754 – 762

Majumdar, D. 2013. Biogeochemistry of N_2O Uptake and Consumption in Submerged Soils and Rice Fields and Implications in Climate Change. *Critical Reviews in Environmental Science and Technology* 43(24): 2653 – 2684

Majumder, R., Livesley, S.J., Gregory, D., Arndt, S.K. 2014. Biosolid Stockpiles are a Significant Point Source for Greenhouse Gas Emissions. *Journal of Environmental Management* 143: 34 – 43

Mariko, S., Harazono, Y., Owa, N., Nouchi, I. 1991 Methane in Flooded Soil Water and the Emission Through Rice Plants to the Atmosphere. *Environmental and Experimental Botany* 31(3): 343 – 350

Moore, T.R., Roulet, N.T. 1993. Methane Flux: Water Table Relations in Northern Wetlands. *Geophysical Research Letters* 20(7): 587 – 590

Müller, C., Stevens, R.J., Laughlin, R.J., Jäger, J., -J. 2004. Microbial Processes and the Site of N_2O Production in a Temperate Grassland Soil. *Soil Biology and Biochemistry* 36(3):453 – 461

Muñoz, C., Paulio, L., Monreal, C., Zagal, E. 2010. Greenhouse Gas (CO_2 and N_2O) Emissions from Soils: A Review. *Chilean Journal of Agricultural Research* 70(3): 485 – 497

Neilsen, G.H., Hogue, E.J., Neilsen, D., Zebarth, B.J. 1998. Evaluation of Organic Wastes as Soil Amendments for Cultivation of Carrot and Chard on Irrigated Sandy Soils. *Canadian Journal of Soil Science* 78(1): 217 – 225

Neue, H.U. 1993. Methane Emission from Rice Fields. *Bioscience* 43(7): 466 – 474

Neue, H.U., Wassman, R., Kludze, H.K, Bujun, W., Lantin, R.S. 1997. Factors and Processes Controlling Methane Emissions from Rice Fields. *Nutrient Cycling in Agroecosystems* 49 (1-3): 111 – 117

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., Liesack, W. 2005. Success of Bacterial Community Structure and Diversity in a Paddy Soil Oxygen Gradient. *Environmental Microbiology* 7(3): 382 – 395

Oertel, C., Matschullat, J., Zurba, K., Zimmermann, F., Erasmi, S. 2016. Greenhouse Gas Emissions from Soils – A Review. *Geochemistry* 76(3): 327 – 352

Payne, W.J. 1973. Reduction of Nitrogenous Oxides by Microorganisms. *Bacteriological Reviews* 37(4): 409 – 452

Patrick, W.H. Jr, Delaune, R.D. 1977. Chemical and Biological Redox Systems Affecting Nutrient Availability in the Coastal Wetlands. *Geoscience and Man* 18: 131 – 137.

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulcke, F. Pesticides and Earthworms. A Review. 2013. *Agronomy for Sustainable Development* 34(1): 199 – 229.

Ponnamperuma, F.N. 1984. Effects of Flooding on Soils. *In*: Flooding and Plant Growth. London; Academic Press, Inc.

Porter, W.M., Cox, W.J., Wilson, I. 1980. Soil Acidity: Is it a problem in Western Australia? *Journal of the Department of Agriculture, Western Australia, Series 4* 21(4): 126 – 133.

Refsgaard, J.C., Christensen, T.H., Ammentorp, H.C. 1991. A Model for Oxygen Transport and Consumption in the Unsaturated Zone. *Journal of Hydrology* 129(1-4): 349 – 369

Rizhiya, E., Bertora, C., van Vliet, P.C.J., Kuikman, P.J., Faber, J.H., van Groenigen, J.W. 2007. Earthworm Activity as a Determinant for N_2O Emission from Crop Residue. *Soil Biology and Biochemistry* 39(8): 2058 – 2069

Roots, B.I. 1956. The Water Relations of Earthworms: II. Resistance to Desiccation and Immersion, and Behaviour when Submerged and when Allowed a Choice of Environment. *Journal of Experimental Biology* 33(1): 29 – 44

Sahrawat, K.L. 1998. Flooding Soil: A Great Equalizer of Diversity in Soil Chemical Fertility. *Oryza* 35(4): 300 – 305

Sahrawat, K. L. 2005. Fertility and Organic Matter in Submerged Rice Soils. *Current Science*, 88: 735-739.

Scheu, S. 1987. Microbial Activity and Nutrient Dynamics in Earthworm Casts. *Biology and Fertility of Soils* 5(3): 230 – 234

Schmidt, O., Curry, J.P., Hackett, R.A., Purvis, G., Clements, R. O. 2001. Earthworm Communities in Conventional Wheat Monocropping and Low-Input Wheat-Clover Intercropping Systems. *Annals of Applied Biology* 138: 377 – 388

Schütz, H., Seiler, W., Conrad, R. 1989a. Processes Involved in Formation and Emission of Methane in Rice Paddies. *Biogeochemistry* 7(1): 33 – 53

Schütz, H., Holzapfel-Pschorn, A., Conrad, R., Rennenberg, H., Seiler, W. 1989b. A 3-Year Continuous Record on the Influence of Daytime, Season, and Fertiliser Treatment on Methane Emissions Rates from an Italian Rice Paddy. *Journal of Geophysical Research: Atmospheres* 94(D13): 16405 – 16416

Sexstone, A.J., Revsbeck, N.P., Parkin, T.B., Tiedje, J.M. 1985. Direct Measurements of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society of American Journal* 49(3): 645 – 651

Sheppard, S.K., Lloyd, D. 2002. Effects of Soil Amendment on Gas Depth Profiles in Soil Monoliths using Direct Mass Spectrometric Measurement. *Bioresource technology* 84(1): 39 – 47

Singer, A.C., Jury, W., Luepromchai, E., Yahng, C.-S., Crowley, D.E. 2001. Contribution of Earthworms to PCB Bioremediation. *Soil Biology and Biochemistry* 33(6): 765 – 776

Singh, R.P., Agrawal, M. 2002. Potential Benefits and Risks of Land Application of Sewage Sludge. *Waste Management* 28(2): 347 – 358

Sizmur, T., Hodson, M.E. 2009. Do Earthworms Impact Metal Mobility and Availability in Soil? – A Review. *Environmental Pollution* 157(7): 1981 – 1989

Takia, Y., Kamura, T. 1966. The Mechanism of Reduction in Waterlogged Paddy Soil. *Folia Microbiologica* 11(4): 304 – 313.

Tanji, K. K., Gao, S., Scardaci, S. C., Chow, A. T. 2003. Characterizing Redox Status of Paddy Soils with Incorporated Rice Straw. *Geoderma*, 114: 333-353.

Terry, R.E., Nelson, D.W., Sommers, L.E. 1979. Carbon Cycling During Sewage Sludge Decomposition in Soils. *Soil Science Society of America Journal* 43(3): 494 – 499.

Terry, R.E., Tate III, R.L., Duxbury, J.M. 1981. The Effect of Flooding on Nitrous Oxide Emissions from an Organic Soil. *Soil Science* 132(3): 228 – 232

Topp, E., Pattey, E. 1997. Soils as Sources and Sinks for Atmospheric Methane. *Canadian Journal of Soil Science* 77(2): 167 – 177

Towprayoon, S., Smakgahn, K., Poonkaew, S. 2005. Mitigation of Methane and Nitrous Oxide Emissions from Drained Irrigated Rice Fields. *Chemosphere* 59(11): 1547 – 1556

Tsadilas, C.D., Matsi, T., Barbayiannis, N., Dimoyiannis, D. 1995. Influence of Sewage Sludge Application on Soil Properties and on the Distribution and Availability of Heavy Metal Fractions. *Communications in Soil Science and Plant Analysis* 26(15 – 16): 2603 – 2619

Wijesekara, H., Bolan, N.S., Thangavel, R., Seshadri, B., Surapaneni, A., Saint, C., Hetherington, C., Matthews, P., Vithangae, M. 2017. The Impact of Biosolids Application on Organic Carbon and Carbon Dioxide Fluxes in Soil. *Chemosphere* 189: 565 – 573

Willén, A., Jönsson, H., Pell, M., Rodhe, L. 2016. Emissions of Nitrous Oxide, Methane and Ammonia after Field Application of Digested and Dewatered Sewage Sludge with or without Addition of Urea. *Waste and Biomass Valorization* 7(2): 281 – 292

Wolters, V. 2000. Invertebrate Control of Soil Organic Matter Stability. *Biology and Fertility of Soils* 31(1): 1 – 19

Ye, R., Doane, T.A., Morris, J., Horwath, W.R. 2015. The Effect of Rice Straw on the Priming of Soil Organic Matter and Methane Production in Peat Soils. *Soil Biology and Biochemistry* 81: 98 – 107

Zou, J., Huang, Y., Jiang, J., Zheng, X., Sass, R.L. 2006. A 3-Year Field Measurement of Methane and Nitrous Oxide Emissions from Rice Paddies in China: Effect of Water Regime, Crop Residue, and Fertiliser Application. *Global Biogeochemical Cycles* 19(2): 1 – 9

7. Conclusions

The primary aim of this thesis was to understand the interactions between earthworms, flooding, and sewage sludge. To attempt this, three key questions were posed at the end of Chapter 2.

1. Does regular flooding impact earthworm populations in arable and pasture soils differently, given the already low earthworm populations in arable soils?

2. Does increasing organic matter content, in the form of sewage sludge application, lead to faster rates of oxygen depletion when soils floods?

3. Are there species specific responses of earthworms to flooding? And, if so, how might these change with the application of sewage sludge to soil?

How the experimental chapters in this thesis answered each of these questions is summarised below.

7.1. Does regular flooding impact earthworm populations in arable and pasture soils differently?

Chapter 3 investigated the differences in earthworm populations in regularly flooded and rarely flooded regions of an arable and pasture field. The chapter found that earthworm populations were generally lower in crop soil than in field margin soil, and that populations of both were lower than those found in pasture sites. This is in agreement with the existing literature about earthworm populations, with a number of causes for the differences between earthworm populations in arable and pasture discussed in Sections 2.6 and 3.3, such as the crushing and mechanical damage caused to individuals by farming machinery and the low organic matter content in arable soils being unsuitable for supporting large earthworm populations. The earthworm population distributions discussed in this chapter were also in accordance with other existing literature: endogeic earthworms, such as *A. chlorotica* and *A. caliginosa* are among the most abundant in the UK, including in agricultural soil (See Sections 2.6 and 3.3), and are present in a variety of habitats, including grassland, field margins, and crop soils (Natural England, 2014).

In both sites, regular flooding led to a reduction of total earthworm abundance and biodiversity. In both sites, the absolute abundance of *A. chlorotica* was not impacted by flooding, while other species were reduced in their overall abundance with regular flooding. Across both field sites, this led to dominance by *A. chlorotica* in regularly flooded regions. This raised the question of whether there were characteristics of *A. chlorotica* which enabled it to better survive these regularly flooded regions than other earthworm species, and whether these characteristics were unique to *A. chlorotica* or shared by other earthworm species. This question was partially addressed in Chapter 5, where the oxygen requirements of three different earthworm species were assessed.

Within the arable field, the differences between the crop and margin populations were emphasised with increased flooding. This suggested that earthworm populations subject to the dual stresses of flooding and intensive soil use under agricultural conditions may not recover as well as populations not subject to the stress of agricultural activity following flooding events. As discussed in Section 2.3, both the intensity and occurrences of flooding in the UK are predicted to increase with climate change. Given that crop soils and regularly flooded regions are likely to be dominated by *A. chlorotica*, which seems to have higher resilience to flooding events, it may be the case that with increased flooding events, the already low presence of other ecotypes in arable soils (Stroud, 2019) is further reduced, leading to even lower earthworm diversity in regularly flooded, crop regions.

The work performed in this thesis suggests that while flooding does not affect the distribution of individual species differently between arable and pasture, earthworm populations subject to the dual stresses of arable soil use and flooding are lower than field margin or pasture soils subject to the same stresses.

7.2. Does increasing organic matter content, in the form of sewage sludge application, lead to faster rates of oxygen depletion when soils floods?

Chapters 4 and 6 examined the effects of sewage sludge addition on soil oxygen concentrations during a flooding event. Chapter 4 examined the changes in oxygen concentration that occurred when a number of soils of different characteristics and uses were flooded after different percentages of the maximum application rate of sewage sludge according to UK regulations (ADAS, 2014) were applied. Chapter 6 examined the fluxes of greenhouse gas emissions and soil oxygen concentration when mesocosms of farmland soil had various combinations of flooding, sewage sludge, and earthworms applied.

Chapter 4 found that, in the short term, there was an effect of the application of sewage sludge on the rate of oxygen concentration depletion in flooded soil. However, when the data were considered over a twenty-four hour time period, soil characteristics such as the soil organic matter content were more informative about the rates of oxygen depletion within the soil. Overall, oxygen concentrations in 600 ml beakers filled with soil depleted to levels below detection within ten hours, regardless of the quantity of sewage sludge applied.

Chapter 6 was performed over a longer time scale, with larger mesocosms of soil than the experiments in Chapter 4. However, oxygen levels within the flooded soils still depleted to below detectable levels within a period of seven days, with no difference between amended and unamended flooded soils. In the unflooded soils, there was high variability in the oxygen concentrations recorded in the mesocosms treated with sewage sludge and earthworms, with oxygen concentrations generally lower in these mesocosms than in the other unflooded mesocosms. This suggests that the addition of sewage sludge may lead to the formation of microsites of reduced oxygen in unflooded soil, perhaps due to the respiration of soil microbes. As discussed in Section 6.2, earthworms contribute to increased microbial populations in soil, and in the mesocosms treated with both earthworms and sewage sludge, this may have led to increased variability in the oxygen concentrations of the mesocosms.

Chapter 6 also investigated greenhouse gas fluxes from soils. Each of the three research elements of this thesis of earthworms, flooding, and sewage sludge are known to increase greenhouse gas emissions in isolation. By studying them in combination, insight could be gained into potential interactions between the three. The results indicated that floodwater suppressed emissions of all greenhouse gases except CO_2. Once surface water was drained from the mesocosms, but the soil remained saturated, fluxes of CH_4 began to increase from soils containing sewage sludge, earthworms, and a combination of the two over a two week period.

The work performed in this thesis suggests that, while there is an effect of the addition of sewage sludge to soils, the impact it has on rates of oxygen depletion is limited. This is likely due to the recalcitrant nature of the organic matter in sewage sludge.

7.3. Are there species specific responses of earthworms to flooding? And, if so, how might these change with the application of sewage sludge to soil?

The experiments performed in Chapter 5, investigating the oxygen requirements of three earthworm species belonging to different ecotypes, aimed to understand and explain the fieldwork observations made in Chapter 3, and to contextualise these observations given the results obtained in Chapter 4. As discussed in Section 7.2, Chapter 4 only found an effect of sewage sludge application on the rate of oxygen concentration depletion in the very short term period immediately after flooding, likely due to the recalcitrant nature of the organic matter content of sewage sludge discussed in Section 4.5.5.

In Chapter 5, the absolute oxygen concentrations at which earthworms died were posited to be, in the case of *L. terrestris* and *L. castaneus,* highly linked to the lifestyle of the ecotype. *L. terrestris,* a largely sedentary, anecic earthworm, required a lower overall oxygen level to survive than *L. castaneus,* a surface foraging, epigeic earthworm. In flooded conditions, therefore, while the high motility of *L. castaneus* on the soil surface would be advantageous to escape flooded conditions, the oxygen concentrations in the flooded soil would drop below lethal levels sooner than for the soil dwelling *L. terrestris*. As discussed in Sections 5.5.4 and 5.5.5, the continued persistence of epigeic species in the regularly flooded sites of the pasture and field margins is likely more due to a high reproductive output and rapid recolonization strategy than survival in flooded events. The results for *A. chlorotica* indicated any lifestyle characteristics that could be linked to *r* or *K* persistence strategies were overridden by an aestivation response. The experiments showed that individuals of *A. chlorotica* survived low oxygen, flooded conditions for twelve days with no earthworm deaths. The high proportion of the population composed of *A. chlorotica* in the regularly flooded regions of the arable and pasture fields can likely be attributed to a number of factors: their ability to respond to extreme soil conditions by entering a state of aestivation, and their small body size allows them to avoid crush damage from farmland machinery in arable soils (see Sections 2.6, 3.3, and 7.1).

In Chapter 6, the experiments were designed to more closely mimic flooded field conditions than in Chapters 4 and 5. In this chapter, only individuals of *L. terrestris* were used, for reasons discussed in Section 6.3.1. The oxygen concentration monitoring performed in Chapter 6 indicated that oxygen concentrations in the flooded soils dropped below detectable levels within seven days. However, when the mesocosms were deconstructed, only two earthworm individuals out of sixteen subject to flooding treatments had died. This was attributed to earthworm behavioural responses, where individuals ‘snorkelled’, maintaining portions of their body on the soil surface, or emerged onto the soil surface. While earthworm burrowing and foraging behaviour was presumably disrupted by these behaviours, there was no difference in earthworm biomass or the mass of casting material produced by the earthworms between flooded and unflooded treatments. This suggests that earthworm behaviour may have a strong effect on their survival and persistence in flooded soils. Earthworms famously emerge from soil after heavy rainfall, a behaviour recorded by Charles Darwin in his 1881 work “The Formation of Vegetable Mould…”. While the reasons for this emergence are disputed, as discussed in Section 2.6, with some attributing emergence to escaping low oxygen conditions and others to the facilitation of movement and migration on the soil surface that wet conditions allow (Butt and Nuutinen, 2005), there is a clear behavioural response of earthworms to flooded soil conditions. The survival of individuals of *L. terrestris* in flooded soil observed in Chapter 6, therefore, is not surprising, and suggests that the low presence of anecic earthworms in regularly flooded areas is more likely due to their emergence from burrows during flooding events, leaving them vulnerable to predation and potentially disrupting foraging activity, than the death of individuals due to low oxygen conditions within flooded soils.

The work performed in this thesis suggests that there are species specific related responses to flooding, with species exhibiting different behavioural responses to flooded conditions: *A. chlorotica* curled up and appeared to aestivate, while *L. terrestris* exhibited ‘snorkelling’ behaviour and evacuation to the soil surface. With little effect of the addition of sewage sludge on the rates of oxygen depletion in flooded soil, the addition of sewage sludge to soil likely has no effect on earthworm survival or responses to flooding events.

7.4. Concluding remarks

This thesis has resulted in a number of novel findings. By comparing the regularly flooded arable and pasture soils, the field work found that earthworm communities subject to the dual stresses of intensive land use and flooding were reduced in comparison to earthworm subject to the stresses in isolation. The study examining the survival of earthworms in flooded conditions found that earthworms able to aestivate exhibited the behaviour as a stress response to low oxygen conditions; while Zorn et al. (2008), found that individuals of *A. chlorotica* were removed from flooded columns in a quiescent state, this study has been the first to provide proof that the aestivation behaviour was directly in response to low oxygen, flooded conditions. The two experiments examining the interactions of flooding and sewage sludge have shown that, counter to the expected responses, the addition of organic matter to soil in the form of sludge had no effect on the rate of oxygen depletion when the soil was flooded. The experiment examining greenhouse gas emissions found that flooding of sludge amended soil had no effect on greenhouse gas emissions, but that the interactions of sludge and earthworms led to emissions of N_2O similar to those observed when soil was amended with animal waste (Saggar et al., 2009, in Muñoz et al., 2010).

This thesis has demonstrated that flooding events reduce earthworm populations, and the addition of flooding stress to low earthworm populations in farmland soils results in reduced abundance and diversity. The addition of sewage sludge to these soils will likely have little impact on these already reduced populations. The most abundant earthworms in UK soils, *A. chlorotica*, are those which are able to withstand flooding stress through a stress response previously believed to be associated with extremes of soil temperature (Evans and Guild, 1947) or very dry soil conditions (Edwards and Lofty, 1972). The findings from Chapters 3 and 5 indicated that increased flooding may lead to reductions in the populations of both epigeic and anecic earthworms in regularly flooded arable fields. The effects that this may have on soil health and crop production need further investigation, with earthworms playing a key role in soil health. However, the effects that increased flooding may have on earthworm populations are likely less impactful on soil health than current farming practices. A study in 2019 found that 42% of UK arable fields were overworked, with very low presences of epigeic and anecic earthworms (Stroud, 2019), with tillage leading to reduced earthworm populations in arable soils (Birkás et al., 2004; Stroud, 2019). When considering the context of this research, increasing flooding is likely to exert less stress on earthworm populations than intensive soil uses.

The work performed in this thesis suggests that, when considering only the interaction between sewage sludge and flooding, no changes to the policy of sewage sludge applications are needed; the application of sewage sludge likely does not pose a greater risk to earthworm communities in regularly flooded regions. However, the key finding of Chapter 6 was the high level of N_2O flux from unflooded soils amended with both earthworms and sewage sludge. This was unexpected: while earthworms are known to increases the emissions of N_2O from soil, the combination of earthworms and sewage sludge unexpectedly led to higher N_2O fluxes than either factor in isolation. As discussed in Section 6.5.2, the increases in denitrifying bacteria caused by earthworms and the low C:N ratio of the sewage sludge as a soil amendment likely contributed to these high fluxes. 79% of sewage sludge in the UK was applied to farmland in 2010 (DEFRA, 2012), with a report by the Air Quality Expert Group (AQEG) in 2018 indicating that the uses of nitrogen fertilisers and livestock wastes in agriculture accounted for 80% of UK N_2O emissions (AQEG, 2018). As a greenhouse gas with a potential residence period of over 100 years in the atmosphere (Ko et al., 1991; Prather et al., 2015), higher emissions of N_2O with soil amendment practices may be of concern in the context of anthropogenic climate change. It may be the case that further work is needed to examine the UK practice of the addition of sewage sludge to agricultural soils from a greenhouse gas emissions policy perspective.

7.5. Limitations of the experiments

While the experiments described in Chapters 4, 5, and 6 each addressed some aspects of the questions posed at the end of the literature review, there were limitations to each experiment. Across the whole thesis, perhaps the key limitation of all the experiments was the small scale of the laboratory experiments. While these allowed control of many environmental factors, they did not necessarily represent what may occur in soils amended with sewage sludge in the field.

In Chapter 4, the sewage sludge amendments to soil were air dried, milled, and hand mixed through air dried soil. While this reduced variation and increased reproducibility of the experiment, it did not represent field applications of sewage sludge, where sludge would applied wet to field wet soil and mechanically mixed into the soil. This limitation was partially addressed in Chapter 6, where field wet sludge was hand mixed through field wet soil.

In Chapter 5, the experiments only investigated three species of earthworm. The experiment was not intended to mimic field conditions, but instead to understand the oxygen requirements of earthworm species which may drive behaviour responses. The experiment did also not vary light levels to allow for diurnal rhythms of earthworms due to limitations in the equipment availability, and to reduce variability between the CT room and CT chamber.

In Chapter 6, the attempt to mimic drying conditions after a flooding event in the laboratory were unsuccessful. Although this led to insight into gas emissions, particularly of CH_4, from soil that was still saturated but without a cushion of floodwater to absorb CH_4 emissions, this was not the planned experimental design. There were limits to the quantifiable measurements of earthworm burrowing behaviour, with only measures of casting activity and burrow openings counted. The experiment also only considered one species of earthworm. While this species was chosen for a number of reasons, such as its ease of procurement through commercial suppliers and its creation of large, permanent burrows to the soil surface, it is not a species that typically persists at high population densities in arable soils.

7.6. Further work

There is the potential for further work derived from each of the experimental chapters.

Section 4.5.5 describes further work to address the limitations of the sewage sludge application methods used in Chapter 4, which was partly resolved through the use of larger mesocosms in Chapter 6. Section 4.5.5 also discusses the need for investigation into soils which have had multiple, sequential applications of sewage sludge under various crop types or land uses.

Section 5.5.6 discusses the need for further work investigating the oxygen requirements for other earthworm species, to see if similar responses occur within ecotypes, and for further investigation into the effect of flooded conditions on earthworm lifecycles, cocoon production, and time to maturity. The section also discusses how further work could involve choice experiments for different earthworm species between flooded and unflooded soils, to examine species specific responses to flooded soil when escape is available. Section 5.5.6 also discusses the need for further experimentation to address the limitations of the experiment, which did not account for diurnal cycles of earthworm behaviour. Although not discussed in Section 5.5.6, further experiments could examine the survival time of earthworms when the solution is maintained at low oxygen concentrations. This experiment was not performed during this thesis, as the oxygen concentration depletion rates observed in the flooded soil in Chapters 4 and 6 suggested that soil was unlikely to remain at low oxygen concentrations, and instead tended rapidly to deplete to levels below detection.

Section 6.5.4 discusses the potential for an increased drying period, to counter the limitation of the soil remaining saturated following the removal of floodwater. Section 6.5.4 also discusses experimental changes to address some of the limitations of the experiment, such as the potential uses of technology to better understand the extent of earthworm burrowing, and the use of multiple earthworm species to better mimic field populations of earthworms.

When considering the thesis as a whole, further work is needed to examine the three elements of earthworms, flooding, and sewage sludge together. The impacts of sewage sludge amendment on soil properties are well understood, but this thesis suggests interactions between the changes earthworms induce in soil and sewage sludge, with N_2O fluxes highest in soils treated with both earthworms and sewage sludge. The flooding events simulated in this thesis were one off, extreme events in laboratory conditions. The natural progression for future work from this thesis, beyond those discussed at the end of each chapter, would be field scale studies. These studies would aim to understand how sewage sludge amendment of soils impacts earthworm populations, and whether the recovery of these populations following flooding events differs from population recovery in unamended soils. Further work into greenhouse gas emissions from these amended, flooded, field soils may also provide insight into whether current practices of sewage sludge disposal as a soil amendment are suitable given the predicted increases in UK flooding with climate change.

7.7. References

ADAS, 2014. Biosolids Nutrient Management Matrix. Leaflet. [Accessed online 22/03/2019. Available at http://www.adas.uk/Downloads/Biosolids_Nutrient_Management_Matrix.pdf].

Air Quality Expert Group (AQEG). 2018. Air Pollution from Agriculture. Report prepared for DEFRA. Available at https://uk-air.defra.gov.uk/assets/documents/reports/aqeg/2800829_Agricultural_emissions_vfinal2.pdf [Accessed online 19/09/2019]

Birkás, M., Jolánkai, M., Gyuricza, C., Percze, A. 2004. Tillage Effects on Compaction, Earthworms, and Other Soil Quality Indicators in Hungary. Soil and Tillage Research 78(2): 185 – 196

Butt, K.R., Nuutinen, V. 2005. The Dawn of the Dew Worm. *Biologist* 52(4): 218 – 223

Darwin, C. 1881. The Formation of Vegetable Mould, Through the Action of Worms: With Observations on their Habits Illustrated Edition. Milton Keynes: Dodo Press

DEFRA, 2012. Waste Water Treatment in the United Kingdom – 2012. Implementation of the European Union Urban Waste Water Treatment Directive – 91/271/EEC. [Leaflet]

Edwards, C.A., Lofty, J.R., 1972. Biology of Earthworms. London: Chapman and Hall, P. 96 – 97.

Evans, A.C., Guild, W.M. 1947. Studies on the Relationships between Earthworms and Soil Fertility: I. Biological Studies in the Field. *Annals of Applied Biology* 34(3): 307 – 330

Muñoz, C., Paulio, L., Monreal, C., Zagal, E. 2010. Greenhouse Gas (CO_2 and N_2O) Emissions from Soils: A Review. *Chilean Journal of Agricultural Research* 70(3): 485 – 497

Natural England, 2014. Earthworms in England: Distribution, Abundance and Habitats (NECR145)

Ko, M.K.W., Sze, N.D., Weisenstein, D.K. 1991. Use of Satellite Data to Constrain the Model-Calculated Atmospheric Lifetime for N_2O: Implications for Other Trace Gases. *Journal of Geophysical Research: Atmospheres* 96(D4): 7547 – 7552

Prather, M. J., Hsu, J., DeLuca, N.M., Jackman, C.H., Oman, L.D., Douglass, A.R., Fleming, E.L., Strahan, S.E., Steenrod, S.D., Søvde, O.A., Isaksen, I.S.A., Froidevaux, L., Funke, B. 2015. Measuring and Modelling the Lifetime of Nitrous Oxide Including its Variability. *Journal of Geophysical Research: Atmospheres* 120(11): 5693 – 5705

Stroud, J.L. 2019. Soil Health Pilot Study in England: Outcomes from an On-Farm Earthworm Survey. *PLoS ONE* 14(2)

Zorn, M. I., Van Gestel, C. A. M., Morrien, E., Wagenaar, M., Eijsackers, H. 2008. Flooding Responses of Three Earthworm Species, *Allolobophora chlorotica, Aporrectodea caliginosa* and *Lumbricus rubellus*, in a Laboratory-Controlled Environment. *Soil Biology & Biochemistry,* 40: 587-593

A1 Method Development

The methods described in Chapter 4, 5 and 6 are the final methods used for the experiments. This chapter details the preliminary experiments used to develop these methods for each chapter.

A1.1 Chapter 4: The availability of oxygen in flooded soil amended with sewage sludge

This chapter examined the broad hypothesis that the addition of sewage sludge to soil leads to faster rates of oxygen depletion when flooded than in unamended soils.

Anaerobically digested sewage sludge was collected from the Esholt water treatment works in West Yorkshire (postcode BD10 0TW). A small sample was dried at 105°C for twenty-four hours and ground. The sewage sludge sample Carbon and Nitrogen content was determined using a Vario Macro C/N Analyser. By determining the nitrogen content of the dried sludge, and by knowing the loss of water that occurred when drying the sludge for analysis, the application rate of wet sludge per hectare could be calculated in order to apply 250 kg of nitrogen; the maximum application rate in the UK (ADAS, 2014). The application rate per hectare could then be scaled down based on the surface area of the beakers. The soil used in the first pilot studies was collected from Spen Farm, near Leeds, as part of another research project. The soil had been collected during earthworm sampling for another experiment and brought back to the laboratory for hand sorting and earthworm identification.

For the preliminary test, sixteen glass beakers of capacity 600 ml were each prepared with two Presens Oxygen Sensitive Spots on opposite sides (a detailed description of the Presens sensor spots can be found in Section 4.3.3). With a diameter of 9.4 cm, the surface area of the soil in the beakers was approximately 69.4 cm^3, or 6.939×10^{-7} Ha. Four of the beakers were filled to a depth of 20 cm with the sample soil; a weight of approximately 500 g of soil. Treating the 20 cm depth of the soil as an active layer, 10 g of sludge was thoroughly mixed into partially dried soil. This 10 g was at the time estimated to represent 100% of the 250 kg N application rate. The fourth beaker was filled only with the soil. The beakers were then flooded with 300 ml deionised water to simulate a flooding event. On the first day, the percentage saturation of oxygen within the beakers was recorded at 10 am, 12 noon, 2 pm and 4 pm. The following day the oxygen percentage saturations were recorded again at 10 am, 12 noon, 2 pm and 4 pm, and the final recording being made at 9 am on the third day.

This preliminary test showed that the oxygen within the unamended soil depleted over this three day period, while the oxygen content in the amended soil appeared to deplete much more rapidly, dropping to very low levels by 9 am on the second day. This lead to the assumption that three days of sampling would be sufficient to accurately capture the rates of oxygen depletion.

However, while reviewing the data from the preliminary tests, it emerged that errors had been made when attempting to calculate the rate of sludge application to the beakers. The sludge had been dried for 24 hours at 105°C. Drying high organic matter samples at high temperatures can lead to a loss of organic matter (Jager and Bruins, 1975), so may have resulted in a lower nitrogen percentage returned than was truly present in the sample. To determine if this was the case, another sample of the sludge was air dried in the laboratory. The sludge was weighed before and after drying in order to determine water loss from the air drying method. The dried sludge was ground in a ball mill and triplicate samples analysed using the Vario Macro C/N analyser.

The calculation used to determine the nitrogen content of the sample was also revisited and corrected. The calculation used to determine the N content of the sludge was as follows:

The dilution factor for the dry weight was calculated as the wet weight of the sample divided by the dry weight, giving a value of 3.0747. In order to determine the percentage N of wet weight, the percentage N of the dry weight was divided by the dilution factor. 4.156% N divided by the dilution factor gave a 1.35% N content in the wet sludge. This means that a metric tonne of the wet sludge would contain 0.0135 tonnes of Nitrogen, or 13.5 kg.

In order to then determine how many tonnes of wet sludge would need to be applied to a hectare to obtain 250 kgN/Ha, 250 divided by 0.0135 gave a total of 18.5 tonnes of sludge per hectare. This value, multiplied by the surface area of the beakers (6.939×10^{-7} Ha) gave a total sludge rate of 12.8 g of sludge per beaker in order to achieve the maximum application rates. Lower rates of application was subsequently achieved at 50% (6.4 g per beaker) and 25% (3.2 g per beaker).

Another factor considered following the preliminary test was the state of the partially dried soil. As the soil had been collected some time before the experiment and hand-sorted for earthworms, the soil had formed into small aggregates, and was not necessarily representative of field soil. Due to the aggregation, it was decided that the soils used in the experiment would first be air dried, then hand ground using a pestle and mortar to break up large aggregates, before being rewetted to resemble field soil. This would also allow the microbial burst of activity that occurs when dried soil is rewetted (Stevenson, 1956) to occur without giving false readings of rapid oxygen depletion within the experiment itself. By treating all collected soils this way, variation between the test soils would also be reduced.

The next study used soil collected from Spen Farm near Leeds. The soil was air dried for approximately 7 days in the laboratory, then ground in a pestle and mortar to remove large aggregates. Large stones and any rubbish, such as pieces of plastic, were removed, but smaller stones (approximately 5 mm or smaller) were left in. A sample of air dried soil was retained for CN analysis. Following grinding, the soil was rewetted using 300 ml of water per kilogram of soil.

500 grams of the prepared soil were weighed out into a container. The container was large enough to allow thorough hand mixing of the sewage sludge into the soil, using a ‘rubbing in’ method. When the sludge was mixed into the soil, with no large aggregates of sludge or soil visible, the soil was transferred into the sensor spot equipped beakers. The soil was packed in sufficiently to prevent the formation of large air spaces and pores behind the sensor spots but not enough to prevent infiltration of the floodwater. Once prepared, the beakers were left for approximately 18 hours in a 15°C CT room.

At 9 am, the beakers were flooded with 300 ml of deionised water. Oxygen concentration (mg O_2 L^{-1}) readings were taken from the sensor spots and the surface water percentage oxygen saturation, oxygen concentration (mg O_2 L^{-1}) and temperature were recorded. Measurements continued every two hours until 7 pm the same day, and then resumed at 9 am the next day, concluding at 11 am if the soil water content had reached anoxic conditions.

Although this method successfully produced well mixed beakers of sewage sludge and soil, the results were very heterogeneous; the same beaker could produce very different readings at each of the two sensor spots. While soil itself is unlikely to be a homogenous environment, this meant that reproducibility of the results was low.

Two points in the method were identified that could be altered to allow better reproducibility of results. The first change was to the preparation of the soil. Following grinding of the soil in the large pestle and mortar, the soils were to be sieved to 2 mm. This allowed removal of large stones and litter, and large aggregates were returned to the pestle and mortar to be broken down again.

The second change was to the method of mixing the sewage sludge into the soil. To determine the most effect method of mixing the sludge into the soil, a small pilot study was conducted. The study compared the mixing of dried and ball mill ground sludge into dried and sieved soil, dried and ground sludge into rewetted soil, field wet sludge into dried and sieved soil, and field wet sludge into rewetted soil, using the same soil collected from Spen Farm. Duplicates of each treatment were produced, mixing the sludge and soil by hand in a separate container before their transfer to the sensor spot fitted beakers. The experiment was run over a twenty-four hour period, with oxygen measurements of the sensor spots taken every two hours. These measurements were compared to determine which combination of soil and sludge produced the most homogenous results across the two sensor spots.

Following the pilot study, it was decided that the method giving the most reproducibility was the combination of dried and ground sludge with dried and sieved soil. The method reported in Section 4.3 was performed following this pilot study.

A1.2 Chapter 5: Earthworm survival in water; the difference in oxygen requirements for an anecic, an endogeic and an epigeic species.

This chapter examined the broad hypothesis that earthworm species of different ecotypes would die at different oxygen levels when maintained in reconstituted groundwater for a period of time.

The first experiment designed was for fifteen individuals each of *L. terrestris*, an anecic earthworm, *A. chlorotica*, an endogeic earthworm, and *L. rubellus*, an epigeic earthworm.

Preliminary studies performed by placing an individual of each of the three earthworm species in a 50 ml centrifuge tube filled with reconstituted groundwater indicated that the time to earthworm death exceeded 36 hours. The reconstituted ground water (as described in Arnold et al., 2007) was an aerated solution of pH 8.2, with a composition of 100 mg L^{-1} $NaHCO_3$, 200 mg L^{-1} $CaCl_2$, 20 mg L^{-1} $KHCO_3$, and 180 mg L^{-1} $MgSO_4$ that had been maintained at 15°C.

Preliminary studies also found that, in a 100 ml beaker containing 50 ml of liquid maintained in the 15°C CT room, 1 ml was lost per 24 hour period. As this is equal to 2% of the volume of liquid per 24 hour period, future experiments would cover the beakers with a tin foil lid.

Fifteen adult individuals of *A. chlorotica* were prepared for depuration over the weekend in a 15°C controlled temperature (CT) room on the Friday, with damp blue roll to be changed 24 hours later on the Saturday, and 24 hours later again on the Sunday, to prevent re-ingestion of gut contents. However, at the blue roll changing, it was found that all individuals of *A. chlorotica* had escaped and died in the unfavourable conditions of the CT room. Following this find on the Saturday, adult individuals of *L. rubellus* were prepared for depuration at 15°C, and the moist blue roll changed on the Sunday. When depurating these earthworms, and in future experiments, the large petri dishes used to contain the earthworms and blue roll were sealed using masking tape, to prevent earthworms escaping through the narrow gap between the base and lid.

Following the 48 hour depuration period, fifteen weighed individuals of *L. rubellus* were placed in 50 ml of reconstituted groundwater in 100 ml beakers, and the total weight of the earthworms and beakers recorded. The beakers were randomly arranged in three rows of ten beakers; of these ten beakers, five were controls and five contained earthworms (Fig. A1-1). This was to control for environmental gradients in the 15°C CT room.

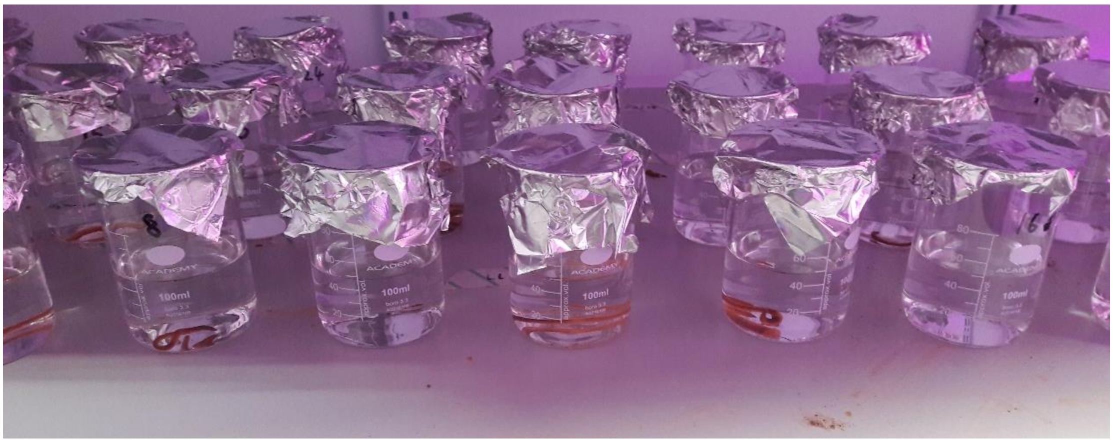

Figure A1-1. Individuals of *L. rubellus* in 50 ml reconstituted groundwater solution in 100 ml beakers in a 15°C CT room. Beakers were covered with a tin foil lid to prevent loss of solution through evaporation.

Once earthworms were placed in solution, the dissolved oxygen concentration of the water was measured using the Thermo Scientific Orion Star A223 and Star A23 Portable Dissolved Oxygen Meter (Fig. A1-2). Following the initial measurement at time zero, the dissolved oxygen measurement was taken twice a day at 9 am and 6 pm, with the intention of continuing measurements until earthworm death (defined as when earthworms do not respond to a gentle prodding with a sharp needle near the sensitive mouthparts (OECD, 1984). Between measurements, the probe was rinsed in deionised water and blotted to remove excess water.

Following earthworm death, the weights of the beakers and earthworms, and the individual weight of each earthworm, were to be recorded again.

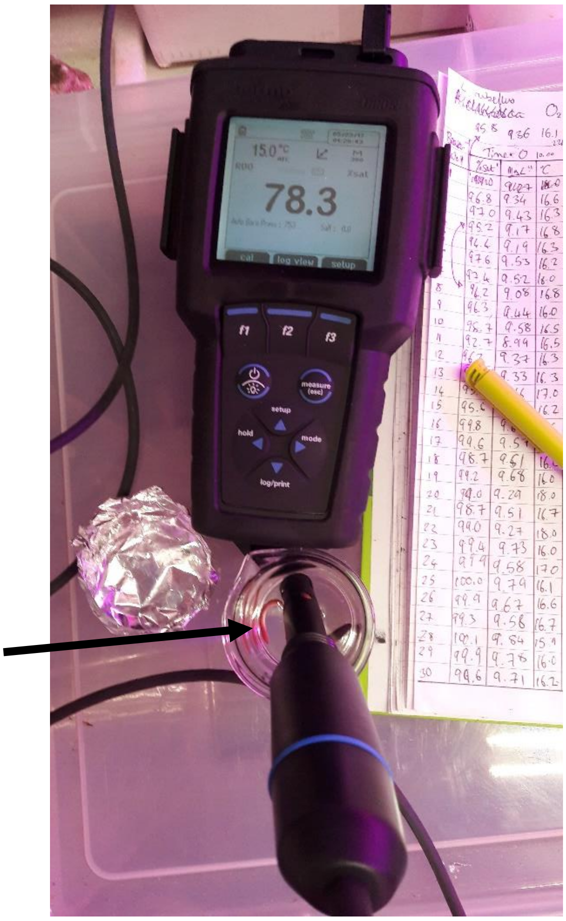

Figure A1-2. Recording the percent oxygen saturation (displayed), oxygen concentration (mg O_2 L^{-1}) and temperature of the reconstituted groundwater solution using the Thermo Scientific Orion Dissolved Oxygen probe. The 50ml of reconstituted groundwater in a 100 ml beaker contains an individual of *L. rubellus* (indicated with an arrow).

However, the length of survival far exceeded the preliminary studies. Individuals of *L. rubellus* survived over fourteen days in the solution, with very little reduction in dissolved oxygen. Of the fifteen earthworms used in the study, only two individuals died. As the reason for this low level of oxygen depletion was unclear, experiments were performed to determine whether there were more effective experimental setup for containing the reconstituted groundwater and earthworm.

For this experiment, eight adult individuals of each experimental earthworm species (*L. terrestris, L. rubellus* and *A. chlorotica)* were depurated on moist blue roll at 15°C for 48 hours. The earthworm weight, width and length were recorded. Earthworm width and length allowed for the calculation of approximate earthworm surface areas by assuming that the earthworms were cylindrical in shape.

Two individuals of each earthworm species were then used for each of the four potential experimental treatments. These treatments were: 50 ml of reconstituted groundwater in a 100 ml beaker; 40 ml of reconstituted groundwater in a 100 ml beaker; 50 ml of reconstituted groundwater in a 50 ml centrifuge tube; and 40 ml of reconstituted groundwater in a 50 ml centrifuge tube. The centrifuge tubes were used as an alternative to the beakers as their tight screw tops and lower diameter compared to the beakers provided a reduced surface area for oxygen diffusion into the solution. Two controls of each treatment were also carried out without earthworms. Before the earthworms were placed into the containers, the weights of the containers, and the weights of the container with the reconstituted groundwater were also recorded. In order to prevent loss of liquid through evaporation, the beakers were covered with a tin foil lid, and the centrifuge tubes were sealed with their screw-top lids. The beakers and tubes were maintained in a 15°C CT room.

Once earthworms were placed in solution, the dissolved oxygen concentration of the water was measured using the Orion portable dissolved oxygen meter. Following the initial measurement at time zero, the dissolved oxygen concentration was taken every 24 hours until earthworm death, following the same method as described above.

All individuals of *L. terrestris* bar one died within the first 24 hours, with the last individual, in 40 ml of liquid in a 100 ml beaker, dying within 48 hours. Mortality rates for the other earthworm species were low in the beakers, with only one individual of *L. rubellus* dying by day fourteen. In the same time period, three of the four individuals of *L. rubellus* within the tubes had died. In both the tubes and the beakers, there were no deaths of individuals of *A. chlorotica*. The lack of deaths of individuals of *A. chlorotica* was, at the time, attributed to the low body size of individuals compared to individuals of *L. terrestris*.

Between the four combinations of container and volume of liquid, it was decided that the 40 ml of reconstituted groundwater in the centrifuge tubes was the most effective combination. The 50 ml of reconstituted groundwater was judged unsuitable, as large quantities of water were displaced when taking measurements with the probe.

When considering the difference in earthworm consumption of different species, it was evident that measuring every 24 hours, while suitable for individuals of *L. rubellus* and *A. chlorotica*, did not sufficiently represent the changes in oxygen concentration for *L. terrestris*. This was because most individuals of that species were found to be dead within the first 24 hours, so provided only two data points to represent the changes in oxygen concentration. A brief experiment with three individuals in 40 ml of solution in 50 ml centrifuge was performed to determine the best way to measure the oxygen consumption over a 24 hour period. Hourly measurements between 8 am and 6 pm, and again the following morning at 8 am, were taken, to determine if this would be sufficient to capture the oxygen consumption. With two test individuals dying within the first ten hours, this was accepted as the intervals of measurements for *L. terrestris* alone, while *L. rubellus* and *A. chlorotica* would be measured at 24 hour intervals.

Following research into soil temperature fluctuations, it was decided that the 15°C CT room represented too great a temperature for UK soils, as this seemed to be more representative of summer temperatures, when earthworms are likely to be aestivating. To counter this, a 10°C CT chamber was sourced for future experiments.

Ten adult individuals of *L. terrestris* were depurated for 48 hours on moist filter paper at 10°C in a CT chamber. Following recording of their weights, lengths and widths, these individuals were then placed into sealable centrifuge tubes containing 40 ml of reconstituted groundwater. An equal quantity of control tubes were prepared, containing 40 ml of reconstituted groundwater, and their weights recorded.

Once earthworms were placed in solution, the dissolved oxygen concentration of the water, the percentage saturation, and the water temperature was measured using the Orion portable dissolved oxygen meter. Following the initial measurement at time zero, the dissolved oxygen concentration was to be taken hourly until earthworm death, following the same method as described above. Tubes not being measured were kept in the 10°C CT chamber.

Unlike the experiments where the earthworms were maintained at 15°C, where the earthworms died within 24 hours, the earthworms in this instance did not die within a four day period. There were a number of potential causes for this earthworm survival. Regular removal of the tubes from the CT cabinet in order to record measurements meant that the temperature of the tubes rose from 10°C to approximately 12°C, and never cooled. Slow probe equalisation may have provided enough time for oxygen to diffuse back into the solution, while the closing and opening of the screw top of the centrifuge top trapped more air into the tube, which could then diffuse back into the water over the hour interval between measurements. The length of the earthworms, accompanied with accidental displacement of water, meant that individuals were able to keep segments of their bodies above the level of the water, effectively ‘snorkelling’. As the skin is the earthworm organ of oxygen exchange (Edwards and Lofty, 1977), this meant that individuals were able to maintain respiration outside of the limited oxygen environment of the reconstituted groundwater. While this may not be a factor in earthworm survival for individuals of smaller species, such as *A. caliginosa* and *L. rubellus,* it indicated that the method needed adapting in order to be suitable for *L. terrestris*.

One method attempted to prevent the earthworms keeping segments of the body above the water and supported on the side of the centrifuge tubes was to line the upper 4 cm of the tubes with sticky backed Velcro. This would provide an uncomfortable surface for the earthworms to use for support, and prevent their prolonged survival. A test was carried out with two individuals of *L. terrestris* under observation for behavioural responses, and it appeared as if the method would be successful. Both individuals of *L. terrestris* died within 24 hours. However, during this test the tubes were not reopened and closed to simulate the experimental method.

The same method as above was carried out, with ten depurated individuals of *L. terrestris* and ten control centrifuge tubes maintained at 10°C, with the upper 4cm of both control and test centrifuge tubes lined with the Velcro. As before, measurements of dissolved oxygen percentage saturation, milligrams per litre and the temperature were taken hourly. However, after approximately three days, the earthworms were still alive.

This led to the conclusion that the key difference between the majority of the smaller preliminary tests and the experiments was the regular opening and closing of the tubes to take the measurements. Opening the tubes exposed the water to more oxygen, and the closing of the tubes again sealed oxygen back into the tubes, which was then able to diffuse into the reconstituted groundwater over the following hour. When oxygen levels dropped in the reconstituted groundwater to sufficiently low levels, this refreshing of the oxygen in the tubes was enough to extend earthworm survival.

The next proposed method was to use a sacrificial replicates system. A pilot study was performed to test the feasibility of the new method, and to see how long the earthworms were likely to survive, as this would dictate how many earthworm replicates would be needed.

Nine individuals of adult *L. terrestris* were depurated overnight at 10°C. At the same time, nine centrifuge tubes were filled to the brim with reconstituted groundwater. The next morning, the earthworm individuals were weighed, their lengths and widths recorded, and the grams of water displaced when the earthworm individuals were placed in the tube recorded.

All earthworms were inserted into the tubes at the same time. At the first time point, eight hours since insertion for the pilot test, three tubes containing earthworms were selected. The dissolved oxygen percent saturation, the milligrams of oxygen per litre, and the temperature of the reconstituted groundwater were recorded. The tubes were emptied, and the earthworms were tested to see if they were alive, and their weights and measurements recorded. The same process was repeated 24 and 48 hours later.

The sacrificial replications method described above was completed using five tubes per timepoint, at timepoints +0 hours since immersion, +3, +6, +9, +24, +33., and so on twice daily until +96 hours. Earthworms reached 100% mortality after 33 hours, but the experiments were continued until all twenty-four earthworms had been sampled.

As the sacrificial replicates method was successful, a control design was needed to ensure that earthworm deaths were occurring due to oxygen depletion, rather than the conditions of being maintained in the centrifuge tubes. Seven holes of diameter approximately 3mm were drilled into the top of the control tubes to allow oxygen exchange with the water surface. Three treatment test tubes and three control test tubes were taken at each of the eight timepoints; +0, +3, +6, +9, +24, +33, +48 and +72 hours following immersion. Following measurements of percentage saturation of oxygen, milligrams of oxygen per litre, and the temperature of the solution, and the pH of the surrounding water was recorded. When the oxygen content of the control tubes was measured, it emerged that the holes in the lids were insufficient for oxygen transfer and the oxygen in the control tubes still depleted, resulting in earthworm death after approximately 48 hours.

As the rates of oxygen depletion in the treatment tubes was very similar to that observed in the first full study using the sacrificial replication method and the pH had been recorded, it was decided that the only element of the experiment that needed repeating was the controls. Lengths of tubing of approximately 2.5 mm internal diameter were inserted into the lid holes from the initial controls attempt. These were attached to a peristaltic pump (Fig. A1-3). An initial test of this setup using an un-depurated individual of *L. terrestris* found that the oxygen content of the tube settled at approximately 75% oxygen saturation when left unmeasured for a period of 48 hours. Following this initial test, 24 individuals were depurated at 10°C, before being placed in filled centrifuge tubes that were connected to the peristaltic pumps. Due to the size and the shape of the pumps, the entire setup could not be maintained at 10°C, and instead were transferred to a 15°C CT room.

Figure A1-3. 50 ml centrifuge tubes, completely filled with reconstituted groundwater and an individual of *L. terrestris*, attached to a peristaltic pump in a 15°C CT room. The plastic tubing pumped ambient air into the tubing, and provided the control earthworm individuals with oxygen. As the pump needed to be connected to the electrical supply, and due to the size of the pump, the setup could not be maintained in a CT chamber.

This method successfully allowed for the survival of control individuals of *L. terrestris*, and was adopted for future work with other species. The experiment was performed again with forty-eight individuals of *A. chlorotica*, collected from Spen Farm, near Leeds. Twenty-four individuals were used in the treatment tubes, and twenty-four in the treatment tubes. Measurements were taken at timepoints +0, +9, +24, +36, +48, +72, +96 and +120 hours after immersion.

However, at the +120 hours timepoint no individuals of *A. chlorotica* had died. The experiment was repeated, using the same methods as described above, but the interval between the timepoints was increased. This was to allow for the much smaller size of individuals of this species compared to *L. terrestris*. When the measurement timepoints were increased to +0, +9, +24, +48, +96, +144, + 216 and +288 hours after immersion, and no individuals died, it was decided that this may be due to the species’ behaviour rather than any problems with the experimental methods.

The experiment was performed for a third time with individuals of *L. castaneus* collected again from Spen Farm. Measurements were taken at the time intervals of +0, +9, +24, +48, +72, +120, +168 and +288 hours following immersion.

The method reported in Chapter 5 is the method used for the final experiments of individuals of *L. terrestris*, *A. chlorotica* and *L. castaneus.*

A1.3 Chapter 6: The effect of flooding on greenhouse gas emissions from sewage sludge amended, earthworm populated mesocosms

This chapter examined the broad hypothesis that greenhouse gas emissions from soil would vary based on treatments of sewage sludge addition, earthworm addition, and flooding.

The experimental mesocosms were 1800 ml ‘Lock and Lock’ plastic containers. These were to be filled with air dried, ground, and rewetted soil collected from the Spen Farm site. Field wet sewage sludge, calculated to be applied at the maximum application rate of 250 kg N ha^{-1} (ADAS, 2014), was to be hand mixed through the rewetted soil. As the experiments described in Chapter 4 had focused on creating a reproducible system for taking oxygen readings across the beakers, this experiment aimed to better replicate field conditions of sludge application, and so the sludge was not air dried and ground before mixing into the soil. As well as soil, two lengths of transparent plastic tubing, at depths of 11 cm and 5 cm respectively, were to be placed in the mesocosms. These tubes had Presens oxygen sensor spots, as used and described in Chapter 4, at their bases, to allow measurements of the dissolved oxygen content of the soils with minimal disturbance. Earthworms were to be added to the mesocosms at the time of setup, to allow some turnover of the soil before measurements began.

The method of taking measurements at the base of the transparent tubes was tested before the experiment took place. As it was difficult to accurately place the fibre optic cable over the spots at the base of the tubes, the cable was stiffened by attaching it to a piece of wire. This allowed better placement of the end of the cable, allowing easier measurements of the dissolved oxygen concentration within the mesocosm soil.

As the measurement taking setup of connecting the ‘Lock and Lock’ containers to the ‘White Dwarf’ EGA61 Multi Channel Gas Exchange System, in turn connected to the Los Gatos Research (LGR) CO_2 and CH_4 and LGR N_2O analysers, had been successfully used in the past by other researchers at the institution, no changes to the existing experimental setup were required.

However, there were problems with the White Dwarf pumps. The pumps needed to draw oxygen over the soil surface at a flow rate of 350 μmol s^{-1} to allow accurate calculation of the greenhouse gas fluxes from the mesocosm contents. However, some of the pumps were failing to reach this flow rate, even after being sent off for repair.

After running blank mesocosms and changing the air filters and the tubing to ensure that there were no blockages leading to the low flow rates, it was suggested that at 7 metres the length of tubing through which the White Dwarf was drawing air may be too long, putting the pumps under pressure and leading them to fail. As this tubing length led from the mesocosms to the White Dwarf and then through the wall from the 15°C CT room into the adjacent laboratory, there were two points at which it could be reduced; between the mesocosms and the White Dwarf, and between the White Dwarf at the adjacent laboratory.

By rearranging the equipment in the CT room, the total tubing length for the setup was reduced to 3 m. Tests using blank mesocosms showed that the pumps were no longer failing, and the experiment continued as described in Chapter 6.

A1.4 References

ADAS, 2014. Sewage sludges Nutrient Management Matrix. Leaflet. [Accessed online 22/03/2019. Available at http://www.adas.uk/Downloads/Sewage sludges_Nutrient_Management_Matrix.pdf].

Arnold, R.E., Hodson, M.E., Comber, S. 2007. Effect of organic complexation on the toxicity of Cu to the earthworm *Eisenia fetida*. *Applied Geochemistry* 22, 2397 – 2405

Edwards, C.A., Lofty, J.R. 1977. Biology of Earthworms (2nd Edition). London: Chapman and Hall.

Jager, G., Bruins, E.H. 1975. Effect of Repeated Drying at Different Temperatures on Soil Organic Matter Decomposition and Characteristics, and on the Soil Microflora. *Soil Biology and Biochemistry* 7(2): 153 – 159

OECD, 1984. Test No. 207: Earthworm, acute toxicity tests. OECD Publishing, Paris.

Stevenson, I.L. 1956. Some Observations on the Microbial Activity in Remoistened Air-Dried Soils. *Plant and Soil* 8(2): 170 – 182